SPANDIDOS PUBLICATIONS

The role of the endothelin axis and microvessel density in bladder cancer - correlation with tumor angiogenesis and clinical prognosis

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Received January 7, 2007; Accepted February 14, 2007

Abstract. Endothelin-1 (ET-1) and its receptors, entothelin-A (ET_AR) and endothelin-B (ET_BR), commonly referred to as the endothelin (ET)-axis, are involved in tumor biology and growth. We investigated the effects of the ET-axis on microvessel density (MVD) and the clinicopathological parameters of patients with invasive bladder cancer. Paraffin tumor sections of 120 patients who had undergone radical cystectomy were assessed immunohistochemically using mono- and polyclonal antibodies for ET-1, ET_AR, ET_BR and CD34 (MVD). Staining intensities were analyzed semiquantitatively and the MVD was calculated as vessels per field. The results were correlated with various pathological and clinical factors, as well as with disease-free and overall survival. Transitional cell carcinomas (MVD = 23.7) were better vascularized than squamous cell carcinomas (MVD = 17.8, p=0.04). Organ-confined tumors (MVD = 32.2) were better vascularized than T3- and T4-tumors (MVD = 21.2, p=0.02) and ET-1 was overexpressed in this subgroup (p=0.027). Patients with metastatic regional lymph nodes (MVD = 20.9) tended to have less MVD than patients without regional lymph node metastases (MVD = 24.1) (p=0.15). The account of MVD did not reveal any significant differences in disease-free or overall survival. Organ-confined tumors and ET-1 overexpression are associated with upregulated microvessel density. These results suggest that MVD and ET-1 could be considered good prognostic factors.

Introduction

Angiogenesis is the development of new vessels from pre-existing vessels. The prognostic value of estimates of angiogenesis in many types of carcinomas has been investigated since Weidner *et al* reported that high vascular scores were associated with distant metastases (1). Several groups have investigated the prognostic significance of angiogenesis in bladder cancer using slightly different methods, and in general, it has been concluded that a highly vascular bladder carcinoma behaves more aggressively than a carcinoma with a low vascular density (2-4).

Endothelin (ET)-1, a vasoactive peptide, is produced primarily in endothelial, vascular smooth muscle, and epithelial cells. ET-1 exerts its physiological effect via two high-affinity, G-protein-coupled receptors, endothelin-A (ET_AR) and endothelin-B (ET_BR). The combination of ET-1 and the two receptors is referred to as the ET-axis, which is associated with tumorigenesis and tumor progression by various mechanisms, including proliferation, invasion, inhibition of apoptosis and angiogenesis (5-8). Conditions of stress such as hypoxia lead to an enhanced production of ET-1. Through ET_BR, it predominantly stimulates endothelial cell growth, while the induction of vascular smooth muscle cell and pericyte mitogenesis are mediated through ET_AR and therefore contribute to the process of angiogenesis in different kinds of tumors (6,9). Although there is not enough data to prove that ET-1 causes angiogenesis directly, this effect is supported by the finding of increased ET-1 expression in ovarian carcinoma (7), colorectal carcinoma (10) and breast cancer (8) in association with an increased expression of VEGF, which in turn stimulates vascular permeability and endothelial cell proliferation by increasing HIF-1α (7) in a time- and dose-dependent manner.

We have recently demonstrated an increased ET-1, ET_AR and ET_BR expression in bladder carcinomas, the latter being the predominant receptor and associated with a more favorable prognosis (11).

The objective of the present study was to assess whether ET expression is related to angiogenesis in bladder carcinoma. Therefore, this study examined for the first time the expression of the endothelin axis and the angiogenic marker microvessel density (MVD) immunohistochemically in representative paraffin tumor sections. Furthermore, we also assessed the effect of ET expression and MVD on the clinical follow-up.

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Key words: microvessel density, endothelin axis, angiogenesis, endothelin receptor, endothelin-A, endothelin-B

Table I. Distribution of pathological and clinical variables in the reported series of bladder cancer specimens (n=120).

Pathological parameters	n (%)
pT stage[a]	
T1	5 (4.2)
T2	12 (10.2)
T3	75 (63.6)
T4	26 (21.7)
pN stage	
N0	78 (65)
N1	18 (15)
N2	23 (19.2)
N3	1 (0.8)
cM stage	
M0	115 (95.8)
M1	5 (4.2)
Histological grade	
G1	3 (2.5)
G2	22 (18.3)
G3	91 (75.9)
G4	4 (3.3)
Histology type	
TCC	99 (82.5)
SCC	14 (11.7)
Other	7 (5.8)

[a]In two cases no tumor was found. TCC, transitional cell carcinoma; SCC, squamous cell carcinoma.

Materials and methods

Patients and tumor specimens. Patients (n=120) treated with radical cystectomy for invasive bladder cancer at our institution were selected from our bladder cancer database. The 78 men and 42 women underwent surgery between 1989 and 1995; median age at surgery was 62 years (range 33-81). None of the patients had received neo-adjuvant treatment before surgery. Histological slides and formalin-fixed, paraffin-embedded tumor tissue blocks from all 120 patients were obtained from the files at the Institute of Pathology. All the slides were reviewed and representative tumor tissue blocks were selected for immunohistochemistry. Histopathological data regarding tumor stage, histological grading and histology type are demonstrated in Table I.

The follow-up assessments with clinical information such as tumor recurrence, progression, overall and disease-free survival and cause of death were evaluated in collaboration with office urologists and were taken from our bladder cancer database (12).

Immunohistochemistry. For immunohistochemical investigations, the paraffin-embedded tumor tissue blocks were cut into 3-μm slices and mounted on poly-L-lysine-coated glass slides. After dewaxing in xylene and rehydrating in a graded series of alcohols, endogenous peroxidase was blocked with 3% H_2O_2. As previously described, staining for ET_AR and ET_BR was run in a multistep semiautomatic procedure (DAKO-Autostainer; DAKO Diagnostics, Hamburg, Germany) (13). The antibodies used were two sheep polyclonal antibodies for ET_AR and ET_BR (ETAR-Antiserum, Product No. 210-506-C250, Affinity Bioregents, Golden, CO, USA), applied at a dilution of 1:100 for 30 min. For ET-1 staining, a monoclonal mouse antibody (Anti-Endothelin-1 MAb, Clone TR.ET.48.5, Affinity Bioreagents) was used. The deparaffinization of the tissue sections was followed by pre-treatment with a steamer (Multi-Gourmet-Steamer, BRAUN, Type 3216) for antigen retrieval (citrate buffer pH 6.0 for 35 min). Endogenous peroxidase was blocked; afterwards the primary antibody was applied at a dilution of 1:500 for 25 min followed by incubation with the secondary antibody (Link-HRP) for 20 min. After the primary antibodies, the sections were incubated with rabbit anti-sheep antibody (DAKO) used as the secondary antibody at a dilution of 1:500 for 25 min. Again, endogenous peroxidase was blocked and then incubated with the Envision Detection Kit for 25 min. The enzyme reaction was developed with Chromogen-DAB (2x5 min). The specimens were then counterstained with hematoxylin and mounted with Kaiser's glycerine. The staining intensities of ET-1 and its two receptors on a high-power field (Fig. 1) were classified according to an arbitrary four-tiered scale (negative = 0, weak = 1, moderate = 2, strong = 3) in a manner consistent with previous investigations (13). Weak, moderate, and strong staining patterns were defined as positive in the subsequent statistical analysis.

CD34 immunohistochemistry was performed as published previously (14). The Class II Clone QBEnd 10 mouse anti-human monoclonal antibody was used (DAKO, Carpenteria, CA, USA). The antigen was unmasked by microwave heating of the slides three times for 5 min in citrate buffer (pH 6.0). After washing in PBS, the primary antibody was applied for 1 h at room temperature. For the detection system, LSAB2 (DAKO, K0675) was used with the secondary antibody as the biotinylated link (30 min) followed by streptavidin-peroxidase (30 min). After every step, the slides were washed in PBS (pH 7.4). The reaction was detected by incubating with diaminobenzidine for 5 min. After counterstaining with H&E, the slides were dehydrated, clarified and mounted. The stained vessels were counted in five consecutive fields from the representative tumor zone and the mean value considered as the MVD.

Data analysis. Staining intensity was evaluated semi-quantitatively in a blind fashion. For statistical analysis SPSS for Windows (Version 13.0) was used. All the histopathological parameters were correlated with the staining results by means of cross-tables, applying the Kruskal-Wallis test. The Kaplan-Meier method was used to derive the recurrence-free survival and the log-rank test to compare curves for two or more groups. For multivariate analysis of the prognostic factors, a Cox regression analysis was

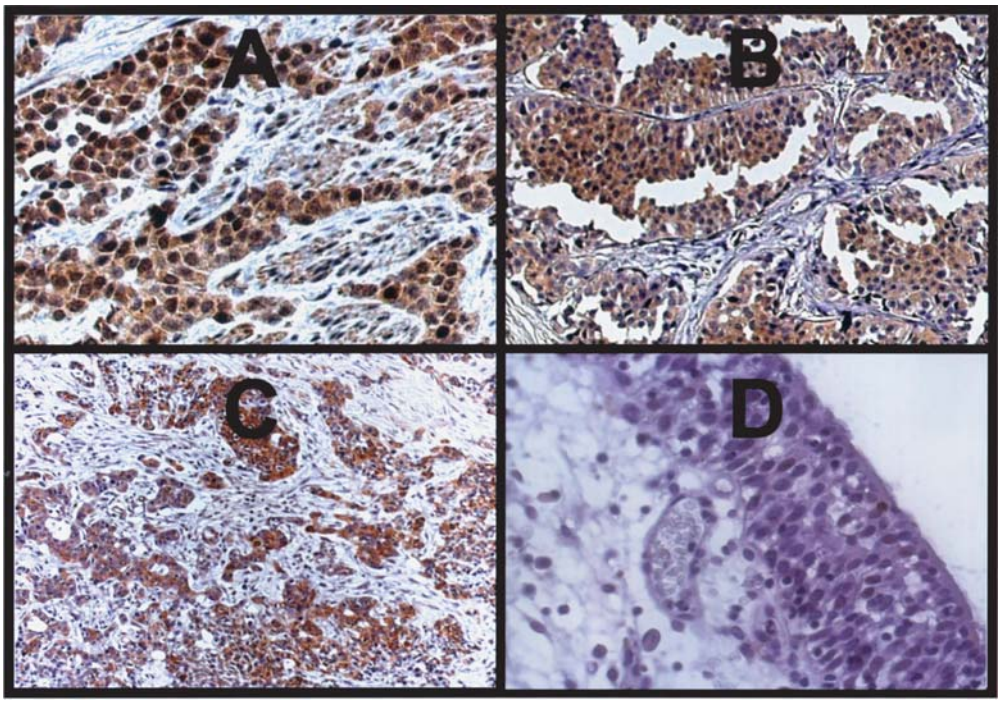


Figure 1. Representative examples of staining for endothelin-1 (A), and its receptors, endothelin-A (B) and endothelin-B (C), in invasive carcinoma of the bladder and negative staining in a normal urothelium (D).

performed. A p-value of $p<0.05$ indicated significant differences between the groups.

Results

From the 120 patients, the ET-1, ET_AR and ET_BR staining status was available in 117 (97.5%) cases. In some of the specimens, tissue had been lost during steamer-pretreatment. The staining intensity of ET-1, ET_AR, and ET_BR among the different samples varied from the complete absence of staining to strong staining. Endothelin-1 staining was identified in 26.5% of the cases, ET_AR in 60.7% and ET_BR in 78.6%, respectively.

For vascular markers, CD34 staining gave a mean MVD of 21.5 for all the investigated tumors. Transitional carcinomas were better vascularized than squamous cell carcinomas (MVD = 23.7 and MVD = 17.8, respectively; p=0.04). Organ-confined tumors were better vascularized than T3- and T4-tumors (MVD = 32.2 and MVD = 21.2, respectively; p=0.02) and ET-1 was overexpressed in this subgroup (p=0.027). There was no association of MVD to histological grading (p=0.47), or metastases (p=0.37). However, patients with metastatic regional lymph nodes (MVD = 20.9) tended to have less MVD than patients without regional lymph node metastases (MVD = 24.1, p=0.15), although this difference was not significant. Tumors with negative ET-1, ET_AR and ET_BR staining had an MVD of 21.7, 21.8 and 23.6, those with faint or positive expression 26.6 (p=0.30), 23.1 (p=0.29) and 23.5 (p=0.37), respectively. A comprehensive summary of the distribution of MVD and its association with clinicopathological parameters and the endothelin axis is given in Table II.

All patients were followed-up for a minimum of 41 months (median, 86; range, 41-170). The median overall survival and disease-free survival times were 24 months [95% Confidence Interval (CI), 17-31 months] and 20 months (95% CI, 13-27 months), respectively. Eighty (66.7%) patients had died of either tumor-related (n=67) or other (n=13) causes. Three patients have recently been diagnosed with local tumor recurrence or metastasis, 33 show no evidence of disease and 4 have been lost to the follow-up.

With the Kaplan-Meier survival analysis and log-rank test for the comparison of the survival curves, the stratification of patients according to MVD > 21.5 (mean value) and MVD < 21.5 did not reveal any significant differences in disease-free or overall survival (Fig. 2).

A multivariate analysis using Cox-regression analysis was performed (Table III). Only tumor stage (p=0.003) and metastatic disease were independent prognostic parameters ($p<0.001$), but not MVD.

Discussion

There is increasing evidence for the emerging role of the ET-axis in several tumor types. Experimental and epidemiological studies have shown that tumor development can be reduced by inhibitors of the ET-axis, which block various mechanisms of tumor growth such as proliferation, invasion, inhibition of apoptosis and angiogenesis (5,6,15,16). In some tumor types, ET-1 production is associated with an increased expression of VEGF and neovascularization, which in turn stimulates vascular permeability and endothelial cell proliferation by increasing HIF-1α (7;13). Endothelial cell growth is stimulated predominantly through ET_BR, while the induction of vascular smooth muscle cell and pericyte mitogenesis is mediated through ET_AR (9,17). In brain tumors, ET-1 expression is correlated with transforming growth factor ß1-expression and

Table II. Mean value of MVD and the expression of ET-1, ET_AR and ET_BR.

Pathological variables	MVD n (mean value)	ET-1 staining n positive total (%)	ET_AR staining n positive/total (%)	ET_BR staining n positive/total (%)
pT stage				
T1	29.9	1/5 (20)	2/5 (40)	4/5 (80)
T2	33.2	5/12 (41.7)	10/12 (83.3)	11/12 (91.7)
T3	21.2	17/72 (23.6)	44/73 (60.3)	56/73 (76.7)
T4	21.4	7/26 (26.9)	14/26 (53.8)	20/26 (76.9)
pN stage				
N0	24.1	23/76 (30.3)	45/76 (59.2)	60/76 (79.8)
N1	20.6	1/17 (5.9)	10/17 (58.8)	16/17 (94.1)
N2	21.3	7/23 (30.4)	15/23 (65.2)	15/23 (65.2)
N3	17.4	0/1 (0)	1/1 (100)	1/1 (100)
cM stage				
M0	23.1	31/112 (27.7)	68/112 (60.7)	89/112 (79.5)
M1	20.7	0/5 (0)	3/5 (60)	3/5 (60)
Histological grade				
G1	22.9	2/3 (66.7)	2/3 (66,7)	1/2 (50.0)
G2	20.8	4/21 (21)	14/22 (63.6)	17/22 (77.3)
G3	23.4	24/89 (27)	53/88 (60.2)	71/89 (79.8)
G4	25.9	1/4 (25)	2/4 (50)	3/4 (75)
Histology type				
TCC	23.7	24/97 (24.7)	60/98 (61.2)	76/97 (78.4)
SCC	17.8	4/13 (30.8)	8/13 (61.5)	11/13 (84.6)
Other	22.8	3/7 (42.9)	3/6 (50)	5/7 (71.4)

MVD, microvessel density; ET-1, endothelin-1; ET_AR, endothelin-A receptor; ET_BR, endothelin-B receptor; TCC, transitional cell carcinoma; SCC, squamous cell carcinoma.

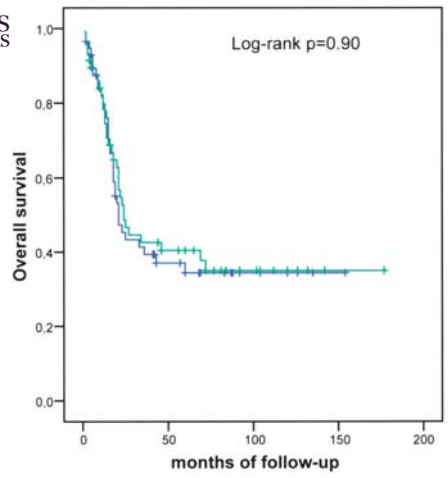
tumor vascularity (18). These preclinical data indicate that the ET-axis is involved in the process of angiogenesis and can accelerate neovascularization in malignancies. For bladder carcinoma there is not enough data to confirm these results - the role of the ET-axis and its direct influence on angiogenesis is still unclear.

Some authors have highlighted that a highly vascular bladder carcinoma behaves more aggressively than a carcinoma with a low vascular density and is associated with a less favorable prognosis (2-4). Few studies have demonstrated the relationship of angiogenesis with improvement in survival (19,20). Offersen *et al* were able to show a positive correlation between inflammation in bladder carcinoma, angiogenic factors and good prognosis. In particular, patients with an increasing degree of inflammation in the tumor were associated with a survival improvement, which could reflect the ability of the patient to mount an immune response (21).

Only two studies have been published that deal with the same angiogenic marker (CD34) as used in our study in order to estimate angiogenesis in cystectomy specimens, identifying MVD as an independent parameter of poor prognosis (2,4). Both studies determined MVD by estimating the number of microvessels in the most vascular areas (so-called 'hot spots') previously described by Weidner *et al* (1). As the main goal of our present study was to compare the local expression of the investigated markers within different areas of representative tumor tissue, we counted the stained vessels in five consecutive fields and considered the mean value as the MVD.

In a former study, we were able to demonstrate the expression of ET-1 and both of its receptors, ET_AR and ET_BR, in bladder cancer patients who had undergone radical cystectomy. ET-1 and ET_AR expression were not correlated with pathological and clinical parameters or with survival curves. However, ET_BR expression was associated with more favorable tumor types (11).

The objective of the current study was to clarify whether the expression of the ET-axis has any influence on angiogenesis and whether upregulated MVD has a prognostic value for bladder cancer patients. A directly proportional increase in the expression of the ET-axis and MVD associated with a poor prognosis was the expected outcome of the study. Applying standard immunohistochemical techniques, we showed upregulated MVD in organ-confined tumors. Furthermore, we demonstrated the overexpression of ET-1 in



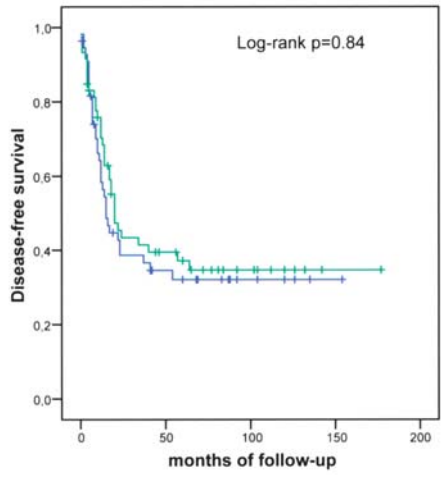


Figure 2. Overall survival and disease-free survival time in 120 bladder cancer patients in relation to microvessel density (MVD) > 21.5 (green line) and MVD < 21.5 (blue line).

this subgroup compared to T3- and T4-tumors. Patients with regional lymph node metastases tended to have less MVD compared to patients without metastatic lymph nodes, although statistically this was not significant. Patients with transitional carcinoma of the bladder had more MVD compared to patients with squamous cell carcinoma. All these suprising results suggest the possibility of a good prognosis for patients with upregulated MVD. However, with the Kaplan-Meier survival analysis and log-rank test for the comparison of the survival curves, the stratification of patients according to MVD did not reveal any significant differences in disease-free or overall survival. In the multivariate analysis, only the tumor stage and metastatic disease were considered independent prognostic parameters, but not MVD.

In 1979, Mihatsch *et al* were the first to report a significantly better 1-year survival when lymphocytes, plasma cells and/or lymph follicles were present in invasive carcinoma of the bladder (22). Flamm was able to confirm these results in superficial bladder carcinoma, reporting that about 428 patients with an inflammatory reaction in their tumor experienced significantly fewer recurrences and cancer-related deaths

Table III. Multivariate analysis of prognostic factors in 120 bladder cancer patients.

Prognostic variables	Overall survival, all patients (n=120)	
	Risk ratio	Statistical significance
MVD	0.634	0.433
ET-1	1.120	0.900
ET_AR	0.975	0.853
ET_BR	0.658	0.128
T	3.073	0.003
N	0.707	0.688
M	4.098	<0.001
G	2.878	0.058
Histology type	1.098	0.974

MVD, microvessel density; ET-1, endothelin-1; ET_AR, endothelin-A receptor; ET_BR, endothelin-B receptor; T, tumor stage; N, lymph node stage; M, metastases; G, histological grade.

compared to those without inflammation (23). In another study, Offersen *et al* showed a survival advantage for patients with intense inflammation in their tumors with a correlation to significantly increased vascular density. MVD was very closely associated to the degree of inflammation and even represented an independent parameter for better survival using overall death as the end-point. The survival advantage was described to reflect a host vs tumor response (20).

Taking these results into account, upregulated MVD in our series, as described in organ-confined tumors, could be due to the angiogenetic stimulation of a local inflammatory reaction generated by the host against the cystectomy specimens. As ET-1 was overexpressed in these specimens and is associated with neovascularization and VEGF expression in various human tumors, it could be involved in the regulation of angiogenesis in bladder cancer. Its loss could lead to a diminished differentiation and an association with unfavorable parameters, such as higher tumor stages, higher histological grades and tumor progression.

In summary, we have shown that ET-1 and its receptors are expressed by bladder carcinoma cells, and that the expression of ET-1 positively correlates with angiogenesis defined as microvessel density in organ-confined tumors. These results suggest that MVD could be considered a good prognostic factor. However, angiogenesis is not yet considered a useful prognostic marker for time to progression or overall survival in patients with invasive carcinoma of the bladder.

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