Endothelin-1 overexpression: a potential biomarker of unfavorable prognosis in non-metastatic muscle-invasive bladder cancer

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<td>Dimakopoulous</td>
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<td>Science and Technology Park of Epirus</td>
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<td>Moutzouris</td>
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<td>General Hospital of Argos</td>
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<td>Vasilios</td>
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<td>Georgios</td>
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Endothelin-1 (ET-1) is a multifunctional peptide exerting its effects via receptors A and B. ET-1 and its receptors, endothelin axis (ET axis), play a promoting role in cancer biology. Alterations of proteins of ET axis have been detected in non-metastatic muscle-invasive bladder cancer (NMMIBC). The objective of this study is to investigate the potential role of ET-1 tumor expression as a biomarker of prognosis, compared to other prognostic parameters (epidemiologic and pathologic), in NMMIBC. We prospectively included 40 consecutive, primary, high-grade NMMIBC patients. Tumor specimens after initial transurethral resection were stained immunohistochemically for ET-1. The ET-1 evaluation of expression was based on staining intensity (SI) of ET-1. SI was classified according to an arbitrary four-tiered scale (negative = 0, mild = 1, moderate = 2, strong = 3). Epidemiologic and pathologic parameters were analyzed, using univariate and multivariate statistics, for disease progression, progression-free survival (PFS), and overall survival (OS). ET-1 overexpression (SI = 3) was the unique parameter which associated significantly, both in univariate (log-rank test, \( p = 0.033 \)) and multivariate (Cox regression analysis, \( p = 0.045, \text{HR} = 4.849, 95 \% \text{CI}: 1.039–22.624 \)) analysis, with an increased hazard ratio of progression. ET-1 overexpression (SI = 3) was also the unique parameter which associated, marginally significantly in univariate analysis (log-rank test, \( p = 0.056 \)) and
highly significantly in multivariate analysis (Cox regression analysis, 
\( p=0.005 \), HR\( =7.001 \), 95 % CI: 1.782–27.501), with an increased 
hazard ratio of death. Overexpression of ET-1 may be a potential 
biomarker of unfavorable prognosis in NMMIBC patients.

81 Keywords

82 Foot note

Endothelin-1 - Overexpression - Biomarker - Muscle-invasive 
bladder cancer - Prognosis

information
Endothelin-1 overexpression: a potential biomarker of unfavorable prognosis in non-metastatic muscle-invasive bladder cancer

Mitrakas Lampros · Gravas Stavros · Karasavvidou Fotini · Dimakopoulos Georgios · Moutzouris Georgios · Tzortzis Vasilios · Koukoulis Georgios · Papandreou Christos · Melekos Michael

Abstract Endothelin-1 (ET-1) is a multifunctional peptide exerting its effects via receptors A and B. ET-1 and its receptors, endothelin axis (ET axis), play a promoting role in cancer biology. Alterations of proteins of ET axis have been detected in non-metastatic muscle-invasive bladder cancer (NMMIBC). The objective of this study is to investigate the potential role of ET-1 tumor expression as a biomarker of prognosis, compared to other prognostic parameters (epidemiologic and pathologic), in NMMIBC. We prospectively included 40 consecutive, primary, high-grade NMMIBC patients. Tumor specimens after initial transurethral resection were stained immunohistochemically for ET-1. The ET-1 evaluation of expression was based on staining intensity (SI) of ET-1. SI was classified according to an arbitrary four-tiered scale (negative=0, mild=1, moderate=2, strong=3). Epidemiologic and pathologic parameters were analyzed, using univariate and multivariate statistics, for disease progression, progression-free survival (PFS), and overall survival (OS). ET-1 overexpression (SI=3) was the unique parameter which associated significantly, both in univariate (log-rank test, \( p = 0.033 \)) and multivariate (Cox regression analysis, \( p = 0.045 \), HR=4.849, 95 % CI: 1.039–22.624) analysis, with an increased hazard ratio of progression. ET-1 overexpression (SI=3) was also the unique parameter which associated, marginally significantly in univariate analysis (log-rank test, \( p = 0.056 \)) and highly significantly in multivariate analysis (Cox regression analysis, \( p = 0.005 \), HR=7.001, 95 % CI: 1.782–27.501), with an increased hazard ratio of death. Overexpression of ET-1 may be a potential biomarker of unfavorable prognosis in NMMIBC patients.

Keywords Endothelin-1 · Overexpression · Biomarker · Muscle-invasive bladder cancer · Prognosis

Introduction

Bladder cancer is the ninth most common cancer affecting humans worldwide [1]. At diagnosis, about 30 % of patients have muscle-invasive bladder cancer (MIBC). Approximately one third of them have undetected metastases at the time of primary treatment with transurethral resection (TUR) of the tumor. Radical cystectomy (RC) with urinary diversion represents the therapeutic gold standard for non-metastatic muscle-invasive bladder cancer (NMMIBC). About 50 % of patients relapse after RC, depending on the pathological stage of the primary tumor and the nodal status, and the 5-year overall survival rate is 45 % [1].
Endothelin-1 (ET-1) is a multifunctional peptide, exerting its effects via receptors A and B (ETAR and ETBR). ET-1 and its receptors, endothelin axis (ET axis), play a role in cancer biology. ET-1 has been demonstrated to stimulate tumor cell proliferation, to facilitate tumor invasion and metastasis, and to have anti-apoptotic and neoangiogenic effects [2]. ETAR leads to critical steps in tumor biology, such as tumor progression (through activation of tumor cell proliferation), inhibition of apoptosis, effects on bone matrix, production of vascular endothelial growth factor (VEGF) leading to endothelial cell proliferation and vascular permeability, by increasing the levels of hypoxia-inducible factor-1α (HIF-1α). ETBR is less involved in tumor biology by inducting proliferation of endothelial cells and migration [3]. A high expression of the ET axis, both on messenger RNA (mRNA) and protein level, has been detected in MIBC [4].

Pathological parameters, such as local tumor invasiveness (T-tumor stage), presence of positive lymph nodes (N-nodal stage), histologic grade, and lymphovascular invasion in pathologically node-negative bladder cancer, are identified as having a prognostic value in MIBC [5]. Clinical parameters, such as tumor size or number of tumors, are used in combination with pathological ones and are mainly considered as prognostic for non-muscle-invasive bladder cancer as described in the European Organization for Research and Treatment of Cancer (EORTC) risk calculator [1]. Up to now, no established molecular markers can be unequivocally recommended for risk assessment in MIBC on a routine basis [5]. Biomedical and oncologic research studies continue to focus on molecular markers in order to achieve a reliable prognosis for patients after RC and to design and test targeted therapeutic approaches. From this point of view, studying of ET-1 as a potential prognostic biomarker for MIBC patients looks intriguing.

**Objectives**

To investigate the potential role of ET-1 tumor expression as a biomarker of prognosis, compared to other prognostic parameters (epidemiologic and pathologic), in NMIBC.

**Methods**

Between January 2004 and May 2006 in our institution (Department of Urology, University Hospital of Larissa, Greece), we prospectively included 40 consecutive bladder cancer patients. The included patients had primary, high-grade (according to the WHO classification 2004), muscle-invasive, transitional cell and non-metastatic carcinoma. Other types of urinary bladder carcinoma were defined as exclusion criterion. Patients were treated initially with a TUR of the tumor and subsequently, within 2 months, with RC and urinary diversion.

Before RC, all patients were submitted to clinical staging with X-ray computed tomographies (CTs) of abdomen, retroperitoneum, and thorax. When clinical (osseous pain) or laboratory (increased levels of alkaline phosphatase) findings were present, staging process was completed with an additional integral bone scintigraphy with γ-camera. After RC, patients were followed up, with laboratory (hematologic and biochemical testing, blood gases) and imaging (ultrasound examination of kidneys, abdomen, retroperitoneum, and thorax CTs) exams, every 6 months for the first 3 years and annually thereafter. Disease progression was defined as detection of distant metastases or enlarged pelvic or extrapelvic lymph nodes, regardless of the dimensions, in abdomen and thorax CTs. Every patient with disease progression was referred to the oncologists for adjuvant chemotherapy.

All pathologic specimens were evaluated for stage according to the TNM classification of malignant tumors (UICC, International Union Against Cancer, seventh edition, 2010) and for grade according to the classification of World Health Organization (WHO, 2004). One pathologist, specialized on genitourinary tract tumors, performed evaluation, grading, and staining of samples. Representative tumor samples from initial TUR of tumor of all patients were obtained for the purpose of immunohistochemical staining. Choice was based on the presence of good morphology and antigenicity; therefore, extremely cauterized or mechanically abused or quantitative insufficient material was not used. For the staining, we applied mouse monoclonal antibody for ET-1 (clone TR.ET.48.5, dilution 1:250, NOVUS Biologicals, Littleton, CO, USA). Staining intensity (SI) of ET-1 (Fig. 1) on a high-power field was classified according to an arbitrary four-tiered scale (negative=0, mild=1, moderate=2, strong=3) in a manner consistent with previous investigations [6].

Epidemiologic (age, gender, smoking) and pathologic parameters (stage T, stage N, concomitant carcinoma in situ at RC) were analyzed, using univariate and multivariate statistics, for disease progression, progression-free survival (PFS), and overall survival (OS). PFS and OS for each individual patient were calculated starting from the day of TUR diagnosis of muscle-invasive disease till the day of diagnosis of disease progression and death, respectively.

Univariate analysis for progression was performed using chi-squared or Fisher’s exact test and multivariate analysis using multiple logistic regression. Univariate analysis for PFS and OS was performed using log-rank test for categorical variables and Cox regression for scale variables. Multivariate analysis for PFS and OS was assessed using Cox regression analysis after checking the proportional hazard assumption. The level of statistical significance was set as $p\leq0.05$. All
analyses were performed with the use of IBM SPSS Statistics version 21 software.

Our study complies with the provisions of Declaration of Helsinki (as revised in Tokyo 2008).

**Results**

We enrolled 40 patients, 33 males (82.5 %), and 7 females (17.5 %), with mean age 69.6±6.78 years (49–79). No patient received neoadjuvant chemotherapy prior enrollment. Of the patients enrolled in our study, 17 (42.5 %) had positive lymph nodes at RC and they received adjuvant chemotherapy after radical surgery. A total of 19 (47.5 %) patients had disease progression and needed chemotherapy. The results of evaluation of intensity of ET-1 staining (SI) are as follows: 0 (0 %) patients scored SI=0, 12 (30 %) patients scored SI=1, 23 (57.5 %) patients scored SI=2, and 5 (12.5 %) patients scored SI=3. No significant association between expression of ET-1 and pathologic parameters was found. Median PFS was 42.7 months. Median OS and duration of follow-up were 51.4 months. Baseline characteristics at RC are presented in Table 1.

Progression

None of the parameters under the study was statistically significantly associated, in univariate and multivariate analysis (Table 2), with disease progression.

Progression-free survival (PFS)

In univariate analysis (Table 3), only ET-1 was statistically significantly associated with PFS ($p=0.033$, log-rank test).

In multivariate analysis (Table 3), none of the parameters under the study was statistically significantly associated with PFS. By further Cox regression analysis and stratification of $t_1$:

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Baseline characteristics of all patients ($n=40$) at radical cystectomy</th>
</tr>
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<tbody>
<tr>
<td>Age (mean, years)</td>
<td>69.6±6.78 (49–79)</td>
</tr>
<tr>
<td>Gender (♂/♀)</td>
<td>33/7 (82.5/17.5 %)</td>
</tr>
<tr>
<td>Smoking (yes/no/former)</td>
<td>25/5/10 (62.5/12.5/25 %)</td>
</tr>
<tr>
<td>Concomitant carcinoma in situ (yes/no)</td>
<td>13/27 (32.5/67.5 %)</td>
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<tr>
<td>Stage T (T2/T3/T4)</td>
<td>13/16/11 (32.5/40/27.5 %)</td>
</tr>
<tr>
<td>Stage N (N0/N1/N2/N3)</td>
<td>23/8/9/0 (57.5/20/22.5/0 %)</td>
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In univariate analysis (Table 4), only ET-1 was marginally significant regarding OS \((p=0.019, \text{Cox regression analysis})\). By further Cox regression analysis and stratification of patients according to all ET-1 expression levels (SI=0, 1, 2, 3), we found that only patients with strong expression (SI=3) of ET-1 (Fig. 1), compared with patients with mild expression (SI=1) of ET-1, had worse clinical outcome, in specific an increased hazard ratio of death during the entire observation period (Cox regression analysis, \(p=0.005, \text{HR}=7.001, 95 \% \text{CI: 1.782–27.501}\), as it is also shown in Fig. 3 by Kaplan-Meier curves. We additionally found that patients with moderate expression (SI=2) of ET-1, compared with patients with strong expression (SI=3) of ET-1 (Fig. 1), had better clinical outcome, in specific a decreased hazard ratio of death during the entire observation period (Cox regression analysis, \(p=0.026, \text{HR}=0.279, 95 \% \text{CI: 0.091–0.856}\), as it is also shown in Fig. 3 by Kaplan-Meier curves.

### Discussion

ET-1 belongs to a family of multifunctional peptides (ET-1, ET-2, ET-3) and was firstly isolated in 1998 from porcine endothelial cells [7]. ET-1 has a strong vasoconstrictive action [6], stimulates tumor cell proliferation, facilitates tumor invasion and metastasis, and has antiapoptotic and neoangiogenic effects [2]. ET-1 and its receptors, ET axis, play an apparently increasingly significant role in cancer biology of several human tumors (prostatic, ovarian, renal, pulmonary, colorectal, cervical, breast, urinary bladder, endometrial carcinoma, sarcoma Kaposi, brain tumor, bone metastases, and melanoma) [2].

Endothelins exert their natural effect via two high-affinity, G-protein-coupled receptors, A (ETAR) and B (ETBR). ETAR is selective for ET-1, ET-2, and ETBR for all isoforms [6]. Endothelial cell proliferation and migration are influenced by ET-1 and mediated through activation of ETBR, whilst stimulation of vascular smooth muscle, mitogenesis of pericytes, and production of VEGF are mainly achieved through ETAR [8–11]. The presence of ET-1 and its receptors in normal urothelium and detrusor muscle is experimentally well documented [12–14].

ET axis is significantly overexpressed in muscle-invasive tumors compared with normal urothelium [4, 6]. Wülfing et al. studied 157 radical cystectomy samples with immunohistochemistry and documented overexpression of the entire endothelin axis (ET-1, ETAR, ETBR). They found significantly better disease-free survival and a trend toward better overall survival only for ETBR (+) tumors [6]. In a preclinical study on bladder tumor xenografts, Wülfing et al. implanted KU-19-19 bladder cancer cells, expressing only ET-1 and ETAR but not ETBR, in nude mice [15]. After tumor growth in the flank, one group was treated with...
intraperitoneal atrasentan (selective ETAR antagonist) and the other with placebo. Atrasentan group showed a diminished tumor growth rate with an increased necrosis in the tumor tissue but no significant cytoreduction. Interestingly, ETAR expression increased (RT-PCR) in the treatment group, implying an escape mechanism to overcome antiproliferative effect caused by targeting ETAR [15]. A subsequent study in a mouse model by Herrmann et al. studied whether inhibition of ETAR with atrasentan leads to antitumor effect in bladder cancer. They also used KU-19-19 bladder cancer cells, which were implanted in nude mice with thymic aplasia. Mice were treated with atrasentan either placebo. For atrasentan group, a trend toward higher values for the necrosis rate as well as receptor density for ETAR was found. This bladder cancer xenograft model failed to show significant antitumor effect [16]. Herrmann et al. studied retrospectively the correlation of ET axis with microvessel density (MVD) and other clinical and pathologic parameters in muscle-invasive tumors. They concluded that ET-1 and MVD could be considered as "good prognostic factors", because ET-1 overexpression correlated significantly with increased MVD and organ-confined disease [17]. However, they included five patients with non-muscle-invasive disease (4.2 %), 25 patients (20.8 %) with low-grade tumors, and 21 cases (17.5 %) of non-transitional cell carcinoma. Eltze et al. performed a retrospective study for the expression of the endothelin axis in non-invasive and superficially invasive bladder cancer and concluded that both lack of ET-1 and ETAR have negative prognostic significance [18]. They used transurethrally resected, formalin-fixed, paraffin-embedded samples from 154 patients with primary bladder cancer. According to tumor stage T, these patients were stratified as follows: 91 patients with pTa, 28 patients with pT1, and 35 patients with pT2 disease. The observed differences between the two studies [17, 18] and the present one might be explained by the different design. The previous studies were retrospective, while our study is prospective. In addition, there is a significant discrepancy in histopathological characteristics (T stage, grade, and histologic type) of the evaluated patients. Eltze et al. enrolled primary Ta, T1, and T2 patients, and Herrmann et al. included few patients with non-muscle-invasive, transitional cell carcinoma (TCC), low- and high-grade tumors with various histological
types without reporting if these tumors were primary or recurrent. This heterogeneity must be taken into consideration when someone interprets and compares the results. Our group consisted of 40 prospectively included, consecutive patients with primary, muscle-invasive, high-grade TCC. We comprehensively present all clinicopathologic studies about the potential role of endothelin axis in bladder cancer in Table 5. To our knowledge, it is reported for the first time a significant negative impact of bladder tumor ET-1 strong expression (overexpression) on progression-free survival and overall survival. However, we found no correlation of ET-1 overexpression with disease progression. Recently, Said et al. evaluated whether tumor ET-1 expression could be utilized as a molecular biomarker for lung metastases and whether it is required for metastatic disease [19]. Evaluation of ET-1 mRNA and protein expression in four

**Table 5** Summary of clinicopathologic studies for potential association of endothelin axis with bladder cancer

<table>
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<tr>
<th>Patients Enrolled Method</th>
<th>Primary Histology</th>
<th>Grade</th>
<th>Tumor stage T</th>
<th>Reported association</th>
</tr>
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<tr>
<td>t5.3 Wülffing (2005) 157 Ret. IHC (ET-1, ETAR, ETBR) NA TCC, SCC, other G1 G2 G3 G4 pT1 pT2 pT3 pT4 Overexpression of ET-1, ETAR, ETBR Predomination of ETBR which is associated with better DFS</td>
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<td>t5.4 Hermann (2007) 120 Ret. IHC (ET-1, ETAR, ETBR) NA TCC, SCC, Other G1 G2 G3 G4 pT1 pT2 pT3 pT4 ET-1 associates with organ-confined disease</td>
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<tr>
<td>t5.5 Eltze (2009) 154 Ret. Tissue microarrays, IHC (ET-1, ETAR, ETBR) Yes NA G1 G2 G3 G4 pT1 pT2 pT3 pT4 Lack of ET-1 may be an independent negative prognostic factor for OS Lack of ETAR may be an independent negative prognostic factor for OS</td>
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<tr>
<td>t5.6 Present study (2015) 40 Pr. IHC (ET-1) Yes TCC High grade (WHO 2004) pT2 pT3 pT4 Overexpression of ET-1 may be a negative prognostic factor for both PFS and OS</td>
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Ret. retrospectively, Pr. prospectively, IHC immunohistochemistry, NA not available, TCC transitional cell carcinoma, SCC squamous cell carcinoma, ET-1 endothelin-1, ETAR receptor A of endothelin-1, ETBR receptor B of endothelin-1, DFS disease-free survival, RFS recurrence-free survival, PFS progression-free survival, OS overall survival
patient cohorts revealed higher levels of ET-1 in muscle-invasive bladder cancer, which are associated with higher incidence of metastasis, and that higher levels of ET-1 are also correlated with decreased disease-specific survival. Consistent with its proinflammatory activity, it was found that tumor-derived ET-1 acts through ETAR to enhance migration and invasion of both cancer cells and macrophages and induces expression of inflammatory cytokines and proteases [19]. Using human and mouse cancer cell lines depleted of ET-1 and pharmacologic blockade of endothelin receptors in lung metastasis models, it was observed that tumor ET-1 expression and ETAR activity are indispensable for metastatic lung colonization [19]. This process is preceded by and dependent on macrophage infiltration of the lung [19]. Contrariwise, tumor ET-1 expression and ETAR activity appeared less important in established primary or metastatic tumor growth [19].

Limitations of our study are the small number of patients and the relatively short duration of follow-up. On the other hand, the strengths of the study are its prospective nature and the homogenous features of the included patients.

Muscle-invasive bladder cancer is a lethal disease that must be aggressively treated, taking into account that 15 % of patients with transitional cell carcinoma will die within 2 years if untreated [4]. In addition, approximately 50 % of patients relapse after RC, depending on the pathological stage of tumor and lymph nodes [1]. Therefore, a biomarker that could help us distinguish the NMMIBC patients who are at greater risk for disease progression and death would be undoubtedly useful in making decisions in our daily clinical practice. Moreover, such a biomarker could also serve as a guide for novel molecular therapies which target ET axis (e.g., atrasentan). Overexpression of ET-1 in NMMIBC may be a potential biomarker of unfavorable prognosis in these particular patients. However, more and larger scale studies are required to clarify the exact importance of ET-1 expression in NMMIBC.

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References


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Q1. Please check authors and their affiliations if presented correctly.