Original Study

Association of Aromatase With Bladder Cancer Stage and Long-Term Survival: New Insights Into the Hormonal Paradigm in Bladder Cancer

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Abstract

Hormones may play a role in bladder cancer. We evaluated the impact of a novel marker, the enzyme aromatase, on characteristics of bladder cancer in 40 patients. We found that expression of this enzyme was associated with higher tumor stage as well as survival outcomes. Our preliminary results should encourage further investigations into the role of hormones in bladder cancer.

Background: Hormonal factors may play a role in bladder cancer (BCa). We investigated the expression of aromatase and estrogen receptor (ER)β and its association with pathological variables and survival outcomes. **Patients and Methods:** BCa specimens from 40 patients were evaluated. Immunohistochemistry was performed for aromatase and ERβ. 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 of 19 (79%) patients with positive staining and in 7 of 18 (39%) patients with negative staining ($P = .02$). Node-positive disease was found in 8 of 19 (42%) patients with positive staining and 1 of 18 (6%) patients with negative staining ($P = .01$). 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, 2.37; confidence interval, 0.92-6.08; $P = .07$) and an almost 4-fold higher risk of cancer-specific death (hazard ratio, 3.66; 95% confidence interval, 1.19-12.06; $P = .02$). Muscle-invasive disease was found in 15 of 18 (83%) ERβ-positive specimens and 4 of 12 (33%) ERβ-negative specimens ($P = .0009$). Hierarchical clustering analysis demonstrated a 4-fold up-regulation of ERβ gene expression in tumor versus adjacent, non-tumor urothelium ($P < .05$). However, no significant association with survival outcomes was found. **Conclusion:** Aromatase expression in BCa may be 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 provide further evidence for the hormonal paradigm in BCa.

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Introduction

Men are 3 to 4 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]

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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 (ER)β, 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 biologic 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.

Patients and Methods

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 years (interquartile range [IQR], 59-73 years) 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.

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 minutes. 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 minutes. 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 hour and with EnVision Plus-labeled Polymer-HRP anti-rabbit antibody (DakoCytomation) for 30 minutes. 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.

Data Analysis and Outcome Measures

Aromatase and ERβ expression were scored semiquantitatively by a single pathologist (B.D.R.) 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 was performed 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.

Gene Expression Profiling

Gene expression analysis was performed in matched tumor and adjacent, non-tumor bladder specimens from 7 patients (14 specimens), of which 5 were men and 2 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) 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