Role of Steroid Hormone Receptors in Formation and Progression of Bladder Carcinoma: A Case-Control Study

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Purpose: To compare the expression rate of sex steroid hormone receptors of estrogen (ER), progesterone (PR) and androgen (AR) in normal urothelium and urothelial bladder cancer (UBC) and to evaluate the possible associations of these receptors expression with cancer progression and patient's survival.

Materials and Methods: We evaluated the clinical data and tumor specimens of 120 patients with pathologically confirmed primary UBC with 132 normal healthy controls. Both patients and controls selected from list of subjects who have been referred to Sina Urology clinic, and had a minimum of one year follow-up duration. Data collected from medical cords. For evaluation of expression, immunohistochemistry was performed on paraffin-embedded tissue sections using a monoclonal antibody for androgen, estrogen and progesterone receptors. Presence of at least 10% positive cells defined as positive expression.

Results: None of the control subjects showed AR expression, while 22% of the patients were AR-positive. ER/PR expressions were observed in 4.2%/ and 2.5% of the cases and in 2.3% and 1.5% of the controls, respectively. A statistically significant correlation was found between AR expression and tumor stage and grade ($P < .001$). AR-positive patients showed a significantly poorer prognosis than AR-negative cases (log-rank test, $P = .02$, hazard ratio = 2.12; 95% confidence interval: 1.36-4.65).

Conclusion: AR expression was significantly associated with higher grade and poorly differentiated tumors with unfavorable outcome. AR expression test might be useful as a diagnostic tool for determining the malignancy and outcome of UBC patients.

Keywords: receptors; androgen; estrogen; progesterone; tumor markers; biological; urinary bladder neoplasms; mortality; neoplasm recurrence; gene expression regulation; survival rate.

INTRODUCTION

Urothelial bladder cancer (UBC) is one of the most common cancers, as it is ranked the 9th most common cancer worldwide.[1] UBC is responsible for the death of 130,000 people annually worldwide[2] and its incidence is 3 times higher in men than women.[1] This cancer ranked the 7th most common cancer in men and the 17th most common in women.[1,3] UBC is the fourth most incident cancer in the USA and it is the 5th most common cancer in Iran.[1,3,4] According to the 2008 report of Iran's National Cancer Registry, the incidence of bladder cancer was 13.03 in males and 3.32 in females per 100,000 population.[5]

Interestingly, in addition to dissimilarity in incidence, the tumor behavior is also different between two sexes. The female subjects tend to have more aggressive tumors with less favorable prognosis than male subjects[6-8]

Although the exact origin of this difference between the genders is unknown, it is assumed that part of this variation comes from higher exposure of male subjects to industrial, environmental and occupational chemicals and also tobacco use.[9,10] However higher incidence in males cannot be fully explained solely by above mentioned factors.[9] A study by Mir and colleagues showed that, even after adjustment for carcinogenic factors, sex-associated differences in UBC risk was still exist.[10] Some studies suggested the hormonal factors as a potential explanation for the gender disparity in the incidence and behavior of bladder cancer.[9] To support this hypothesis, some experimental animal studies showed that development of chemically induced UBC was less in female than in male animals.[10] Moreover, these an-

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imal studies generally demonstrate that hormonal manipulation changes the natural course of the tumors and that animals who have been treated with androgen inhibitors had better survival and more benign courses.[11,12] In agreement with these observations, some epidemiologic studies demonstrated that postmenopausal women have a greater risk for development of UBC as well as breast cancer than premenopausal women.[13,14] On the basis of these findings, sex steroid hormones and subsequently their receptors have been considered as a potential explanation for the different biologic behavior of UBC between men and women.[10]

Sex steroid hormones act by binding to their receptors including androgen receptors (ARs), estrogen receptors (ERs) and progesterone receptors (PRs) in target cells.[7] It is well known that steroid hormone receptors are expressed in normal bladder urothelium,[15,16] although physiological functions of these receptors in the bladder are not completely understood.[17,18] On the other hand, it has been reported that the expression of ARs, ERs and PRs play an essential role during development, growth and progression of several malignancies.[9,19-22] Shen and colleagues showed that ERs are expressed in human bladder cancers and their expressions augment with increase of the stage and grade of cancer. Their results also demonstrated a strong inhibitory effect of antiestrogen treatment on UBC growth in vitro.[23] Miyamoto and colleagues used N-butyl-N-(4-hydroxybutyl) nitrosamine to induce bladder cancer in both wild-type and ARs knockout male and female mice, and showed that 92% of the wild-type male, 42% of the wild-type female mice and none of the ARs knockout male and female mice developed tumors.[12]

Despite the importance of steroid hormone receptors in the initiation, progression and outcome of bladder cancer in experimental animal studies, its role in human UBC is still controversial.[24] In this regard, in present research we aimed to further clarify the role of sex steroid receptors (AR, PR and ER) expression in development and progression of UBC in human subjects and to investigate whether or not there is an association between grade of the cancer and sex steroid receptor expression. To our knowledge this is the first study from our region in which the role of these receptors in outcome and prognosis of patients with UBC has been addressed.

MATERIALS AND METHODS

Study Subjects and Tissue Specimens

This is a retrospective case-control study, in which 252 subjects including 120 pathologically confirmed UBC patients and 132 non-UBC individuals were recruited. The study protocol was approved by Medical Ethics Committee of Tehran University of Medical Sciences.

Participants were drawn from the list of patients who attended the Urology clinic at Sina Hospital, and had a bladder specimen through either transurethral resection of bladder tumor (TURBT), cystectomy or cystoscopy.

According to the sample size calculation and the literature review, there should be 133 subjects in each group, however due to nature of retrospective studies, we only could recruit 120 subjects in case group and 132 subjects in control group which provides a ratio of control/case of 1.1/1.

All of the subjects were between 18-85 years old. Individuals who had pathologically confirmed UBC were placed in patient group. Controls were subjects who did not have pathologically confirmed UBC and have been referred to urology clinic because of other causes such as hematuria, benign prostatic hyperplasia (BPH), urethral stricture, bladder stones, pelvic trauma or chronic cystitis. Patients who had concomitant or previous malignancies, or a history of hormonal therapy, chemotherapy and/or radiotherapy were excluded from the study. Two groups were sex matched. The anthropometric characteristics of patients and medical history were obtained from medical records. Definition for diabetes mellitus was use of anti-diabetic medication or at last two fast blood sugar levels of higher than 120 mg/dL. Definition for hypertension was use of anti-hypertensive medication or the average blood pressure in two readings before admission was > 140/90 mmHg. Patients were considered to have dyslipidemia when the serum total cholesterol level was ≥ 200 mg/dL, high density lipoprotein cholestrol was < 40 mg/dL, or triglyceride was ≥ 150 mg/dL. Patients who were taking lipid lowering medications were also categorized in this group. Smokers were those who had smoked at least 100 cigarettes in their lifetime, while those who consumed less than 100 cigarettes were defined as non-smokers.

Tissue specimens were examined by blinded pathologists to determine the grade, stage and other histopathological characteristics. Grading of samples was performed according to the World Health Organization/International Society of Urologic Pathology classification of urothelial neoplasia.[25] Pathological T stage (depth of invasion) was assessed according to American Joint Committee on Cancer Classification.[26]

Immunohistochemistry

Immunohistochemical (IHC) staining was performed on fixed paraffin embedded (3 μm) sections. Briefly, after deparaffinization in xylene and rehydration by graded concentrations of alcohol to distilled water, the specimens were washed with phosphate buffered saline (PBS). Endogenous peroxidase was blocked by 10-15 min incubation of specimens in 5% H_2O_2. The specimens were washed again with PBS and then antigen retrieval was performed in citrate buffer under 126°C and 2 atmosphere for 30 min. After washing with PBS, in order to decrease nonspecific antibody binding, protein blocking was carried out by incubation in protein block serum-free (code X0909, Dako, Glostrup, Denmark) for 10 min at room temperature. The sections were then incubated with the following primary antibodies: anti-estrogen receptor (clone 1D5, 1/50 dilution, Dako, Glostrup, Denmark), anti-androgen receptor (clone AR441, 1/50 dilution, Dako, Glostrup, Denmark) and anti-progesterone receptor (clone PgR 636, 1/50 dilution, Dako, Glostrup, Denmark). After washing with PBS, the slides were incubated with a dextran polymer reagent conjugated with peroxidase and secondary antibody (Envision+, Dako, Glostrup, Denmark) for staining detection for 1 h. We also used 3,3'-diaminobenzidine as a chromogen for color development and subsequently counterstained them with Carazzi's hematoxylin.

Table 1. Anthropometric characteristics and steroid hormone expression in study groups.*

Variables	Control (n = 132)	Case (n= 120)	*P* Value
Age (year), mean ± SD	60.4 ± 15.54	66.2 ± 12.10	.001
Male/female	111/21 (84/16)	105/15 (87.5/12.5)	.47
Smokers	67 (50.8)	73 (60.8)	.12
Hypertension	43 (32.6)	52 (43.3)	.09
Hyperlipidemia	7 (5.3)	15 (12.5)	.04
Diabetes mellitus	35 (26.5)	42 (35)	.17
Family history of cancer	1 (0.8)	5 (4.2)	.10
AR expression	0	26 (21.7)	<.0001
PR expression	3 (2.3)	5 (4.2)	.48
ER expression	2 (1.5)	3 (2.5)	.67

Abbreviations: AR, androgen receptor; PR, progesterone receptor; ER, estrogen receptor.
* Data are presented as no (%).

Immunostained sections were evaluated under a light microscope by two experienced pathologists in blinded fashion. The immunoreactivity was scored on a four-point scale as follows: negative (<10% of cells with nuclear staining), weak (10-50% of cells with nuclear staining), moderate (51-80% of cells with nuclear staining) and strong (> 80% of cells with nuclear staining).

Statistical Analysis

For statistical analyses, we used Statistical Package for the Social Science (SPSS Inc, Chicago, Illinois, USA) version 21. Data were presented as mean ± SD. Fisher's exact test, chi-square test and the independent sample *t*-test were used to investigate the association between steroid hormone receptors expression with pathological and clinical factors. In all statistical analyzes, *P* values less than .05 was considered as statistically significant.

Table 2. Tumor characteristics (n = 120).*

Variables	Values
Stage	
T1	61 (50.8)
T2	21 (17.5)
T3	18 (15)
T4	20 (16.7)
Grade	
Low	20 (16.7)
High	100 (83.3)
Recurrence	
Yes	57 (47.5)
No	63 (52.5)
Metastasis	
Yes	14 (11.7)
No	106 (88.3)
Mortality	
Yes	10 (8.3)
No	110 (91.7)
Chemotherapy	
Yes	44 (36.7)
No	76 (63.3)
Radiotherapy	
Yes	11 (9.2)
No	109 (90.8)

* Data are presented as no (%).

RESULTS

The mean age of the patients was 66.2 ± 12.10 years in case group and 60.4 ± 15.54 years in control group (*P* = .001). Over all the majority of subjects (85.7%, n = 216) were male. This rate in case and control groups was 87.5% (n = 105) and 84% (n = 111), respectively, however the ratio of male to female in case and control groups was not significantly different (*P* = .47). The mean follow-up period was 24.5 months (range, 12-60 months). **Figure 1** shows examples of AR/PR/ER positive cases and four-point scale of staining score is shown in **Figure 2**. Evaluation of steroid hormone receptor expression revealed that only AR expression is significantly different between case and control groups (*P* = .0001). Moreover, we could not find any significant difference between AR, PR and ER expression in two genders in both case and control groups. The anthropometric and clinical characteristics of patients as well as steroid hormone receptor expression are presented in **Table 1**. Clinical and demographic characteristics of patients with UBC are summarized in **Table 2**.

We also assessed the possible associations between steroid hormone receptors expression and tumor recurrence, tumor progression, tumor metastasis, tumor grades and stages, death because of UBC and family history of UBC. We found that only AR expression had a significant association with tumor stage (*P* < .001) and

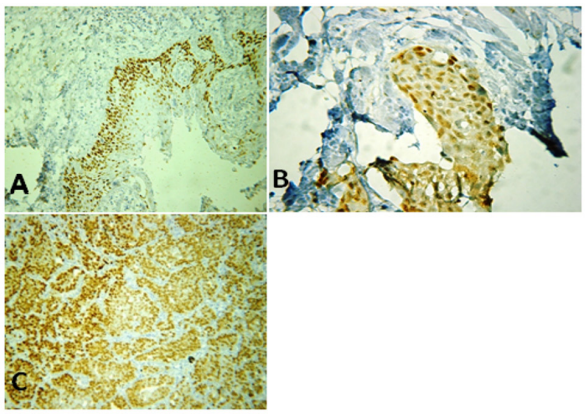

Figure 1. Immunohistochemical staining of steroid hormone receptors in primary bladder cancer. (A) Estrogen receptor, (B) progesterone receptor, (C) androgen receptor.

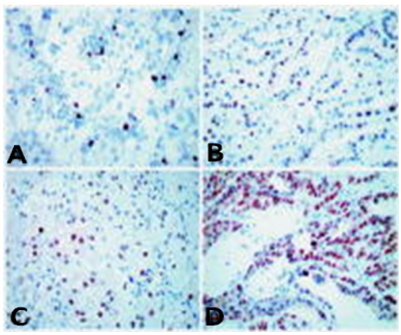

Figure 2. Staining score of androgen receptor in primary bladder cancer. (A) < 10% positive tumor cells, (B) 10-50% positive tumor cells, (C) 51-80% positive tumor cells, (D) > 80% positive tumor cells.

tumor grade ($P < .0001$). Moreover, the rate of AR expression was higher in patients with family history of UBC ($P = .04$). Interestingly, AR/PR-positive patients had a higher rate of metastasis in comparison to AR/PR-negative patients ($P < .05$). Also, AR-positive patients showed a poorer prognosis than AR-negative cases (log rank test, $P = .08$), while survival was not affected by PR/ER expression.

DISCUSSION

The incidence of bladder cancer is higher in men (about three times) than women.[1] It seems that this difference is primarily due to a difference in chemical exposure and smoking.[1,9] However, animal studies have shown that chemically and spontaneous bladder cancer development is significantly higher in male rats than females.[12] Epidemiological studies have also shown that the development of bladder cancer in postmenopausal women is more common than women who are premenopausal.[13] It has been shown that in bladder cancer cells with AR-positive, cell growth is promoted by androgens,[12,16,18] or in another study, it has been found that antiestrogens can inhibit urothelial carcinoma of the bladder in ER-positive bladder cancer cell lines.[23] In another study on breast cancer, PR and ER were considered as prognostic factors which play a role in the identification of patients who may benefit from hormonal therapy.[14]

It should be noted that previous studies that assess the relationship between AR/PR/ER expression and histopathological characteristics of the tumors have led to conflicting results.[7] So, the results of such studies cannot be clearly identified prognostic significance of AR/PR/ER expression in patients with bladder cancer. Variability seen in results of such studies could be due to differences in sample size, study methods or interpretation of the results.[7] Moreover, there is no sufficient or strong evidence to establish epidemiological links between steroid hormone receptors and observed gender differences in development of cancer.[24]

In the present study, we found that AR/PR/ER expressions were similar in both sexes with UBC. Similar results have also been reported in other studies.[24,27-29] In this study there is no correlation between AR/PR/ER expression and gender differences in subjects with UBC.

In a study by Kirkali and colleagues loss of AR expression in malignant bladder urothelium is reported. They concluded that AR did not have a direct role in malignant transformation.[30] Boorjian and colleagues reported that loss of AR expression might lead to invasive bladder cancer as they found a decreased AR expression in high stage tumors; also they observed AR expression in 53% of urothelial carcinoma and in 86% of normal urothelium cases.[27] Another study showed a significant decrease in the expression of AR in bladder cancer compared to nonneoplastic bladder specimens.[7] In present study AR was positive in 22% of the 120 patients with BC, but none of the 132 normal urotheliums showed AR-positivity as observed in the studies by Tuygun and colleagues,[24] and Ruizeveld de Winter and colleagues.[31] Birtle and colleagues studied AR expression in 17 cases of high grade transitional cell carcinoma (TCC) of the bladder. They showed that AR staining was negative in all areas of normal urothelium, although AR was positive in 9/17 (52%) cases.[32] Although, Zhuang and colleagues reported nuclear immunoreactivity of AR in 7/9 (77%) urinary bladder cancers, they failed to detect positive immunohistochemical staining in normal urinary bladder. They mentioned that AR expression can be used as a diagnostic marker.[17]

According to the available data, the prognostic role of the AR expression in bladder cancer is controversial. Tuygun and colleagues reported a significant decrease in AR expression in higher grades and invasive tumors, which is consistent with the findings of Boorjian and colleagues and Miyamoto and colleagues.[17,24,27] In contrast, Mir and colleagues in a study involving 472 patients, showed that AR-positivity was higher in muscle–invasive tumors (15%) compared to non-muscle invasive one (9%).[10] In another study with 33 superficial bladder cancers, authors reported that patients with high AR expression tended to have a higher recurrence rate, compared to patients with low AR expression.[12] In present study, we found a significant correlation between AR expression and high grade and high stage tumors ($P < .0001$ and $P < .001$, respectively). Also, the present study demonstrated a significantly higher rate of metastasis in AR-positive patients compared to AR-negative patients ($P = .009$). Moreover, relapse-free survival in AR-positive patients was lower than AR-negative patients (log-rank test, $P = .08$). Therefore AR could be used as a prognostic factor (hazard ratio: 2.12; 95% confidence interval: 1.36-4.65).

In this study, PR and ER were positive in 2.5% and 4.2% of the UBC specimens, respectively. Our results confirmed that PR/ER expression is not associated with aggressiveness of UBC. Similar to our findings, Bolenz and colleagues reported that PR expression cannot be a prognostic factor in patients with UBC. In their study PR was not expressed in any of the UBC specimens.[9] In a study by Basakci and colleagues ER was positive in 12.4% of the superficial TCC specimens. They concluded that ER does not play any direct role on the prognosis of superficial bladder TCC.[33] Also in our study, ER and PR expression do not have any direct roles in formation and progression of UBCs.

This study has some limitations. First, since this study was a retrospective one, some of the data were not avail-

able. Also, due to complex nature of the malignancies, there might be some confounders and effect modifiers that might interfere with the obtained results. We tried to control and limit the bias by using the blinded pathologist and including the control subjects from same population cohort. Finally two groups were not age matched.

CONCLUSION

We concluded that there was no significant difference in steroid hormone receptors expression between two sexes. Of studied steroid hormone receptors, only AR expression had significant association with stage and grade of bladder cancer. Based on study results, AR could be used as a prognostic factor in UBC.

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CONFLICT OF INTEREST

None declared.

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