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Association of aromatase with bladder cancer stage and long-term survival: new insights into the hormonal paradigm in bladder cancer

Daniel P. Nguyen[a,b], Padraic O'Malley[a], Bashir Al Hussein Al Awamlh[a], Marc-Alain Furrer[b], Nigel P. Mongan[c], Brian D. Robinson[d], Gerald J. Wang[e], Douglas S. Scherr[a]

[a] Department of Urology, New York Presbyterian Hospital-Weill Cornell Medical College, New York, NY, United States
[b] Department of Urology, Bern University Hospital, Bern, Switzerland
[c] Department of Pharmacology, Weill Cornell Medical College, New York, NY, United States
[d] Department of Pathology, New York Presbyterian Hospital-Weill Cornell Medical College, New York, NY, United States
[e] Department of Urology, New York Hospital Queens, Flushing, NY, United States

Corresponding author: Daniel Nguyen, MD
Department of Urology
University Hospital Bern
CH - 3010 Bern
Switzerland
Phone number: +41 31 632 36 21
Fax number: +41 31 632 21 80
E-mail address: danielphatnguyen@hotmail.com

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Author contributions:

Study concept and design: Nguyen, Scherr.

Acquisition of data: Nguyen, O'Malley, Al Hussein Al Awamlh, Robinson, Wang.

Analysis and interpretation of data: Nguyen, O'Malley, Al Hussein Al Awamlh, Furrer, Mongan, Robinson, Wang.

Drafting of the manuscript: Nguyen, O'Malley.

Critical revision of the manuscript for important intellectual content: Al Hussein Al Awamlh, Furrer, Mongan, Robinson, Wang, Scherr.

Abstract

Objectives: To investigate the expression of aromatase and estrogen receptor (ER) β in bladder cancer and its association with pathological variables and survival outcomes.

Methods: Bladder cancer specimens from 40 patients were evaluated. Immunohistochemistry was performed using antibodies for aromatase and ERβ. Expression levels of each protein was determined. Descriptive statistics and univariate analyses assessed the association of these markers with pathologic variables and survival outcomes.

Results: Aromatase expression was significantly associated with tumor stage; muscle-invasive disease was found in 15/19 (79%) patients with positive staining and in 7/18 (39%) patients with negative staining (p=0.02). Node-positive disease was found in 8/19 (42%) patients with positive staining and 1/18 (6%) patients with negative staining (p=0.01). After a median follow-up of 112 months, Cox regression analysis demonstrated that aromatase expression was associated with a more than 2-fold risk of cancer recurrence (hazard ratio [HR] 2.37, confidence interval [CI] 0.92-6.08, p=0.07) and an almost 4-fold higher risk of cancer-specific death (HR 3.66, 95% CI 1.19-12.06, p=0.02). Muscle-invasive disease was found in 15/18 (83%) ERβ-positive specimens and 4/12 (33%) ERβ-negative specimens (p=0.0009). Hierarchical clustering analysis demonstrated a four-fold up-regulation of ERβ gene expression in tumor versus adjacent, non-tumor urothelium ($p < 0.05$). However, no significant association with survival outcomes was found.

Conclusions: Aromatase expression in bladder cancer is associated with advanced tumor stage and poorer survival outcomes. ERβ is upregulated in malignant tissue and its expression is associated with muscle-invasive disease. These findings strengthen the hormonal paradigm in bladder cancer.

Key words: aromatase, bladder cancer, estrogen receptor beta, hormones, survival

1. Introduction

Men are three to four times more likely to develop bladder cancer (BCa) [1], while women present with more advanced disease [2] and worse survival rates independent of stage [3]. Differential exposure to tobacco and occupational carcinogens has been postulated to explain the gender-related disparity in incidence. However, the difference persisted in a population-based analysis that controlled for these factors [4]. Genetic and anatomical factors, referral patterns for hematuria, and differences in treatment practices have also been advanced as contributors of differences in outcome between men and women with BCa [5].

More recently, hormonal factors have been postulated to explain the differential behavior of BCa between genders. The incidence of spontaneous and chemically induced bladder tumors in animals is greater in males than females and decreases with androgen deprivation [6–8]. The androgen receptor (AR) is expressed in human urothelium, and a progressive loss of expression with increasing pathologic stage of BCa has been reported [8–11].

Against this background, some data suggest that estrogen receptor-β (ERβ), the main subtype of ER in the bladder urothelium [12], may play a role in bladder carcinogenesis [12–14]. However, the available evidence on the biological and prognostic significance of ERβ is inconsistent [10–12,15,16]. To further expand on the subject, in this study we hypothesized that the enzyme aromatase, which is responsible for estrogen synthesis from androgen precursors [17], may be a component of the hormonal paradigm in BCa. This has not been previously evaluated. Specifically, we assessed the association between aromatase and

ERβ expression in BCa with tumor pathology and long-term survival outcomes. In addition, we compared ERβ gene expression in matched tumor and adjacent, non-tumor urothelium using DNA microarray technology.

2. Methods

2.1 Patients

Upon study approval by our Institutional Review Board, we evaluated tumor specimens from patients treated for urothelial carcinoma of the bladder from June 2002 to April 2005. A total of 40 patients with median age of 67 yrs (interquartile range [IQR] 59 to 73) were included, of which 27 (68%) were men and 13 (32%) were women. Tissue was collected at the time of transurethral resection (n = 14, 35%) or radical cystectomy (n = 26, 65%). Tumors were classified according to the 2002 TNM classification system [18] and graded using the 1998 World Health Organization/International Society of Urological Pathology consensus classification [19]. The study investigators complied with the provisions of the Declaration of Helsinki and its subsequent modifications.

2.2 Immunohistochemistry

Immunohistochemistry for aromatase expression was performed on paraffin tissue sections with a monoclonal mouse anti-aromatase antibody (Serotec, Oxford, UK). Tissue localization of the ERβ protein was performed on paraffin tissue sections using a polyclonal rabbit anti-ERβ antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Staining was performed on the automated

immunostainer TechMate^{500} (Ventana Medical Systems, Tucson, AZ). Briefly, formalin-fixed, paraffin-embedded tissue blocks were sectioned, deparaffinized, and rehydrated in a graded series of ethanol. Endogenous peroxidase activity was blocked with hydrogen peroxide in methanol. For aromatase, antigen retrieval was performed by heat with Antigen Unmasking Solution (Vector Laboratories, Burlingame, CA) in a pressure cooker for 2 min. Nonspecific binding was blocked with 5% horse serum. Slides were then incubated with the primary mouse anti-aromatase antibody overnight at 4°C. Sections were then incubated with the secondary biotinylated affinity-purified horse anti-mouse IgG and treated with an avidin-peroxidase conjugated complex. Color was developed with 3,3'-diaminobenzidine substrate for 10 min. For ERβ, antigen retrieval was performed in an autoclave using DAKO Target Retrieval Solution (DakoCytomation, Carpinteria, CA). Slides were incubated with the primary rabbit anti-ERβ antibody for 1 hr and with EnVision Plus-labeled Polymer-HRP anti-rabbit antibody (DakoCytomation) for 30 min. The peroxidase reaction was developed using DakoCytomation Liquid DAB plus Substrate Chromogen System (DakoCytomation). For both aromatase and ERβ, sections were counterstained with hematoxylin, dehydrated, and mounted with Cytoseal-XYL (Richard Allan Scientific, Kalamazoo, MI).

Negative controls were treated identically, except that mouse IgG was used instead of primary antibody for aromatase, and rabbit IgG was used instead of primary antibody for ERβ. Negative controls for both aromatase and ERβ showed an absence of staining. For aromatase positive control, formalin-fixed,

paraffin-embedded placental tissue was used. For ERβ, breast tissue was used. Immunohistochemistry for both aromatase and ERβ was not performed on all patients because in some cases, not enough tissue was available for research purposes.

2.3 Data analysis and outcome measures

Aromatase and ERβ expression were scored semiquantitatively by a single pathologist using standard light microscopy. A minimum of 500 cells from each tumor was evaluated. For aromatase, distinct, brown cytoplasmic staining, either granular or homogenous, was considered positive. For ERβ, distinct, brown nuclear staining was considered a positive result. The percentage of cells staining positive was recorded. Results were categorized as negative (<10% cell staining) or positive (>10% cell staining) [12]. Immunohistochemical analysis and pathologic evaluation were performed independently in a blinded fashion.

Clinical data were reviewed retrospectively from a prospectively maintained database. Our follow-up protocol comprised history, physical examination, urine cytology and laboratory measurements every 3 to 4 months the first year, semi-annually for the second year, and annually thereafter. Additionally office cystoscopy for patients who underwent transurethral resection only. Diagnostic imaging was performed at least annually or when clinically indicated. Based on clinical and radiologic findings, cancer recurrence was defined as development of local recurrence after cystectomy, diagnosis of a new tumor after transurethral resection, or evidence of metastases after any

intervention. For patients who died during the duration of the study, the cause of death was determined through review of medical records or death certificates.

2.4 Gene Expression Profiling

Gene expression analysis was performed in matched tumor and adjacent, non-tumor bladder specimens from seven patients (14 specimens), of which five were men and two were women. Specimens were compared using DNA microarray technology as described previously [20]. Briefly, bladder specimens were placed in the embedding medium Tissue-Tek (Sakura Finetek, USA) and stored at –70°C. RNA extraction was obtained using TRIzol reagent (Invitrogen, Carlsbad, CA) and Rneasy clean-up (Qiagen, Chatsworth, CA). RNA processing and hybridization protocols, as recommended by Affymetrix (Santa Clara, CA), were followed and are described in the Genechip® Expression Analysis Technical Manual. RNA samples were fragmented randomly to approximately 200 bp. Each fragmented RNA sample was hybridized to the Affymetrix Human U133 A microarrays set. The microarray was washed, stained, and amplified according to the Affymetrix protocol.

2.5 Statistical analyses

The Chi-square test (or Fisher's exact test) and Mann-Whitney U test were used to compare clinicopathological variables of patients according to expression of aromatase and ERβ. The Kaplan-Meier method was used to illustrate recurrence-free and cancer-specific survival probabilities stratified by expression of aromatase and ERβ, and statistical significance was computed with the log-

rank test. Univariate Cox regression models were used to evaluate whether expression of aromatase or ERβ was associated with disease recurrence or cancer-specific death. The relatively small sample size and number of events precluded multivariable analyses. All p-values are two-sided with statistical significance evaluated at the 0.05 alpha level. All analyses were performed using SPSS Version 22.0 (IBM, Armonk, NY, USA).

For gene expression profiling, data normalization, log transformation, statistical analysis and pattern study were performed using GeneSpring™ v 6.1 software (Silicon Genetics, Redwood City, CA). Statistical comparison between tumor and adjacent, non-tumor urothelium was performed using the Welch t-test with log transformed data. A two-way hierarchical clustering by distance measure was used to group genes that were differentially expressed between tumor and non-tumor tissue.

3. Results

3.1 Aromatase expression

Aromatase immunoreactivity was evaluated in 37 of the 40 patients and was found primarily in the cytoplasm (Fig. 1A,C,E). Table 1 shows the patients' clinicopathological characteristics and their association with aromatase staining. Of the 37 patients, 19 (51%) had some degree of staining for aromatase expression. There was no difference in aromatase expression between females and males, and between low- and high-grade tumors. However, aromatase

expression was significantly associated with advanced stage. Muscle-invasive disease was found in 15/19 (79%) patients with positive staining vs. 7/18 (39%) patients with negative staining (p=0.02). Furthermore, aromatase expression was significantly associated with node-positive disease (8/19 [42%] patients with positive staining vs. 1/18 [6%] patients with negative staining, p=0.01).

After a median follow-up of 112 months for living patients (IQR 50-123), 19 (51%) patients experienced disease recurrence and 15 (41%) patients died of disease. The Kaplan-Meier curves are shown in Fig.S1. Patients with aromatase-expressing tumors had greater probability of cancer recurrence (log rank p=0.057) and cancer-specific death (log-rank p = 0.02). Univariate Cox regression analysis demonstrated that aromatase expression was associated with a more than 2-fold risk of cancer recurrence, although this did not reach conventional levels of significance (hazard ratio [HR] 2.37, confidence interval [CI] 0.92-6.08, p=0.07) (Table 2). Furthermore, aromatase expression was significantly associated with an almost 4-fold higher risk of cancer-specific death (HR 3.66, 95% CI 1.19-12.06, p=0.02).

3.2 ERβ expression

We evaluated 30 of 40 primary urothelial tumors for ERβ expression. ERβ immunoreactivity was localized to the nuclei, with weak staining evident in the cytoplasm (Fig. 1 B,D,F). Of the 30 patients, 18 (60%) had some degree of staining for ERβ expression (Table 3). Muscle-invasive disease was found in 15/18 (83%) ERβ-positive specimens vs. 4/12 (33%) ERβ-negative specimens

(p=0.0009). There were no association between ERβ expression and tumor grade or nodal stage.

After a median follow-up of 89 months for living patients (IQR 45-121), 17 of 30 (57%) patients experienced disease recurrence and 13 (43%) patients died of disease. The Kaplan-Meier curves are shown in Fig.S2. ERβ expression was not significantly associated with recurrence-free survival (log-rank p=0.4) or cancer-specific survival (p=0.2). Univariate Cox regression analysis showed that ERβ expression was associated with a 1.5.fold higher risk of cancer recurrence, and a 1.9-higher risk of cancer-specific death, although this did not reach statistical significance (Table 4).

3.3 DNA Microarray Analysis

Hierarchical clustering analysis demonstrated distinct gene expression patterns in tumor and adjacent, non-tumor urothelium from seven patients (Fig. 2). We tested 22,283 genes, of which 496 demonstrated a significant difference between tumor and non-tumor urothelium. ERβ gene expression demonstrated a four-fold up-regulation in tumor versus adjacent, non-tumor urothelium ($p < 0.05$).

4. Discussion

It is unknown whether a molecular basis for the gender difference in BCa presentation [2] and behavior [3] exists. Previous research focused mainly on the AR [8–10,21]. Recent evidence suggested that the expression of ERβ, which is the

predominant receptor in bladder urothelium [12], may represent another aspect of hormone-related bladder carcinogenesis [10–14,16]. We extended these studies by demonstrating that the expression of aromatase is associated with advanced pathologic tumor classification and nodal disease. Accordingly, with a median follow-up of almost 10 yrs aromatase expression was associated with increased risk of recurrence and cancer-specific death.

Aromatase is responsible for estrogen synthesis from androgen precursors [17], and its expression in normal human bladder tissue has been previously suggested [22,23]. Previous studies evaluating the role of AR in BCa generated conflicting results. While some authors reported loss of AR protein expression with increased stage and grade [8–10], suggesting a role for AR in the early stages of BCa, others failed to demonstrate an association [21]. It could be postulated that it is not AR that is of importance in hormone-related BCa. We anticipated that the aromatase/ERβ concept may represent another aspect of the hormonal paradigm in BCa. The present data confirmed that ERβ is associated with muscle-invasive tumors [11,12]. In addition, our study is the first to document that ERβ expression at the gene level is significantly upregulated in tumor vs. adjacent, non-tumor urothelium. These findings suggest that ERβ may have a promoting role during tumorigenesis. Unfortunately, despite our long follow-up the sample size chosen for the study was likely too small to detect an association between ERβ expression and survival outcomes. Tuygun et al, evaluating specimens from 139 patients with BCa, found no association between ERβ expression levels and tumor grade and stage. However, only 33 (23%) patients

had T2 classification, and none had > T2 disease. Nevertheless, the authors could show that lower ERβ-expressing tumors had significantly better progression-free survival [10]. Along these lines, Kauffman et al [16] demonstrated an association between high ERβ expression and poorer recurrence-free and overall survival outcomes independently of stage with short follow-up following radical cystectomy (median 21 months). Miyamoto et al [11] further reported that ERβ expression was associated with BCa progression in all tumors and cancer-specific mortality in muscle-invasive disease.

Our results are consistent with experimental studies suggesting a role for estrogens in BCa. Shen et al [12] demonstrated that administration of estradiol stimulated BCa cell growth *in vitro*. Supportively, other authors showed that the selective ER modulator raloxifene inhibited tumor growth *in vitro* and *in vivo* [24,25]. Estrogen synthesized by aromatase from testosterone in extragonadal tissues is locally active in a paracrine and intracrine fashion [17]. Thus, aromatase action can generate high local tissue concentrations of estrogen while circulating estrogen level remain unaffected [17]. It has been documented that estrogen concentrations in breast tumors from post-menopausal patients are at least 20-fold greater than serum levels [26]. Further studies in breast cancer suggested that local estrogen production by aromatase is increased by overexpression of factors derived from the tumor stromal environment [27]. Our hypothesis is also supported by a previous study that demonstrated 5-alpha-reductase (5αR) expression in normal urothelium and urothelial carcinoma [28]. In this study, 5αR expression was found to be lower in cases of high-grade and high-stage urothelial carcinoma.

Since 5αR metabolizes testosterone to dihydrotestosterone, a picture is emerging in which biologically aggressive BCa occurs in the setting of low 5αR and high aromatase expression, leading to elevated local estrogen levels (Fig. 3). Further studies investigating local tissue levels of androgens and estrogens in both normal and tumor urothelium in men and women will shed light on this issue.

Thus, locally increased estrogen could activate one or several pathways that modulate tumor behavior. Glucuronidation catalyzed by uridine diphosphate-glucuronosyltransferases (UGTs) represents a pathway of elimination of estrogens and androgens and, importantly of detoxification of carcinogens in BCa [29]. It was recently shown that androgen-dependent activation of the AR reduces the activity of UGTs located in the bladder urothelium, and that UGTs are upregulated in AR knockout mice [30]. These findings suggested BCa promotion through hormonal regulation of UGTs. They provide a background for similar experiments using anti-estrogens and aromatase inhibitors in *in vitro* and *in vivo* models of urothelial carcinoma.

Our study has notable limitations. Due to the relatively small sample size, we were unable to perform multivariable analysis to determine the independent predictive value of aromatase on survival outcomes. As mentioned above, insufficient number of patients may also explain the lack of an association between ERβ and survival. Our analysis is also limited by the inherent reliability of immunohistochemistry techniques such as choice of antibody, technical procedures and interpretation criteria. These limitations notwithstanding, this

study is the first to (1) evaluate aromatase in BCa and (2) show that ERβ gene expression is significantly upregulated in tumor vs. adjacent, non-tumor urothelium. Our results present an entirely new concept and add another piece to the puzzle; they thus indicate that additional investigations into the potential role of estrogens in BCa are warranted.

In conclusion, aromatase expression in the urothelium of patients with BCa is associated with muscle-invasive disease, nodal metastases and worse survival outcomes. In addition, ERβ expression is associated with higher tumor stage. The expression of these proteins in BCa may be a marker of estrogen action and further suggest a potential role for hormonal regulation in this disease.

Conflicts of interest: Daniel P. Nguyen is a research fellow and is supported by research grants from the Nuovo-Soldati, the Arnold U. und Susanne Huggenberger-Bischoff, the Bangerter Foundations and the Swiss Urological Association (Switzerland).

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6. Figure legends

Fig 1. Immunohistochemical staining of aromatase and ERβ from primary bladder tumors. A, aromatase positive control with placental tissue showing intense cytoplasmic staining. B, ERβ positive control with breast tissue showing dense nuclear staining. C, high-grade T4 lesion showing diffuse, granular cytoplasmic staining for aromatase. D, high-grade T4 lesion demonstrating intense nuclear staining for ERβ. E, high-grade T1 tumor with absent aromatase staining. F, low-grade Ta lesion demonstrating no ERβ expression.

Fig 2. Hierarchical clustering analysis demonstrating a four-fold up-regulation of estrogen receptor β gene expression in tumor versus non-tumor urothelium ($p < 0.05$). Red color indicates up-regulation of gene expression, green indicates down-regulation.

Fig 3. Postulated hormonal paradigm in bladder cancer.

7. List of supporting information

Supporting Fig 1. Kaplan-Meier estimates of (A) recurrence-free survival and (B) cancer-specific survival probabilities according to aromatase staining in 37 patients with bladder cancer. The dashed line indicates positive staining for aromatase expression, the solid line indicates negative staining for aromatase expression.

Supporting Fig 2. Kaplan-Meier estimates of (A) recurrence-free survival and (B) cancer-specific survival probabilities according to estrogen receptor β staining in 30 patients with bladder cancer. The dashed line indicates positive staining for estrogen receptor β expression, the solid line indicates negative staining for estrogen receptor β expression.

Table 1. Clinico-pathological characteristics of the patient population and correlation with aromatase staining (n=37).

		Negative for aromatase (n=18)	Positive for aromatase (n=19)	p value
Age, median (interquartile range)		64 (59-72)	67 (58-71)	>0.9
Female gender, n (%)		6 (33)	7 (37)	0.8
Tumor grade, n (%)	Low	6 (33)	4 (21)	0.4
	High	12 (67)	15 (79)	
Pathologic stage, n (%)	Ta/Tis	6 (33)	3 (16)	0.08 †
	T1	5 (28)	1 (5)	
	T2	3 (17)	5 (26)	
	T3	0	4 (21)	
	T4	4 (22)	6 (32)	
Muscle-invasive tumor	no	11 (61)	4 (21)	0.02
	yes	7 (39)	15 (79)	
Pathologic nodal stage, n (%)	Nx/No	17 (94)	11 (58)	0.01
	N+	1 (6)	8 (42)	

† Fischer's exact test; other p values calculated with the chi-Square test.

Table 2. Univariate Cox regression analyses predicting bladder cancer recurrence and cancer-specific death in 37 patients who had aromatase expression evaluated.

Characteristic		Cancer recurrence			Cancer-specific death		
		HR	95%CI	P value	HR	95%CI	P value
Age (continuous)		0.98	0.93-1.03	0.4	1.00	0.94-1.07	>0.9
Female gender		1.08	0.42-2.75	0.9	1.4	0.51-4.07	0.5
Aromatase expression		2.37	0.92-6.08	0.07	3.79	1.19-12.06	0.02
High-grade tumor	no	-	referent	0.6			0.056
	yes	1.27	0.46-3.53		7.23	0.95-55.11	
Muscle-invasive disease	no	-	referent	0.01	-	referent	0.04
	yes	4.37	1.43-13.34		73.19	1.12-4794.53	
Nodal stage	N0/Nx	-	referent	0.001	-	referent	0.007
	N+	5.50	1.97-15.37		4.39	1.51-12.78	

HR, hazard ratio.

Table 3. Clinico-pathological characteristics of the patient population and correlation with estrogen receptor β (ER β) staining (n=30).

		Negative for ER β (n=12)	Positive for ER β (n=18)	p value
Age, median (interquartile range)		71 (61-74)	67 (59-70)	>0.9
Female gender, n (%)		5 (42)	6 (33)	0.7
Tumor grade, n (%)	Low	4 (33)	2 (11)	0.2
	High	8 (67)	16 (89)	
Pathologic stage, n (%)	Ta/Tis	5 (42)	1 (6)	0.07 †
	T1	3 (25)	2 (11)	
	T2	2 (17)	4 (22)	
	T3	1 (8)	4 (22)	
	T4	1 (8)	7 (39)	
Muscle-invasive tumor	no	8 (67)	3 (17)	0.0009
	yes	4 (33)	15 (83)	
Pathologic nodal stage, n (%)	Nx/No	10 (83)	12 (67)	0.4
	N+	2 (17)	6 (33)	

† Fischer's exact test; other p values calculated with the chi-Square test.

Table 4. Univariate Cox regression analyses predicting bladder cancer recurrence and cancer-specific death in 30 patients who had estrogen receptor β evaluated.

Characteristic		Cancer recurrence			Cancer-specific death		
		HR	95%CI	P value	HR	95%CI	P value
Age (continuous)		0.98	0.93-1.04	0.5	1.00	0.94-1.07	0.9
Female gender		0.98	0.36-2.66	>0.9	1.26	0.41-3.87	0.7
Estrogen receptor β expression		1.49	0.57-3.91	0.4	1.94	0.64-5.84	0.2
High-grade tumor	no	-	referent	0.4	-	referent	0.2
	yes	1.66	0.47-5.88		33.57	0.18-6202.90	
Muscle-invasive disease	no	-	referent	0.03	-	referent	0.07
	yes	3.53	1.13-11.04		63.85	0.71-5751.83	
Nodal stage	N0/Nx	-	referent	0.002	-	referent	0.01
	N+	5.77	1.86-17.88		4.75	1.42-15.87	

HR, hazard ratio.

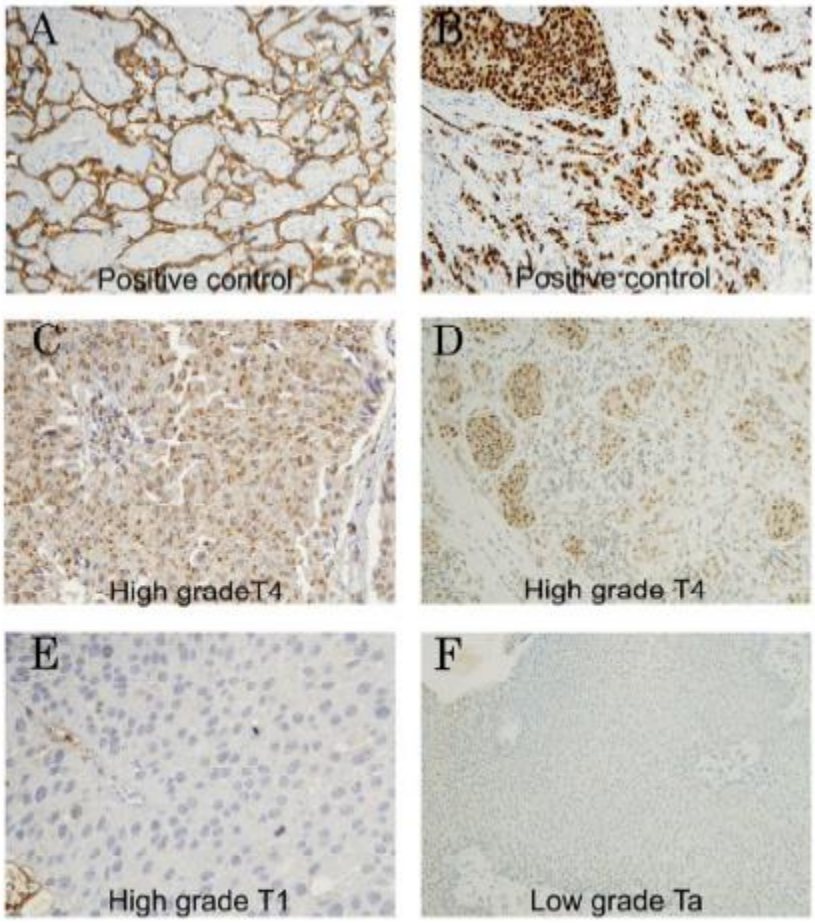


Figure 1. Immunohistochemical staining of aromatase and ERβ from primary bladder tumors. A, aromatase positive control with placental tissue showing intense cytoplasmic staining. B, ERβ positive control with breast tissue showing dense nuclear staining. C, high-grade T4 lesion showing diffuse, granular cytoplasmic staining for aromatase. D, high-grade T4 lesion demonstrating intense nuclear staining for ERβ. E, high-grade T1 tumor with absent aromatase staining. F, low-grade Ta lesion demonstrating no ERβ expression.
209x234mm (120 x 120 DPI)

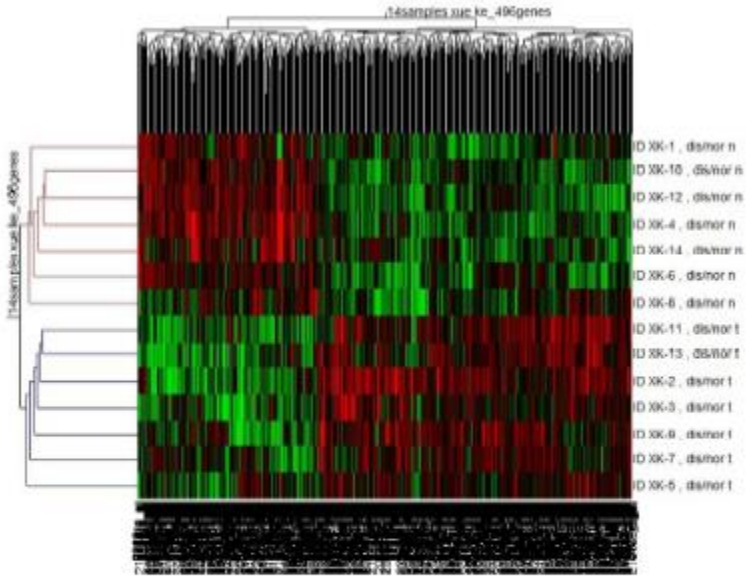


Figure 2. Hierarchical clustering analysis demonstrating a four-fold up-regulation of estrogen receptor β gene expression in tumor versus non-tumor urothelium ($p < 0.05$). Red color indicates up-regulation of gene expression, green indicates down-regulation.
254x190mm (96 x 96 DPI)

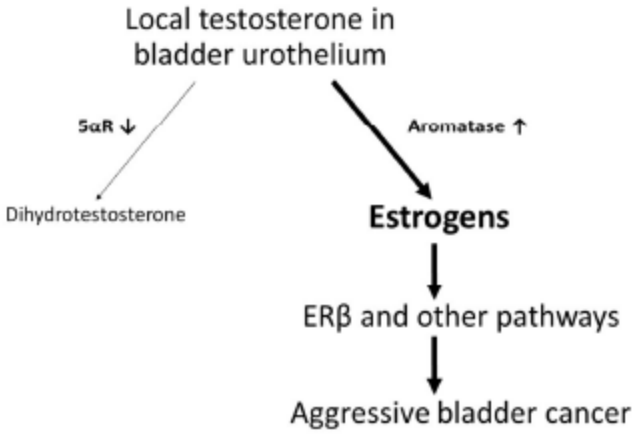
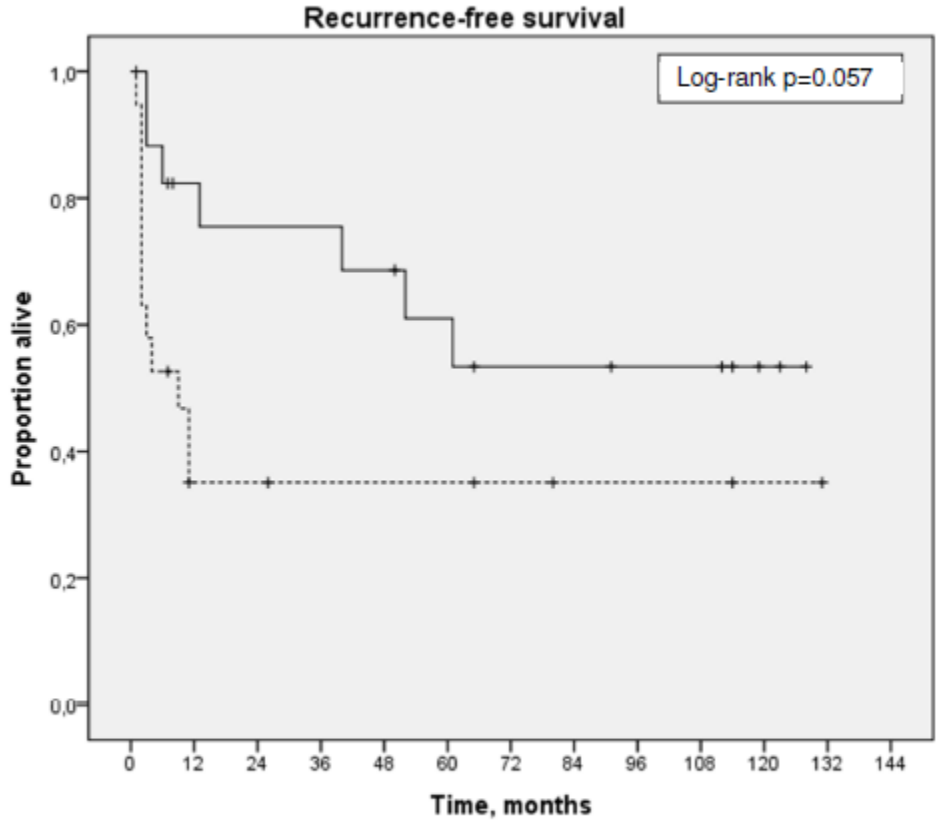


Figure 3. Postulated hormonal paradigm in bladder cancer.

338x190mm (96 x 96 DPI)

Supporting Figure 1

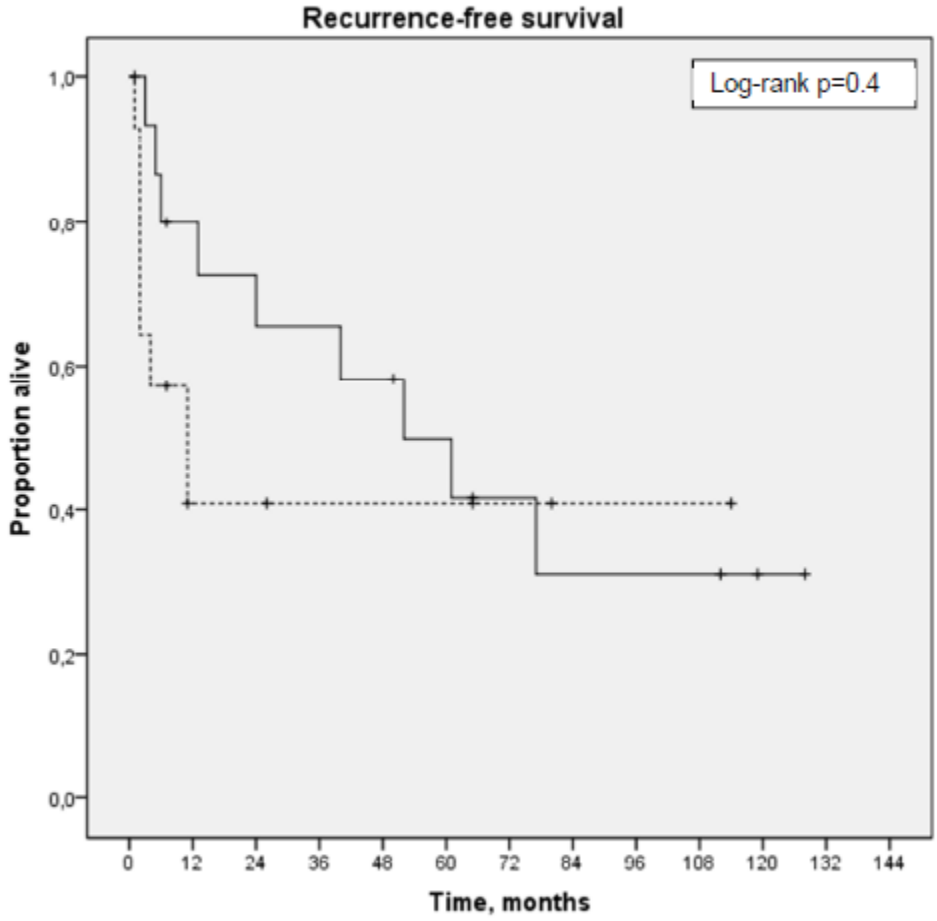
(A)



		Baseline	12 mths	24 mths	36 mths	48 mths	60 mths	72 mths	84 mths	96 mths	108 mths	120 mths
Number at risk	neg	18	12	11	11	10	8	6	6	5	5	2
	pos	19	5	5	4	4	4	3	2	2	2	1

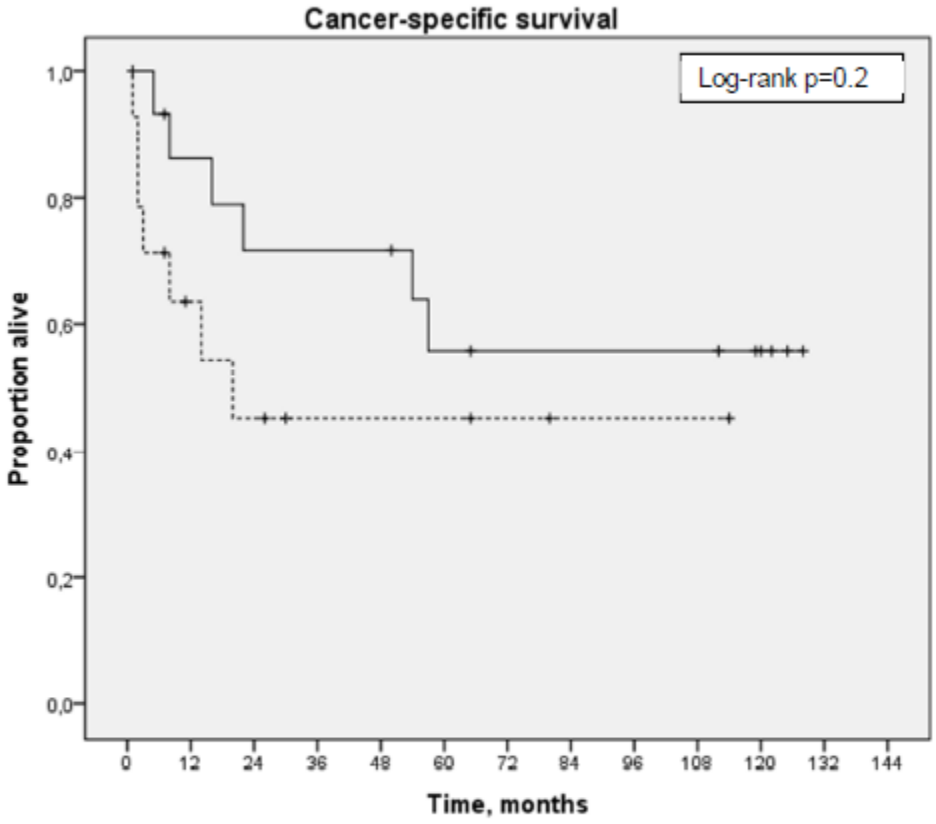
Supporting Figure 2

(A)



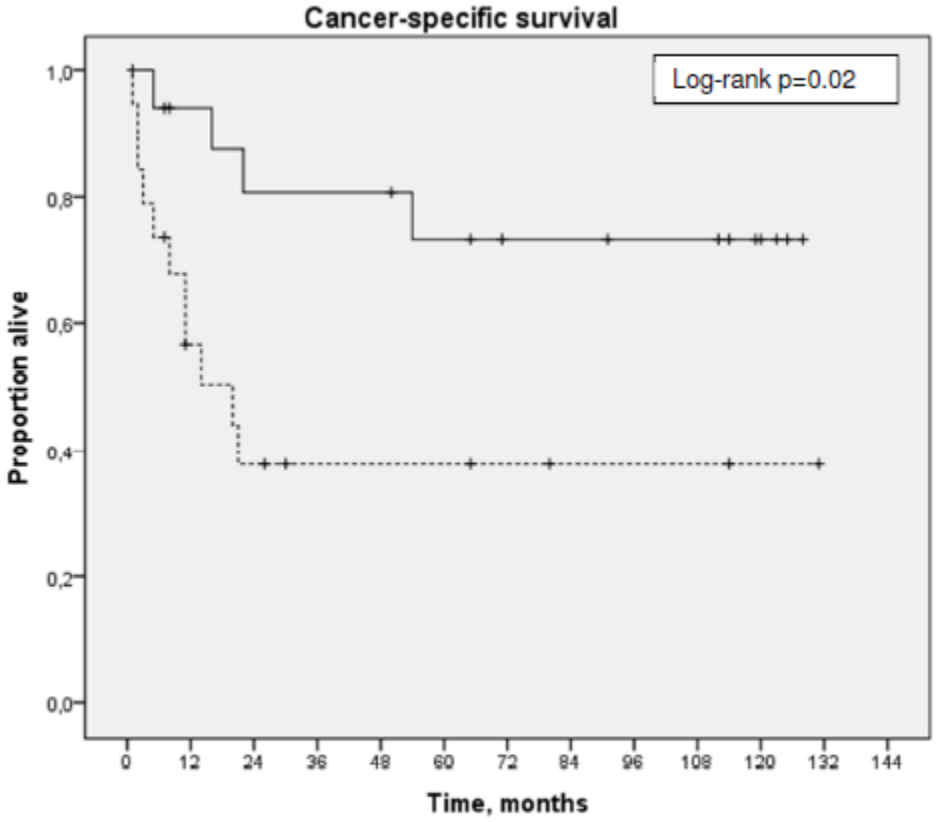
		Baseline	12 mths	24 mths	36 mths	48 mths	60 mths	72 mths	84 mths	96 mths	108 mths	120 mths
Number at risk	neg	16	11	10	9	8	6	4	3	3	3	1
	pos	14	4	4	3	3	3	2	1	1	1	-

(B)



		Baseline	12 mths	24 mths	36 mths	48 mths	60 mths	72 mths	84 mths	96 mths	108 mths	120 mths
Number at risk	neg	16	12	10	10	10	7	6	6	6	6	4
	pos	14	7	5	3	3	3	2	1	1	1	-

(B)



		Baseline	12 mths	24 mths	36 mths	48 mths	60 mths	72 mths	84 mths	96 mths	108 mths	120 mths
Number at risk	neg	18	14	12	12	12	10	8	8	7	7	4
	pos	19	9	6	4	4	4	3	2	2	2	1