Int J Clin Exp Pathol 2015;8(5):4427-4443
www.ijcep.com /ISSN:1936-2625/IJCEP0007226

Original Article

Alterations in ubiquitin ligase Siah-2 and its corepressor N-CoR after P-MAPA immunotherapy and anti-androgen therapy: new therapeutic opportunities for non-muscle invasive bladder cancer

Patrick Vianna Garcia[1], Letícia Montanholi Apolinário[1], Petra Karla Böckelmann[1], Iseu da Silva Nunes[2], Nelson Duran[2,3,4], Wagner José Fávaro[1,2]

[1]Department of Structural and Functional Biology, Laboratory of Urogenital Carcinogenesis and Immunotherapy, University of Campinas (UNICAMP), Campinas, SP, Brazil; [2]Farmabrasilis R&D Division, Campinas, SP, Brazil, [3]Institute of Chemistry, Biol Chem Lab., University of Campinas (UNICAMP), Campinas, SP, Brazil; [4]Institute of Chemistry, NanoBioss, University of Campinas (UNICAMP), Campinas, SP, Brazil

Received February 20, 2015; Accepted April 13, 2015; Epub May 1, 2015; Published May 15, 2015

Abstract: The present study describes the role of the ubiquitin ligase Siah-2 and corepressor N-CoR in controlling androgen receptor (AR) and estrogen receptors (ERα and ERβ) signaling in an appropriate animal model (Fischer 344 female rats) of non-muscle invasive bladder cancer (NMIBC), especially under conditions of anti-androgen therapy with flutamide. Furthermore, this study describes the mechanisms of a promising therapeutic alternative for NMIBC based on Protein aggregate magnesium-ammonium phospholinoleate-palmitoleate anhydride (P-MAPA) intravesical immunotherapy combined with flutamide, involving the interaction among steroid hormone receptors, their regulators and Toll-like receptors (TLRs). Our results demonstrated that increased Siah-2 and AR protein levels and decreased N-CoR, cytochrome P450 (CYP450) and estrogen receptors levels played a critical role in the urothelial carcinogenesis, probably leading to escape of urothelial cancer cells from immune system attack. P-MAPA immunotherapy led to distinct activation of innate immune system TLRs 2 and 4-mediated, resulting in increase of interferon signaling pathway, which was more effective in recovering the immunosuppressive tumor immune microenvironment and in recovering the bladder histology features than BCG (*Bacillus Calmette-Guerin*) treatments. The AR blockade therapy was important in the modulating of downstream molecules of TLR2 and TLR4 signaling pathway, decreasing the inflammatory cytokines signaling and enhancing the interferon signaling pathway when associated with P-MAPA. Taken together, the data obtained suggest that interferon signaling pathway activation and targeting AR and Siah-2 signals by P-MAPA intravesical immunotherapy alone and/ or in combination with AR blockade may provide novel therapeutic approaches for NMIBC.

Keywords: Bladder cancer, immunotherapy, P-MAPA, toll-like receptor, flutamide, Siah2, N-CoR, androgen receptor, estrogen receptor

Introduction

Bladder cancer (BC) is the second most common genitourinary malignancy and leads to considerable morbidity and mortality worldwide [1, 2]. In 2013, 10.510 men and 4.370 women died from BC in the USA, representing 18.9% and 24.4% of cases, respectively [3]. More than 70% of BC is superficial; however, 50% of non-muscle invasive bladder cancers (NMIBC) return within 4 years of treatment, and 11% progress to an invasive phenotype [4]. NMIBC is classified into 3 stages: pTis (flat carcinoma *in situ*), pTa (papillary carcinoma *in situ*) and pT1 (tumor invading mucosa or submucosa of the bladder wall) [5].

Estrogen receptors (ER), subtypes ERα and ERβ, have been implicated in bladder carcinogenesis because these receptors act as ligand-activated transcription factors [6, 7]. Although both ERα and ERβ have been identified in human bladder cells, ERβ is the predominant ER type [7, 8]. Some studies suggest a protec-

tive role of estrogens against prostate, breast and ovarian carcinogenesis [7, 9]. However, in BC, the expression of ERs and their co-regulators, including p300 and the nuclear receptor corepressor (N-CoR), has not been studied. N-CoR is a 270-kDa protein that contains distinct functional domains responsible for interaction with nuclear receptors and activation of de-acetyltransferase proteins, ultimately resulting in targeted repression of transcription [7, 10]. In the absence of a ligand, N-CoR binds to ER and mediates the transcriptional repression of ER target genes [7].

Previous investigators have shown that signaling mediated by androgen and its receptor (androgen receptor-AR) plays an important role in the genesis and development of BC, which may explain some of the differences between tumors in males and females [11]. Additionally, this observation may indicate a potential therapeutic target [12]. AR expression was verified in both normal bladder epithelium and bladder carcinomas from male and female patients [13]. However, AR expression and the exact functional mechanism of androgen/androgen receptor signaling in BC development have remained unclear to date. One important interaction of AR is with the ubiquitin/proteasome pathway, which regulated AR stability [14]. The ubiquitin/proteasome pathway consists of ubiquitin ligases that control the specificity and rate of protein degradation. The protein degradation occurs in the proteasome and requires three types of enzyme: ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2) and ubiquitin ligases (E3), which catalyze the binding of the small protein ubiquitin to lysines on a protein target by forming complexes with ubiquitin-conjugating enzymes, resulting in the directing of modified protein to the proteasome for degradation [14]. The Siah (seven in absentia homolog) proteins regulate ubiquitination-dependent degradation of multiple substrates, including N-CoR, β-catenin, TRAF2 and α-ketoglutarate dehydrogenase, resulting in resistance to apoptosis and affecting on mitochondrial function [15, 16].

Bacillus *Calmette-Guerin* (BCG) is considered the gold standard for the first line treatment of high grade flat carcinoma *in situ* and is used as prophylaxis and in the treatment of papillary carcinoma *in situ* (high grade) and tumor invading mucosa or submucosa of the bladder wall [17]. BCG evokes a complex and robust systemic immune response, comprising CD4+ T cells stimulation and inducing T helper type (Th) 1 immune response [18]. BCG antigens exerts a direct effect cell surfaces of urothelial, leading to a massive immune response characterized by the induction of cytokine and an influx of inflammatory cells into the bladder wall [18, 19]. In addition, many studies have demonstrated an important antiproliferative effect of BCG on urothelial tumor cells [20, 21]. However, undesirable side events related with BCG therapy are observed up to 90% of patients and range from cystitis and irritative voiding symptoms to major complications such as sepsis and death related to the treatment [22, 23].

Based on this background, compounds that activate the immune system, including vaccines, biological response modifiers and tumor environment modulators of steroid hormones, can be considered potential candidates for the development of new treatments of NMIBC aiming to obtain greater therapeutic effect combined with lower toxicity. In this context, toll-like receptors (TLRs) agonist compounds may represent a potential antitumor therapeutic approach, because TLRs are implicated in the pathogenesis of some tumors, including NMIBC [24]. TLRs are key elements of the innate immune system, and their signaling pathways consist of two pathways: the MyD88 (myeloid differentiation primary response 88)-dependent and MyD88-independent (or TRIF-dependent) pathways [25]. The MyD88-dependent pathway is common to all TLRs and activates NF-kB, resulting in the induction of inflammatory chemokines such as Tumor Necrosis Factor α (TNF-α) and interleukin-6 (IL-6). Conversely, the MyD88-independent pathway is peculiar to the TLR3 and TLR4 signaling pathways and activates Interferon (IFN) and Interferon Regulatory Factor 3 (IRF-3) [25].

As cited, immunotherapy using compounds that act as TLR agonists could be a valuable approach for the treatment of cancer, whether used alone or in combination with existing therapies. In this context, P-MAPA (developed by Farmabrasilis, a nonprofit research network) has emerged as a potential candidate for intravesical therapy for NMIBC. P-MAPA is a biological response modifier obtained by fermentation

from *Aspergillus oryzae* that demonstrates important antitumor effect in several animal models of cancer, including NMIBC [26, 27]. Recent advances of our research group in the understanding of its mechanism of action demonstrated that P-MAPA modulates TLR 2 and 4 in both infectious diseases and cancer [26, 27].

Here, we describe the role of the Siah-2 ubiquitin ligase and its corepressor N-CoR in controlling AR, ERα and ERβ signaling in an appropriate animal model of NMIBC, in particular under conditions of anti-androgen therapy with flutamide. Furthermore, we describe the possible mechanisms of a promising therapeutic alternative for NMIBC based on P-MAPA intravesical immunotherapy combined with flutamide, a strategy that involves the interaction of steroid hormone receptors and their regulators, Siah2 and N-CoR, with immune system receptors. BCG immunotherapy was used as positive control in this animal model of NMIBC.

Methods

Experimental proceedings

In this study it was used 35 female rats Fischer 344 strain, aged 7 weeks, weighing on average 150 grams, obtained from the University of Campinas (CEMIB/UNICAMP). For the experiments the protocol followed strictly the ethical principles in animal research (CEUA/IB/UNICAMP-protocol number: 3339-1). For the induction of NMIBC, 30 female rats were anesthetized with both 60 mg/Kg dose of ketamine 10% (Vibra® Roseira, São Paulo, Brazil) and 5 mg/Kg dose of xylazine 2% (Vibra® Roseira, São Paulo, Brazil) intraperitoneally (i.p.), and maintained in this state for 45 min to prevent spontaneous micturition. After, these animals received 1.5 mg/kg dose of *n-methyl-n-nitrosourea* (MNU; Sigma Chemical Co., St. Louis, MO, USA) dissolved in 0.30 mL of sodium citrate (1 M pH 6.0) intravesically (i.v.), via a 22-gauge angiocatheter, every other week for 8 weeks [27]. The other 5 animals (Control group) received a 0.30 mL dose of 0.9% physiological saline i.v. every other week for 8 weeks [27]. Two weeks after the last dose of MNU, all animals were submitted to cystography to evaluate the occurrence of tumor and divided into 7 groups (5 animals per group). The control group (Group 1) received a 0.3 mL dose of 0.9% physiological saline intravesically every other week for 6 weeks. The MNU group (Non-Muscle Invasive Bladder Cancer; Group 2) received the same treatment as Group 1. The MNU-BCG group (Group 3) received a 10^6 CFU (40 mg) dose of BCG intravesically every other week for 6 weeks. The MNU-P-MAPA group (Group 4) received 5 mg/kg dose of P-MAPA (Farmabrasilis, Campinas, SP, Brazil) intravesically every other week for 6 weeks. The MNU-flutamide group (Group 5) received a 10 mg/kg dose of flutamide (Sigma Chemical Co., St. Louis, USA) subcutaneously every other day for 6 weeks for blockade of the androgen receptor. The MNU-BCG-flutamide group (Group 6) received the same treatment as the Groups 3 and 5. The MNU-P-MAPA-flutamide group (Group 7) received the same treatment as Groups 4 and 5.

After 16 weeks of treatment, the animals were euthanized, and their urinary bladders were collected and processed for histopathological, immunological and Western Blotting analysis.

Histopathological analysis

Fragments of the urinary bladders were collected from 5 animals in each group and fixed in Bouin's solution for 12 hours. After fixation, the fragments were washed in 70% ethanol, followed by dehydration in an ascending series of alcohols. Subsequently, the fragments were diaphanized in xylene for 2 hours and embedded in plastic polymer (*Paraplast Plus*, ST. Louis, MO, USA). Then, the specimens were cut on a rotary microtome *Leica RM 2165* (Leica, Munich, Germany) with a thickness of 5 μm, stained with hematoxylin-eosin and photographed with a *Zeiss Axiophot* (Zeiss, Hamburg, Germany) photomicroscope. The urinary bladder lesions were diagnosed according to World Health Organization/International Society of Urological Pathology [5] by a senior uropathologist.

Immunodetection of Siah-2 and N-CoR

Fragments of the urinary bladders were collected from 5 animals in each group, the same used for histopathological analysis, were cut 6 μm thick sections for immunological analysis. The antigens were retrieved in microwave oven by boiling the sections in a 10 mM citrate buffer. After that, the sections were incubated in

0.3% H_2O_2 for 15 minutes to block endogenous peroxidase. Nonspecific binding was blocked by incubating the sections in a blocking solution for 1 hour at room temperature. Primary goat polyclonal antibody sc-5507 (N-14) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) specific for Siah-2 and goat polyclonal sc-1611 (N-19) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) specific for N-CoR were diluted in 1% BSA and applied to the sections overnight at 4ºC. Bound antibody was detected with an *Advance™ HRP kit* (Dako Cytomation Inc., USA). Secondary labeled polymer (Advance™ HRP kit) was applied for 40 min at room temperature. Peroxidase activity was detected using a diaminobenzidine chromogen mixture (Advance™ HRP kit). Sections were lightly counterstained with Harris' hematoxylin and photographed with a *Zeiss Axiophot* (Zeiss, Hamburg, Germany) photomicroscope.

To evaluate the intensity of antigen immunoreactivity, the percentage of positive-staining urothelial cells and/or lamina propria and muscularis layers cells was examined in ten fields for each antibody under high magnification (400×). The staining intensity was graded on a scale of 0-3, and expressed as 0 (no immunoreactivity), 0% positive urothelial cells; 1(weak immunoreactivity), 1-35% positive urothelial cells; 2 (moderate immunoreactivity), 36-70% positive urothelial cells; 3 (intense immunoreactivity), > 70% positive urothelial cells [21].

Western blotting analysis

Fragments of the urinary bladders were collected from 5 animals in each group, frozen in liquid nitrogen, weighed and homogenized in 50 µl/mg of *RIPA* lysis buffer (EMD Millipore Corporation, Billerica, MA, USA). The tissue homogenates were centrifuged, and a sample of each extract was used for protein quantification by Bradford's method. Aliquots containing 70 µg of protein were separated by SDS-PAGE on 10% or 12% polyacrylamide gels under reducing conditions. After electrophoresis, the proteins were transferred to *Hybond-ECL* nitrocellulose membranes (Amersham, Pharmacia Biotech, Arlington Heights, IL., USA). The membranes were blocked with TBS-T containing 1% BSA (bovine serum albumin) and incubated at 4°C overnight with primary rabbit polyclonal antibody 251110 (Abbiotec, San Diego, CA, USA) specific for TLR2, rabbit polyclonal ab2064 (Abcam, Cambridge, MA, USA) specific for MyD88, rabbit polyclonal ab7970 (Abcam, Cambridge, MA, USA) specific for NF-kB, rabbit polyclonal ab6671 (Abcam, Cambridge, MA, USA) specific for TNF-α, rabbit polyclonal antibody 251111 (Abbiotec, San Diego, CA, USA) specific for TLR4, rabbit polyclonal ab13810 (Abcam, Cambridge, MA, USA) specific for TRIF, rabbit polyclonal ab25950 (Abcam, Cambridge, MA, USA) specific for IRF3, mouse monoclonal 507802 (Biolegend, San Diego, CA, USA) specific for IFN-γ, rabbit polyclonal sc-816 (N-20) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) specific for AR, rabbit monoclonal 04-227 (E115) (EMD Millipore Corporation, Billerica, MA, USA) specific for ERα, rabbit polyclonal 06-629 (EMD Millipore Corporation, Billerica, MA, USA) specific for ERβ, goat polyclonal sc-5507 (N-14) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) specific for Siah-2, goat polyclonal sc-1611 (N-19) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) specific for N-CoR and rabbit polyclonal NBP1-19551 (Novus Biologicals, Littleton, CO, USA) specific for Cytochrome P450 (CYP50) diluted in 1% BSA. The membranes were then incubated for 2 h with rabbit or mouse secondary HRP-conjugated antibodies (diluted 1:3,000 in 1% BSA; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Peroxidase activity was detected by incubation with a diaminobenzidine chromogen (Sigma Chemical Co., St Louis, USA). Western blots were run in duplicate, and urinary bladder samples were pooled from 5 animals per group for each repetition. The semi-quantitative densitometry (IOD-Integrated Optical Density) analysis of bands was conducted using *NIH ImageJ 1.47v* software (*National Institute of Health, USA. Available in: http://rsb.info.nih.gov/ij/*), followed by statistical analysis. β-actin was used as endogenous positive controls for standardization of the readings of band staining intensity. The results were expressed as the mean ± standard deviation of the ratio of each band's intensity to β-actin band intensity.

Statistical analysis

For the statistical analysis, analysis of variance (ANOVA) was used followed by Tukey's test for comparison of means. All analyses were performed with a significance level of $P<0.01$. The

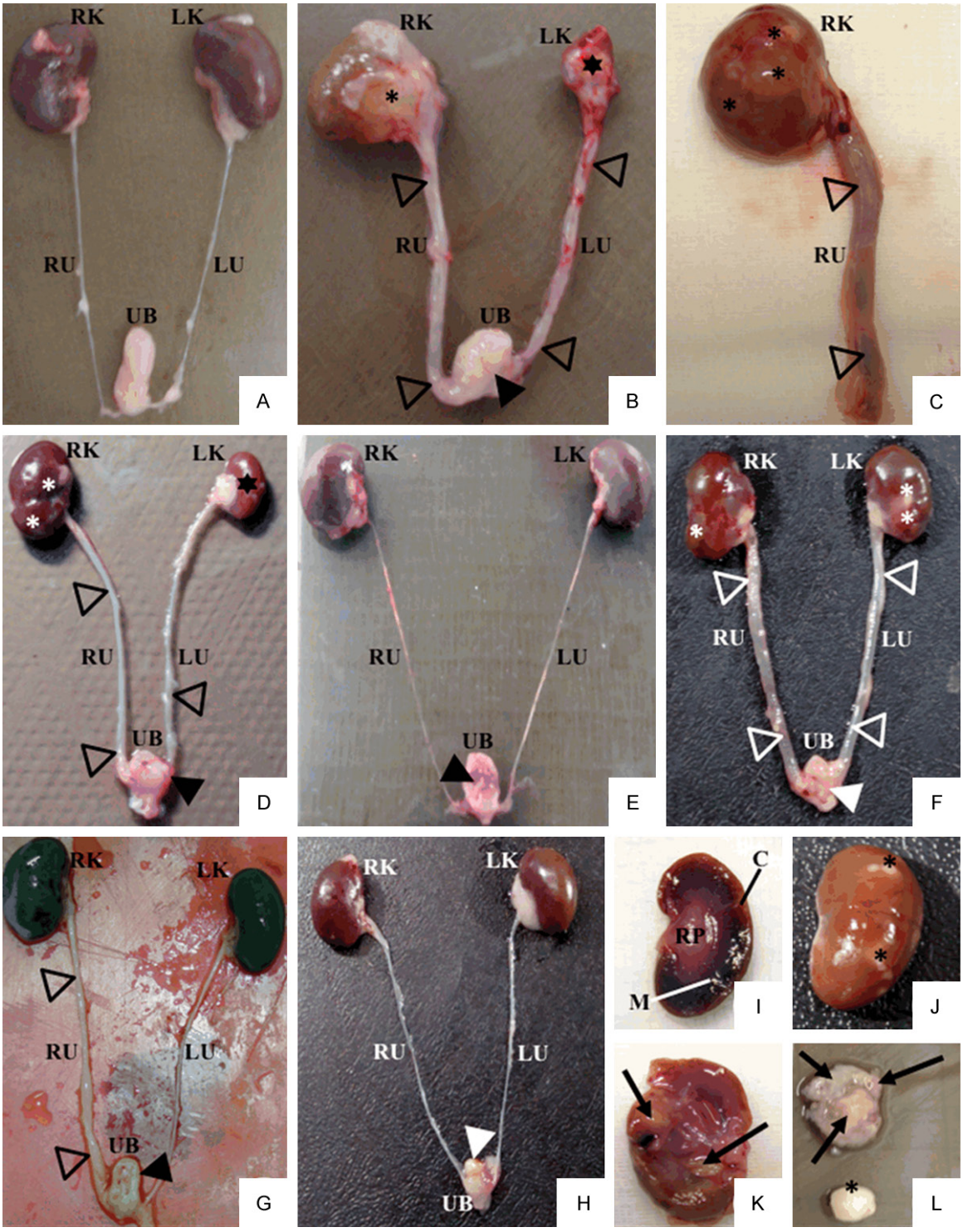

Figure 1. Urinary tract of the female Fischer 344 rats. Group 1 (A, I), Group 2 (B, C, J, K, L), Group 3 (D), Group 4 (E), Group 5 (F), Group 6 (G) and Group 7 (H). In (A), (E) and (H) the urinary tract showed normal features. (B and C) Lesions widespread in different regions of the urinary tract: hydronephrosis; nodular lesions in the kidneys (asterisks) in the kidneys; hydroureter (open arrowheads); renal atrophy (star); and thickening of the urinary bladder wall (solid arrowhead). (D and F) Lesions widespread in different regions of the urinary tract: nodular lesions in the kidneys (asterisks); renal atrophy (star); hydroureter (open arrowheads); and thickening of the urinary bladder wall (solid arrowhead). (E and H) Areas of scarring (fibrosis) in the urinary bladder wall (solid arrowhead). (G) Discrete thickening of the ureters (hydroureter) (open arrowheads) and urinary bladder (solid arrowhead). (I) Frontal section through the left kidney: renal cortex (C), renal medulla (M) and renal pelvis (RP). (J) Nodular lesions (asterisks) evident on the

kidney external surface. (K) Frontal section through the right kidney demonstrating hydronephrosis and neoplastic papillary lesions (arrows) in the renal parenchyma. (I) Intravesical neoplastic papillary lesions (arrows) in the urinary bladder; urinary calculi (asterisks). (A-L) LK-left kidney, LU-left ureter, RK-right kidney, RU-right ureter, UB-urinary bladder.

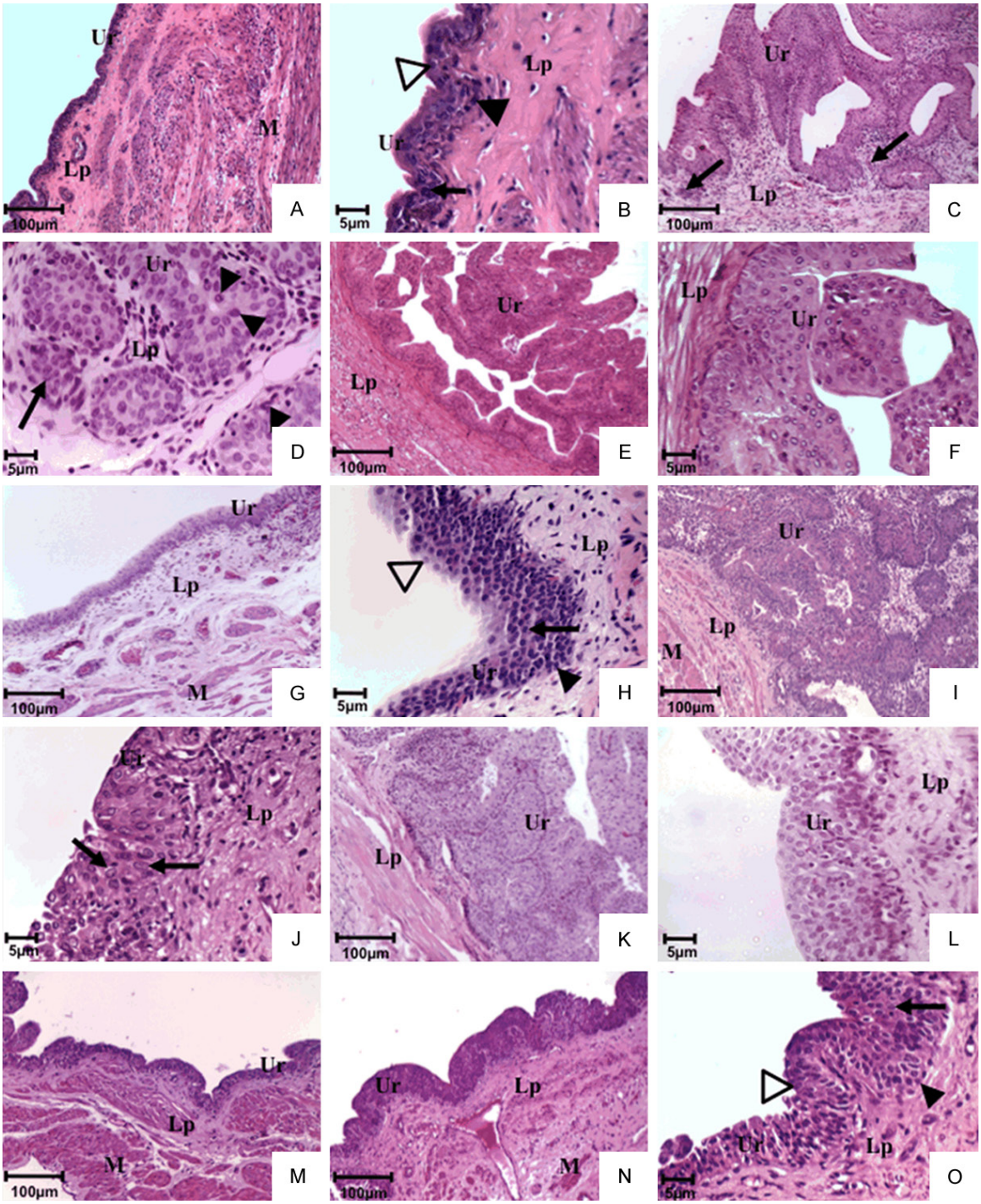

Figure 2. Photomicrographs of the urinary bladder. Group 1 (A, B), Group 2 (C, D), Group 3 (E, F), Group 4 (G, H), Group 5 (I, J), Group 6 (K, L) and Group 7 (M, N, O). (A, B) and (M) Normal urothelium composed of 2-3 layers: a basal cell layer (solid arrowhead), an intermediate cell layer (arrow) and a superficial or apical layer composed of umbrella cells (open arrowhead). (C and D) Tumor invading mucosa or submucosa of the bladder wall (pT1): neoplastic cells arranged in small groups (arrows) invading the lamina propria; mitotic figures (solid arrowheads). (E, F, I and K)

Low-grade papillary carcinoma *in situ* (pTa) characterized by a fibrovascular stalk and frequent papillary branching, with increased cellular size. (G, H, L and N) Flat hyperplasia composed of several layers: a basal cell layer (solid arrowhead), intermediate cell layer (arrow), and a superficial or apical layer composed of umbrella cells (open arrowhead). (J) Flat carcinoma *in situ* (pTis) characterized by cellular atypia: bulky nuclei (arrows) with reduced cytoplasm and prominent nucleoli. (O) Papillary hyperplasia characterized by a markedly thickened mucosa without cytological atypia. (A-O) Lp-lamina propria, M-muscular layer, Ur-urothelium.

Table 1. Percentage of histopathological changes in the urinary bladder of rats from experimental

Histopatology	Groups						
	Group 1 (n=5)	Group 2 (n=5)	Group 3 (n=5)	Group 4 (n=5)	Group 5 (n=5)	Group 6 (n=5)	Group 7 (n=5)
Normal	05 (100%)	-	01 (20%)	02 (40%)	-	-	03 (60%)
Flat Hyperplasia	-	-	-	02 (40%)	-	02 (40%)	01 (20%)
Papillary Hyperplasia	-	-	-	-	-	-	01 (20%)
Flat carcinoma *in situ* (pTis)	-	-	-	-	01 (20%)	01 (20%)	-
Low-grade Papillary Carcinoma *in situ* (pTa)	-	-	04 (80%)	01 (20%)	03 (60%)	02 (40%)	-
High-grade Papillary Carcinoma *in situ* (pTa)	-	01 (20%)	-	-	01 (20%)	-	-
Tumor invading mucosa or submucosa of the bladder wall (pT1)	-	04 (80%)	-	-	-	-	-

Control group: Group 1, MNU group: Group 2, MNU-BCG group: Group 3, MNU-P-MAPA group: Group 4, MNU-Flutamide group: Group 5, MNU-BCG-Flutamide group: Group 6 and MNU-P-MAPA-Flutamide group: Group 7.

results are expressed as the mean ± standard deviation.

Results

Histopathological analysis

The urinary tracts from Group 1 showed no macroscopic (**Figure 1A**, **1I**) or microscopic changes (**Figure 2A**, **2B**; **Table 1**). The normal urothelium was composed of 2-3 layers: a basal cell layer, an intermediate cell layer, and a superficial or apical layer composed of umbrella cells (**Figure 2A**, **2B**).

In contrast, the urinary tracts from Group 2 showed deep macroscopic and histopathological changes, including hydronephrosis, hydroureter, thickening and papillary lesions in the urinary bladder, nodular lesions in the kidneys and renal atrophy (**Figure 1B**, **1C**, **1J-L**). Urinary bladders with tumor invading mucosa or submucosa of the bladder wall (pT1) and high-grade papillary carcinoma *in situ* (pTa) were found in 80% and 20% of the animals, respectively (**Figure 2C**, **2D**; **Table 1**). In addition, keratinizing squamous metaplasia was found in 60% of the animals from Group 2.

Low-grade papillary carcinoma *in situ* (pTa) (80%) was the histopathological change most frequent in the urinary bladders from Group 3, and 20% of the animals in this group showed normal histology of the urinary bladder (**Figure 2E**, **2F**; **Table 1**). In addition, the urinary tracts from Group 3 showed macroscopic changes, including nodular lesions in the kidneys, hydroureter, thickening and papillary lesions in the urinary bladder and renal atrophy (**Figure 1D**).

The macroscopic features of the urinary tract from Group 4 were similar to those found in the Group 1 (**Figure 1E**). However, the urinary bladder wall showed areas of scarring (fibrosis) (**Figure 1E**). In the urinary bladder, normal urothelium was found in 40% of the animals (**Figure 2G**; **Table 1**). Flat hyperplasia (40%) and low-grade papillary carcinoma *in situ* (pTa) (20%) were the most frequent histopathological changes in Group 4 (**Figure 2H**; **Table 1**).

Low-grade papillary carcinoma *in situ* (60%), high-grade papillary carcinoma *in situ* (20%) and flat carcinoma *in situ* (pTis) (20%) were the most common histopathological changes in the urinary bladders from Group 5 (**Figure 2I**, **2J**; **Table 1**). Keratinizing squamous metaplasia was found in 40% of the animals. In addition, the urinary tracts from Group 5 showed macroscopic changes, including hydronephrosis, hydroureter, thickening and papillary lesions in the urinary bladder and nodular lesions in the kidneys (**Figure 1F**).

The incidence of macroscopic urinary tract lesions was significantly lower in Group 6 than in Group 3 and Group 5 (**Figure 1G**). The most

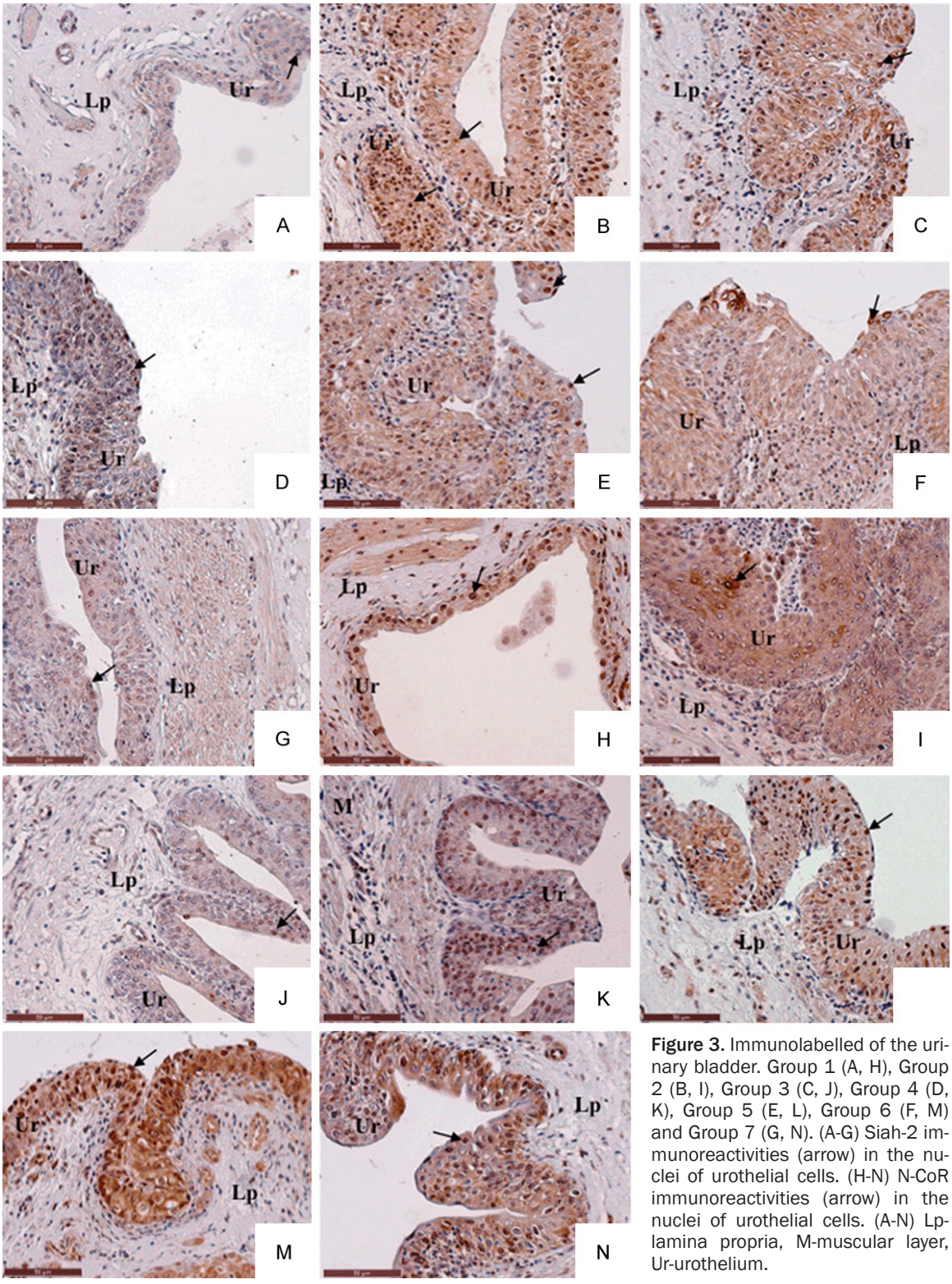

Figure 3. Immunolabelled of the urinary bladder. Group 1 (A, H), Group 2 (B, I), Group 3 (C, J), Group 4 (D, K), Group 5 (E, L), Group 6 (F, M) and Group 7 (G, N). (A-G) Siah-2 immunoreactivities (arrow) in the nuclei of urothelial cells. (H-N) N-CoR immunoreactivities (arrow) in the nuclei of urothelial cells. (A-N) Lp-lamina propria, M-muscular layer, Ur-urothelium.

common macroscopic changes in the urinary tracts from Group 6 were discrete thickening of the ureter (hydroureter) and urinary bladder (**Figure 1G**). Urinary bladders with low-grade papillary carcinoma *in situ* (pTa), flat carcinoma *in situ* (pTis) and flat hyperplasia were found in 40%, 20% and 40% of the animals, respectively (**Figure 2K**, **2L**; **Table 1**).

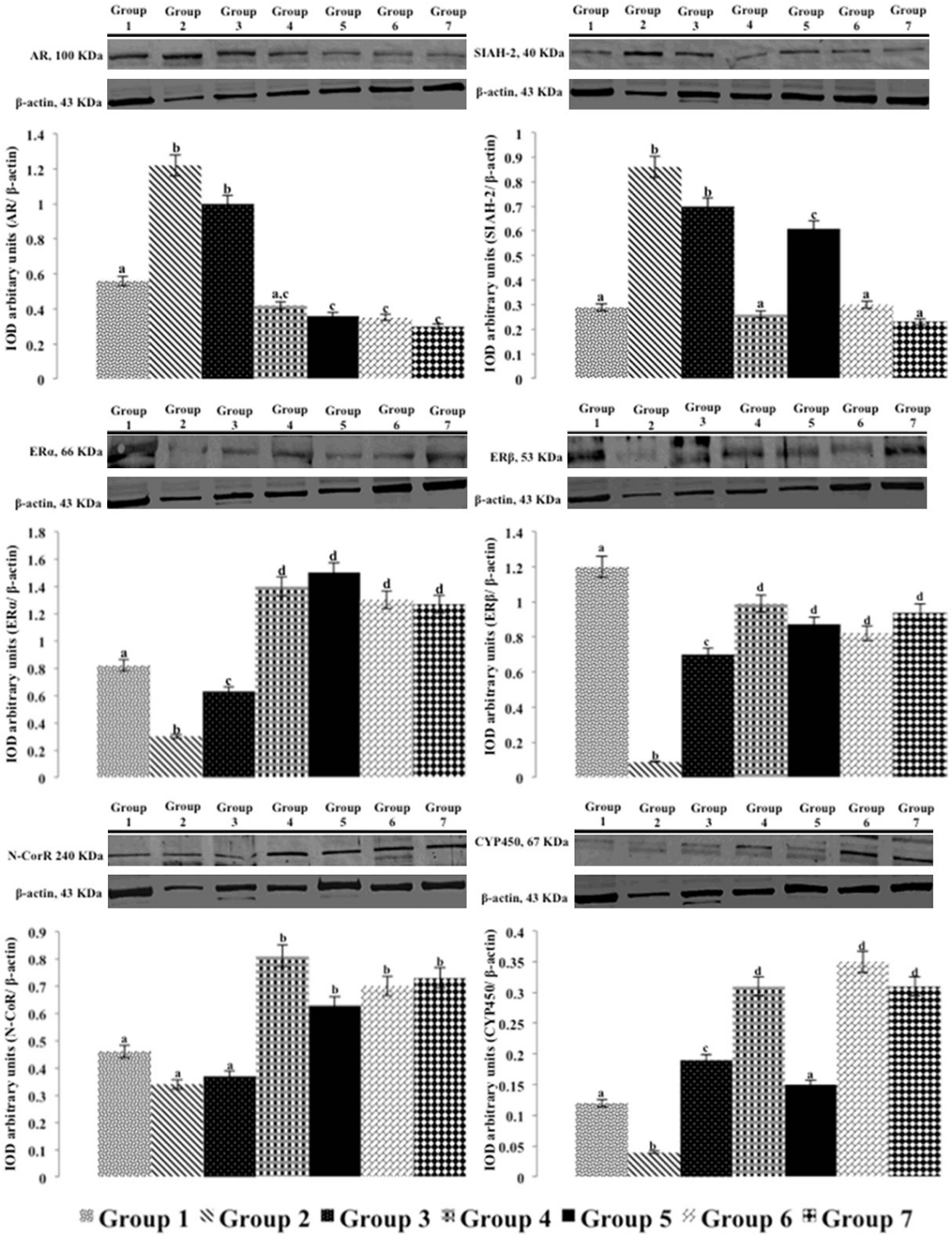

Figure 4. Representative Western Blotting of AR, Siah-2, ERα, ERβ, N-CoR and CYP450 protein levels. Samples of urinary bladder were pooled from 5 animals per group for each repetition (duplicate) and used for semi-quantitative densitometry (IOD-Integrated Optical Density) analysis of the AR, Siah-2, ERα, ERβ, N-CoR and CYP450 levels following normalization to the β-actin. All data were expressed as the mean ± standard deviation. Different lowercase letters (A-D) indicate significant differences ($P<0.01$) between the groups after Tukey's test.

The macroscopic features of the urinary tract from the Group 7 were similar to those found in Group 1 and Group 4, showing only areas of scarring (fibrosis) in the urinary bladder wall

Table 2. Immunolabelled antigens intensity of the urinary bladder of rats in the different experimental groups

	Groups						
Antigens	Group 1 (n=5)	Group 2 (n=5)	Group 3 (n=5)	Group 4 (n=5)	Group 5 (n=5)	Group 6 (n=5)	Group 7 (n=5)
Siah-2	1 (5.4%)	3 (86.7%)	3 (77.8%)	1 (18.3%)	2 (55.0%)	1 (27.3%)	1 (14.9%)
N-CoR	3 (81.3%)	1 (20.7%)	1 (18.9%)	3 (78.6%)	3 (76.1%)	3 (93.7%)	3 (90.0%)

Control group: Group 1, MNU group: Group 2, MNU-BCG group: Group 3, MNU-P-MAPA group: Group 4, MNU-Flutamide group: Group 5, MNU-BCG-Flutamide group: Group 6 and MNU-P-MAPA-Flutamide group: Group 7. Staining intensity grade: 0 (no immunoreactivity), 0% positive urothelial cells; 1(weak immunoreactivity), 1-35% positive urothelial cells; 2 (moderate immunoreactivity), 36-70% positive urothelial cells; 3 (intense immunoreactivity), >70% positive urothelial cells.

(**Figure 1H**). In the urinary bladder, normal urothelium was found in 60% of the animals (**Figure 2M**; **Table 1**). The most often histopathological changes in the MNU-P-MAPA group were flat hyperplasia (20%) and papillary hyperplasia (20%) (**Figure 2N**, **2O**; **Table 1**).

Urinary calculi (**Figure 1L**) and macroscopic hematuria were only observed in Group 2, Group 3, Group 5 and Group 6 but were absent in Groups 4 and 7.

Immunodetection of Siah-2 and N-CoR and Western blotting analysis

The Siah-2 protein levels were significantly higher in Group 2 (0.86) and Group 3 (0.70) than in the other experimental groups, showing intense immunoreactivities in the nuclei of urothelial cells (**Figures 3B**, **3C**, **4**; **Table 2**). In addition, these levels were significantly higher in Group 5 (0.61) than in Group 6 (0.30), Group 1 (0.29), Group 7 (0.23) and Group 4 (0.26), which exhibiting moderate and weak immunoreactivities, respectively (**Figures 3A**, **3D-G**, **4**; **Table 2**). In contrast, the N-CoR protein levels were significantly higher in Group 4 (0.81), Group 7 (0.73), Group 6 (0.70) and Group 5 (0.63) than in the other experimental groups, showing intense immunoreactivities in the nuclei of urothelial cells (**Figures 3H-3N**, **4**; **Table 2**).

The highest AR protein levels were found in Group 2 (1.22) and Group 3 (1.00) (**Figure 4**). Additionally, these levels were significantly higher in the Group 1 (0.56) in relation to Group 4 (0.42), Group 5 (0.36), Group 6 (0.35) and Group 7 (0.30) (**Figure 4**). In contrast, ERα and ERβ protein levels were significantly higher in Group 4 (1.40; 0.99), Group 5 (1.50; 0.87), Group 6 (1.30; 0.82) and Group 7 (1.27; 0.94) than in the other experimental groups (**Figure 4**) Additionally, these levels were significantly higher in the Group 1 (0.82; 1.20) in relation to Group 2 (0.30; 0.09) and Group 3 (0.63; 0.70) (**Figure 4**).

The highest CYP450 protein levels were found in Group 4 (0.31), Group 6 (0.35) and Group 7 (0.31) (**Figure 4**). Additionally, these levels were significantly higher in Group 3 (0.19) than in Group 1 (0.12) and Group 2 (0.04) (**Figure 4**).

The highest TLR4 protein levels were found in Group 7 (1.53) and Group 4 (1.30), which were higher than in Group 6 (1.02) (**Figure 5**). Additionally, these levels were significantly higher in Group 3 (0.93), Group 5 (0.86) and Group 1 (0.85) than in Group 2 (0.57) (**Figure 5**).

The TRIF and IRF3 protein levels were significantly higher in Group 7 (3.04; 0.80) and Group 4 (2.04; 0.69) than in Group 3 (1.90; 0.23) and Group 6 (1.57; 0.21) (**Figure 5**). In addition, these levels were significantly higher in Group 1 (1.18; 0.15) and Group 5 (0.88; 0.12) than in Group 2 (0.59; 0.08) (**Figure 5**).

The IFN-γ protein levels were significantly higher in Group 7 (3.14) and Group 4 (2.41) than in Groups 3 (1.27) and 6 (1.16) (**Figure 5**). In addition, these levels were significantly higher in Group 2 (1.00) than in Group 1 (0.75) and Group 5 (0.68) (**Figure 5**).

The highest TLR2 protein levels were found in Group 4 (1.50) and Group 7 (1.37) (**Figure 6**). Furthermore, these levels were significantly higher in Group 3 (1.16) and Group 6 (1.02) than in Groups 5 (0.82), 1 (0.77) and 2 (0.77) (**Figure 6**).

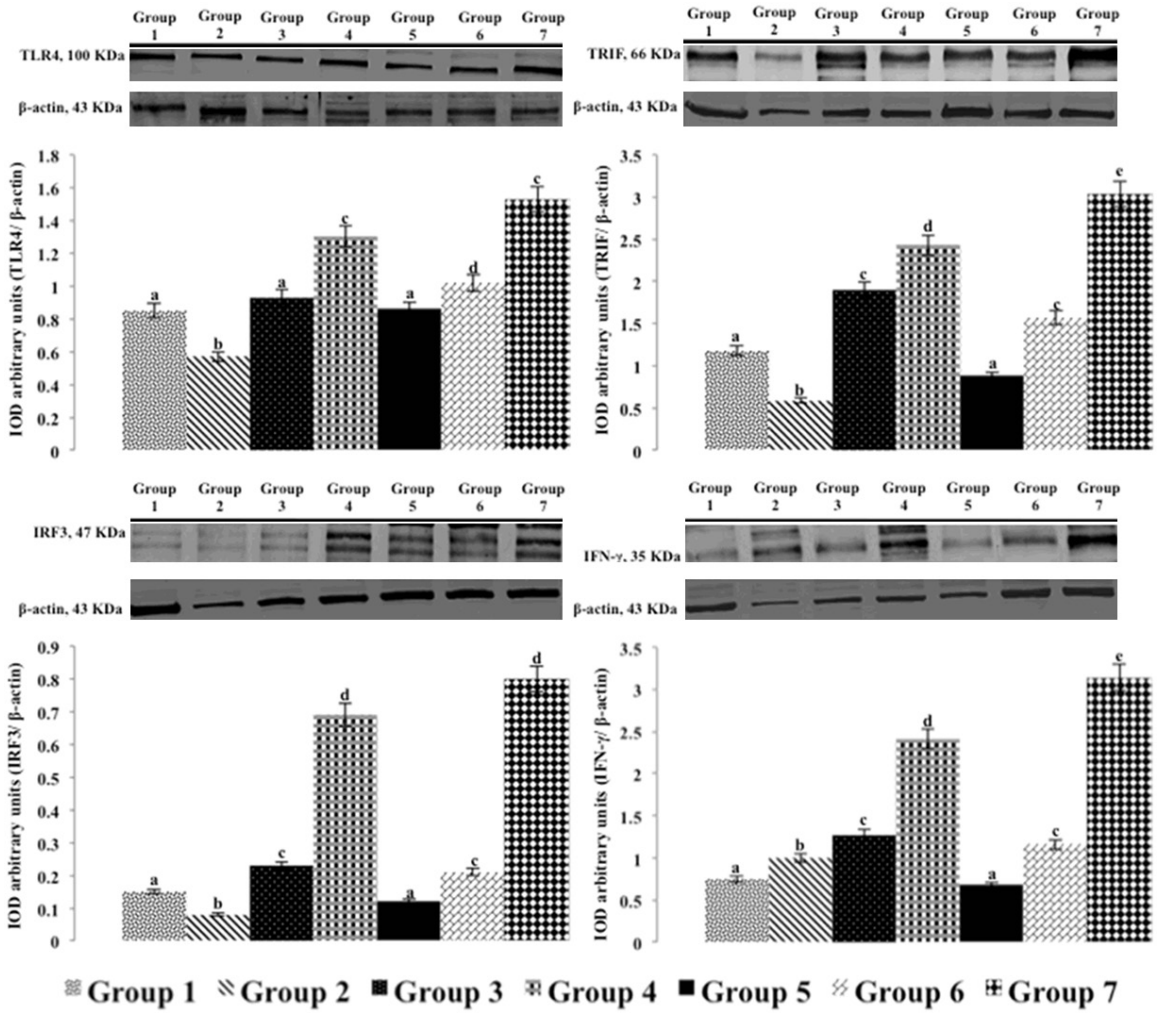

Figure 5. Representative Western Blotting of TLR4, TRIF, IRF3 and IFN-γ protein levels. Samples of urinary bladder were pooled from 5 animals per group for each repetition (duplicate) and used for semi-quantitative densitometry (IOD-Integrated Optical Density) analysis of the TLR4, TRIF, IRF3 and IFN-γ levels following normalization to the β-actin. All data were expressed as the mean ± standard deviation. Different lowercase letters (A-D) indicate significant differences ($P<0.01$) between the groups after Tukey's test.

The MyD88 protein levels were significantly higher in Group 3 (2.28) and Group 4 (2.24) than in Groups 1 (1.91), 6 (1.74), 7 (1.72), 2 (1.30) and 5 (1.11) (**Figure 6**).

The highest NF-kB protein levels were found in Group 3 (2.70) in relation to Group 6 (1.90) (**Figure 6**). In addition, these levels were significantly higher in Group 2 (1.40) than in Group 4 (0.90), Group 5 (0.81), Group 1 (0.70) and Group 7 (0.40) (**Figure 6**).

The TNF-α protein levels were significantly higher in Group 1 (1.12), Group 4 (1.06) and Group 3 (1.00) than in Groups 6 (0.75), 5 (0.35), 2 (0.31) and 7 (0.28) (**Figure 6**).

Discussion

Steroid hormone receptors are involved in the development and progression of bladder cancer (BC). The AR and ERα/ERβ expression has been evaluated in BC and showed contradictory effects in relation to the histopathological characteristics of the tumors [13, 28-30].

Our results demonstrated deep macroscopic and histopathological changes in the MNU group, including hydronephrosis, hydroureter, nodular lesions in the kidneys, tumor invading mucosa or submucosa of the bladder wall (pT1) and keratinizing squamous metaplasia. The combined treatment with BCG and flutamide

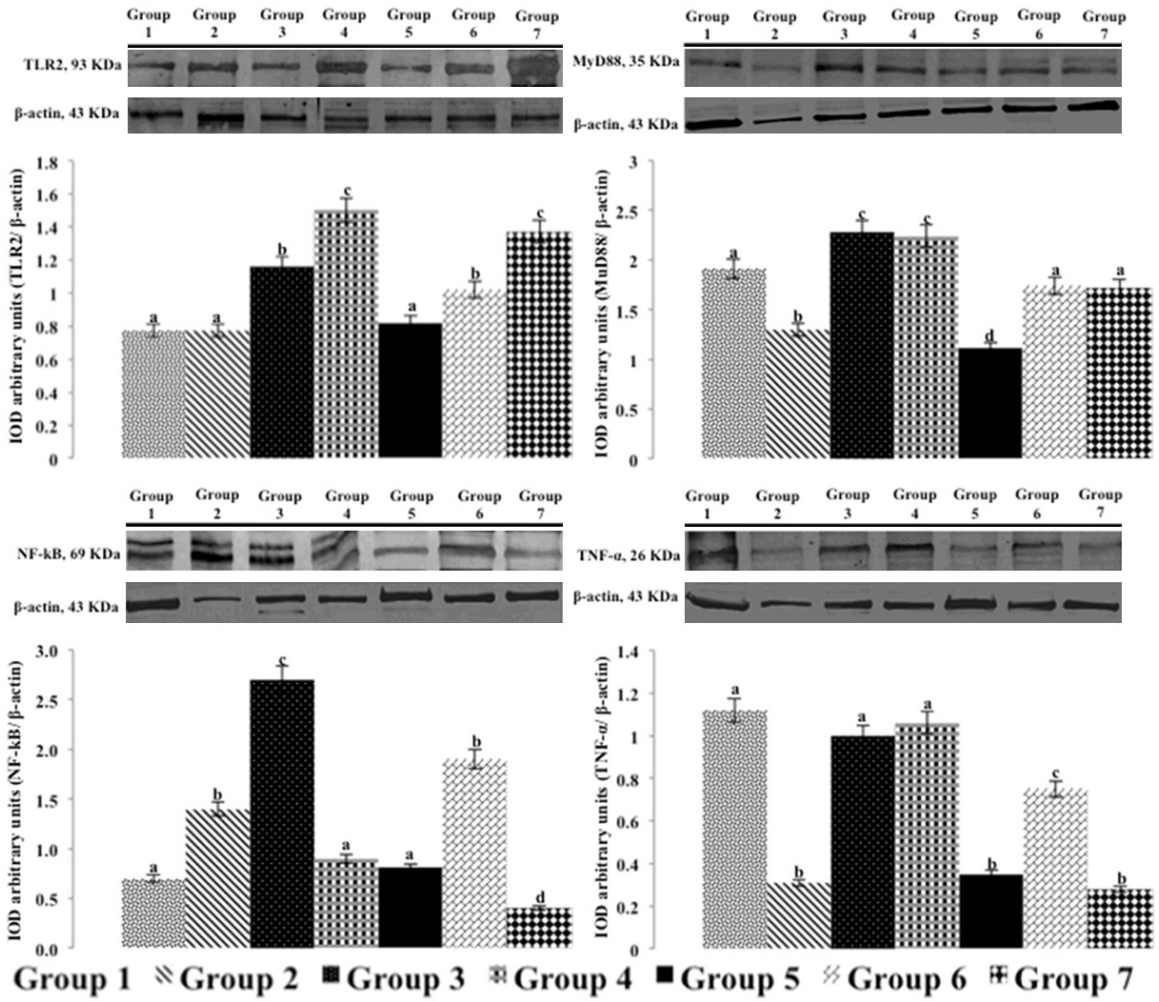

Figure 6. Representative Western Blotting of TLR2, MyD88, NF-kB and TNF-α protein levels. Samples of urinary bladder were pooled from 5 animals per group for each repetition (duplicate) and used for semi-quantitative densitometry (IOD-Integrated Optical Density) analysis of the TLR2, MyD88, NF-kB and TNF-α levels following normalization to the β-actin. All data were expressed as the mean ± standard deviation. Different lowercase letters (A-D) indicate significant differences ($P<0.01$) between the groups after Tukey's test.

was more effective than either therapy alone and resulted in benign lesions (flat hyperplasia), low-grade pTa and pTis. However, P-MAPA immunotherapy showed better histological recovery than did BCG and BCG with flutamide treatments; 40% of the animals showed complete recovery of neoplasic lesions, 40% had benign lesions (flat hyperplasia) and 20% had low-grade pTa; therefore, 80% of the animals were tumor-free. In this study, the best histopathological recovery resulted from the combined treatment with P-MAPA and flutamide, which led to complete recovery of neoplastic lesions in 60% of the animals and benign lesions (flat and papillary hyperplasia) in 40% of the animals; therefore, 100% of the animals were tumor-free.

In regards to steroid hormone receptor protein levels, the highest AR protein levels were found in the MNU and BCG groups, whereas the level was significant lower in the groups that received flutamide. Interestingly, P-MAPA immunotherapy was able to reduce AR protein levels independently of flutamide. In contrast, the ERα and ERβ protein levels were significantly higher in the P-MAPA group and in the groups that received flutamide. Similarly, P-MAPA immunotherapy was able to increase the ERα and ERβ protein levels independently of flutamide.

The outstanding reduction in AR protein levels, accompanied by the proportional reduction in ERα and ERβ levels, in the animals treated with flutamide was previously described in other

studies that used the same antagonist for AR [28-30]. In rodents, stimulatory and inhibitory effects of estrogen signaling were observed in both *in vitro* and *in vivo* experiments [28, 29], including the selective modulation of ERs in urothelial cell carcinoma growth via ERα and ERβ that may be directly or indirectly under the influence of AR [30]. Several studies detected positive AR expression in 52-100% of bladder cancer samples [30, 31]. In these studies, AR levels showed no significant difference in both men and women tumors. Miyamoto et al. [13] and other authors [30, 32] analyzed superficial bladder tumors using qRT-PCR and demonstrated that several urothelial cell carcinoma were AR-positive, and high AR-expressing tumors tended to have a higher risk of recurrence than those with low AR-expressing tumors.

Several studies have shown reduced estrogen receptor expression (12-33%) in bladder cancer samples [30, 33]. ERα expression has been shown to be decreased in BC [34]. Miyamoto et al. [30] showed that ERβ expression was positive in 49.0% of bladder tumor samples in relation to benign urothelium, which was significantly lower. Also, Kontos et al. [7] demonstrated that ERβ expression levels were significantly higher in normal urothelium in relation to malignant urothelium.

Thus, it could be concluded that the different steroid hormone receptor protein levels in the NMIBC result in different signals to the dynamics of the urinary bladder, and the importance of AR signals in the growth and progression of NMIBC is also clear. Furthermore, ERα and ERβ were fundamental in maintaining the structure of the benign urothelium. Here, we present a new therapeutic approach for bladder cancer based on anti-androgen therapy with flutamide and immunotherapy with P-MAPA and/or BCG. The combined treatment with P-MAPA and flutamide showed a better recovery of bladder histology features and ERα/ERβ protein levels than observed in the BCG and BCG with flutamide treatment groups.

In general, the interactions between co-activators and co-repressors (also named as co-regulators) are responsible by control of AR activity [35]. These co-regulators can induce alterations in chromatin structure through posttranslational modifications of the core histone amino-termini [36]. Corepressor N-CoR, a high molecular multiprotein complex, is related with the modulation of HDAC3 (histone deacetylase) enzymatic activity [36]. Kontos et al. [7] showed that expression of both ERβ and its co-repressor N-CoR were inhibited in urothelial cells carcinoma. Atltintas et al. [37] demonstrated that after androgen stimulation, AR target genes were expressed in the G1 phase of the cell cycle in prostate cells, and that AR overexpression inhibited N-CoR activity leading to stimulation AR-dependent expression of genes. These same authors showed that cell cycle-dependent expression of N-CoR is responsible of the non-productive binding of AR to the promoter of target genes during the S and G2-M phases, since N-CoR is expressed only during these phases. Interestingly, AR targeting occurs at chromatin sites at which AR is bound to the transcriptional corepressor N-CoR [16].

The selective degradation of repressed AR complexes results in their replacement with AR bound to co-activators, resulting in up-regulation of AR- and Siah2-dependent genes [16]. Studies have demonstrated that Siah-2 acts as an E3 ubiquitin ligase for the AR, repressing AR chromatin complex for degradation and activating several AR-regulated cellular processes such as tumor cell proliferation, cell motility and lipid metabolism [14]. Under low androgen concentration, Siah-2 activity is required for prostate cancer cells growth, whereas castration therapy leads to inhibition of Siah-2 activity, resulting in tumor growth suppression [14, 16]. Experimental studies have also shown that the relation between Siah-2 and AR is involved in characteristic malignant behaviors such as anchorage-independent growth, cell motility and growth of castration-resistant prostate tumors [14, 39]. Thus, these results demonstrated a new mechanism of AR regulation in the carcinogenesis, based on Siah-2 targeting [14]. Qi et al. [16] verified that knockout of Siah-2 in the TRAMP transgenic mouse model of prostate cancer led to decreased prostate size and suppression of tumor formation. In addition, the same authors showed that regulation of AR transcriptional activity by Siah2 was important for castration-resistant prostate cancer development.

In the present study, we demonstrate the control of AR, ERα and ERβ activity by the Siah-2

ubiquitin ligase and the co-repressor N-CoR in NMIBC. Our results show that the Siah-2 protein levels were significantly higher in the MNU and BCG groups. Treatment with flutamide alone and/or in association with BCG reduced these protein levels. However, treatment with P-MAPA alone and/or in association with flutamide showed the smallest reduction in the Siah-2 protein levels. In contrast, increased N-CoR protein levels were verified in the groups that received flutamide in association with P-MAPA and/or BCG. However, the P-MAPA treatment was able to increase N-CoR protein levels independently of flutamide. Thus, it can be concluded that P-MAPA immunotherapy downregulates Siah-2 protein levels and upregulates N-CoR levels, which were correlated with decreased AR protein levels and increased expression of estrogen receptors. These results demonstrated that the control of Siah-2 and N-CoR activities by P-MAPA were essential for histopathological recovery from the cancer state.

The activation of immune system receptors (e.g., TLRs) may represent a new therapeutic strategy for cancer control. Many studies showed that the use of TLRs agonists led to an important antitumor effect *in vivo* and suppressed the growth of urothelial cancer cell lines, *in vitro* [40-42]. Thus, TLR-based immunotherapies might also be successful for the treatment of NMIBC. TLRs expressions are significantly higher in benign urothelial tissue in relation to tumor cells, although their expressions persist in these cells at low levels, enabling the use of TLR agonists for cancer therapy [40-42]. BCG is the main immunotherapy for NMIBC, acting as TLRs 2 and 4 agonists through its mycobacterial components [43]. Based on experimental findings that certain antigens present in the mycobacterial cell wall are recognized by macrophages and dendritic cells, a cellular immune response against the mycobacteria related with TLR signaling is pointed [43]. For instance, in the TLR signaling pathway the factor MyD88 is essential for induction of inflammatory response to *M. tuberculosis* [44]. Scanga et al. [44] demonstrated that *M. tuberculosis* infection was more susceptible in MyD88-deficient mice than mice controls. The Hu35E6E7 urothelial cell line expresses many TLRs in its surface and induces expression of MyD88 factor in the presence of BCG lipoproteins [44]. Also, the susceptibility to *M. tuberculosis* has been associated with TLR2 polymorphisms [45]. In addition to TLR2, TLR4 is also involved in the response to mycobacteria [42, 46]. BCG immunotherapy induced the activation of ERK1/2 MAPK pathway and secretion of interleukin-8 via TLRs 2 and 4 signaling on human epithelial cells in response to mycobacteria infection [42, 46].

We have demonstrated in the present study that BCG treatment increased TLR2 protein levels, resulting in increased MyD88 and NF-kB protein levels. The induction of the MyD88-dependent pathway by BCG treatment led to increased protein levels of inflammatory cytokines (TNF-α). However, MyD88, NF-kB and TNF-α protein levels were reduced in the animals that received only flutamide treatment or flutamide in addition to BCG. Moreover, treatment with P-MAPA increased TLR4 protein levels, resulting in increased TRIF and IRF3 protein levels. The induction of the TRIF-dependent pathway by P-MAPA led to increased IFN-γ protein levels. Furthermore, TLR4, TRIF, IRF3 and IFN-γ protein levels were increased in the animals that received flutamide in addition to P-MAPA.

Earlier studies from our research group showed that P-MAPA significantly increased TLR2, TLR4 and p53 protein levels in an NMIBC animal model, and these increases were correlated with cancer regression [27]. In addition, it was demonstrated that this immunomodulator was more effective for the treatment of NMIBC than was BCG [27]. Santiago et al. [47] investigated the immunotherapeutic potential of P-MAPA on canine visceral leishmaniasis and verified that in peripheral blood mononuclear cell cultures, supernatants showed a decrease in IL-10 levels and an increase in IL-2 and IFN-γ as well as an increase in CD8 T cells after P-MAPA treatment.

In accordance with our data, we conclude that P-MAPA immunotherapy led to distinct activation of the innate immune system through TLRs 2 and 4 in relation to BCG, resulting in increased interferon signaling, which was more effective for the treatment of NMIBC. The flutamide treatment reduced the ability of BCG to induce the MyD88-dependent pathway and potentiated the P-MAPA induction of interferon produc-

tion (TRIF-dependent pathway). In addition, the interferon signaling pathway was correlated to increase N-CoR and decreased Siah-2 protein levels, which affected the steroid hormone receptor signals in urinary bladder carcinogenesis.

We demonstrated that cytochrome P450 (CYP450) protein levels were significantly higher in the flutamide treatment co-administered with P-MAPA and/or BCG immunotherapies. However, only P-MAPA treatment was able to increase CYP450 protein levels independently of flutamide treatment.

CYP450 is involved in the synthesis and metabolism of sex steroid hormones, and this activity may play a major role in both the development and progression of certain cancers, such as those of the breasts and prostate [48]. In addition, CYP in the rat bladder may also be regulated by growth hormone as well as by androgens. CYPs activities are related to metabolism of carcinogens, as well as in the modulation of tumors response to chemotherapeutic agents [49]. Furthermore, elevated expression of several CYP family members were verified in tumors relative to normal human tissues, and, therefore, these CYPs could be considered as molecular targets for the cancer treatment [50]. In this regard, a potential approach for bladder cancer is to use the oxidation functionality of CYP1A1 to restore the potential toxicity to an inactivated chemotherapeutic selectively within the tumor, thereby facilitating reduced systemic toxicity and improving the therapeutic index [50].

In the present study, the decreased AR protein levels and the action of P-MAPA immunotherapy might have contributed to the increased CYP protein levels and therefore favored stimulation of the innate immune system, especially eliciting an increased production of IFN-γ.

In conclusion, our results demonstrated that increased Siah-2 and AR protein levels and decreased N-CoR, CYP450 and estrogen receptors levels played a critical role in the urothelial carcinogenesis, probably leading to escape of urothelial cancer cells from immune system attack. P-MAPA immunotherapy led to distinct activation of innate immune system TLRs 2 and 4-mediated, resulting in increase of interferon signaling pathway, which was more effective at recovering the immunosuppressive tumor immune microenvironment and at recovering the bladder histology features than BCG treatments. The AR blockade therapy was important in the modulating of downstream molecules of TLR2 and TLR4 signaling pathway, decreasing the inflammatory cytokines signaling and enhancing the interferon signaling pathway when associated with P-MAPA. Taken together, the data obtained suggest that interferon signaling pathway activation and targeting AR and Siah-2 signals by P-MAPA intravesical immunotherapy alone and/or in combination with AR blockade may provide novel therapeutic approaches for NMIBC.

Acknowledgements

Support by Farmabrasilis-Brazil, CNPq-Brazil (Process number 490519/2011-3) and FAPESP-Brazil (Process numbers 2012/20706-2; 2012/13585-4) are also acknowledged.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Wagner José Fávaro, Laboratory of Urogenital Carcinogenesis and Immunotherapy, University of Campinas (UNICAMP), CP-6109, 13083-865, Campinas, SP, Brazil. Tel: +55 (19) 3521-6104; E-mail: wjfavaro@gmail.com

References

[1] Goebell PJ, Knowles MA. Bladder cancer or bladder cancers? Genetically distinct malignant conditions of the urothelium. Urol Oncol 2010; 28: 409-428.

[2] Miyamoto H, Brimo F, Schultz L, Ye H, Miller JS, Fajardo DA, Lee TK, Epstein JI, Netto GJ. Low-grade papillary urothelial carcinoma of the urinary bladder: a clinicopathologic analysis of a post-World Health Organization/International Society of Urological Pathology classification cohort from a single academic center. Arch Pathol Lab Med 2010; 134: 1160-1163.

[3] Siegel R, Naishadham D, Jemal A. Cancer statistics 2013. CA Cancer J Clin 2013; 63: 11-30.

[4] Shimada K, Fujii T, Anai S, Fujimoto K, Konishi N. ROS generation via NOX4 and its utility in the cytological diagnosis of urothelial carcinoma of the urinary bladder. BMC Urol 2011; 11: 01-12.

[5] Epstein JI, Amin MB, Reuter VR, Mostofi FK. The World Health Organization/International Society of Urological Pathology consensus classification of urothelial (transitional cell) neoplasms of the urinary bladder. Bladder Consensus Conference Committee. Am J Surg Pathol 1998; 22: 1435-1448.

[6] Dechering K, Boersma C, Mosselman S. Estrogen receptors alpha and beta: two receptors of a kind? Curr Med Chem 2000; 7: 561-576.

[7] Kontos S, Papatsoris A, Kominea A, Melachrinou M, Tanoglidi A, Kachrilas S, Karavitakis M, Balampani E, Sotiropoulou-Bonikou G. Expression of ERβ and its co-regulators p300 and NCoR in human transitional cell bladder cancer. Urol Int 2011; 87: 151-158.

[8] Shen SS, Smith CL, Hsieh JT, Yu J, Kim IY, Jian W, Sonpavde G, Ayala GE, Younes M, Lerner SP. Expression of estrogen receptors-alpha and -beta in bladder cancer cell lines and human bladder tumor tissue. Cancer 2006; 106: 2610-2616.

[9] Swedenborg E, Power KA, Cai W, Pongratz I, Rüegg J. Regulation of estrogen receptor beta activity and implications in health and disease. Cell Mol Life Sci 2009; 66: 3873-3894.

[10] Cunliffe VT. Eloquent silence: developmental functions of class I histone deacetylases. Curr Opin Genet Dev 2008; 18: 404-410.

[11] Rahmani AH, Alzohairy M, Babiker AY, Khan AA, Aly SM, Rizvi MA. Implication of androgen receptor in urinary bladder cancer: a critical mini review. Int J Mol Epidemiol Gent 2013; 4: 150-155.

[12] Wu JT, Han BM, Yu SQ, Wang HP, Xia SJ. Androgen receptor is a potential therapeutic target for bladder cancer. Urology 2010; 75: 820-827.

[13] Miyamoto H, Yang Z, Chen YT, Ishiguro H, Uemura H, Kubota Y, Nagashima Y, Chang YJ, Hu YC, Tsai MY, Yeh S, Messing EM, Chang C. Promotion of bladder cancer development and progression by androgen receptor signals. J Natl Cancer Inst 2007; 99: 558-568.

[14] Freeman MR. The ubiquitin ligase Siah2 is revealed as an accomplice of the androgen receptor in castration resistant prostate cancer. Asian J Androl 2013; 15: 447-448.

[15] Kim H, Scimia MC, Wilkinson D, Trelles RD, Wood MR, Bowtell D, Dillin A, Mercola M, Ronai ZA. Fine-tuning of Drp1/Fis1 availability by AKAP121/Siah2 regulates mitochondrial adaptation to hypoxia. Mol Cell 2011; 44: 532-544.

[16] Qi J, Tripathi M, Mishra R, Sahgal N, Fazli L, Ettinger S, Placzek WJ, Claps G, Chung LW, Bowtell D, Gleave M, Bhowmick N, Ronai ZA. The E3 ubiquitin ligase Siah2 contributes to castration resistant prostate cancer by regulation of androgen receptor transcriptional activity. Cancer Cell 2013; 23: 332-346.

[17] Askeland EJ, Newton MR, O'Donnell MA, Luo Y. Bladder cancer immunotherapy: BCG and Beyond. Adv Urol 2012; 18: 01-12.

[18] Böhle A, Brandau S. Immune mechanisms in bacillus Calmette Guerin Immunotherapy for superficial bladder cancer. J Urol 2003; 170: 964-969.

[19] DiPaola RS, Lattime EC. Bacillus Calmette-Guerin mechanism of action: role of immunity, apoptosis, necrosis and autophagy. J Urol 2007; 178: 1840-1.

[20] Seow SW, Rahmat JN, Mohamed AA, Mahendran R, Lee YK, Bay BH. Lactobacillus species is more cytotoxic to human bladder cancer cells than Mycobacterium Bovis (bacillus Calmette-Guerin). J Urol 2002; 168: 2236-2239.

[21] Reis LO, Ferreira U, Billis A. Anti-angiogenic effects of the superantigen staphylococcal enterotoxin B and bacillus Calmette-Guerin immunotherapy for non-muscle invasive bladder cancer. J Urol 2012; 2: 438-445.

[22] Perabo FG, Willert PL, Wirger A, Schmidt DH, Von Ruecker A, Mueller SC. Superantigen-activated mononuclear cells induce apoptosis in transitional cell carcinoma. Anticancer Res 2005; 25: 3565-3573.

[23] Berry DL, Blumenstein BA, Magyary DL, Lamm DL, Crawford ED. Local toxicity patterns associated with intravesical bacillus Calmette-Guerin: a Southwest Oncology Group study. Int J Urol 1996; 3: 98-100.

[24] Killeen SD, Wang JH, Andrews EJ, Redmond HP. Exploitation of the Toll like receptor system in cancer: a doubled-edged sword? Br J Cancer 2006; 95: 247-252.

[25] Takeda K, Akira S. TLR signaling pathways. Semin Immunol 2004; 16: 03-09.

[26] Durán N, Gowen BB, Costa FT, Justo GZ, Brocchi M, Nunes OS, Nunes IS. A biotechnological product and its potential as a new immunomodulator for treatment of animal phlebovirus infection: Punta Toro virus. Antiviral Res 2009; 83: 143-147.

[27] Fávaro WJ, Nunes OS, Seiva FR, Nunes IS, Woolhiser LK, Durán N, Lenaerts AJ. Effects of P-MAPA immunomodulator on Toll-like receptors and p53: Potential therapeutic strategies for infectious diseases and cancer. Infect Agent Cancer 2012; 7: 01-15.

[28] Sonpavde G, Okuno N, Weiss H, Yu J, Shen SS, Younes M, Jian W, Lerner SP, Smith CL. Efficacy of selective estrogen receptor modulators in nude mice bearing human transitional cell carcinoma. Urology 2007; 69: 1221-1226.

[29] Teng J, Wang ZY, Jarrard DF, Bjorling DE. Roles of estrogen alpha and beta in modulating urothelial cell proliferation. Endocr Relat Cancer 2008; 15: 351-364.
[30] Miyamoto H, Yao JL, Chaux A, Zheng Y, Hsu I, Izumi K, Chang C, Messing EM, Netto GJ, Yeh S. Expression of androgen and oestrogen receptors and its prognostic significance in urothelial neoplasm of the urinary bladder. BJU Int 2012; 11: 1716-1726.
[31] Zhuang YH, Bläuer M, Tammela T, Tuohimaa P. Immunodetection of androgen receptor in human urinary bladder cancer. Histopathology 1997; 30: 556-562.
[32] Wu JT, Han BM, Yu SQ, Wang HP, Xia SJ. Androgen receptor is a potential therapeutic target for bladder cancer. Urology 2010; 75: 820-827.
[33] Basakci A, Kirkali Z, Tuzel E, Yorukoglu K, Mungan MU, Sade M. Prognostic significance of estrogen receptor expression in superficial transitional cell carcinoma of the urinary bladder. Eur Urol 2002; 41: 342-345.
[34] Shen SS, Smith CL, Hsieh JT, Yu J, Kim IY, Jian W, Sonpavde G, Ayala GE, Younes M, Lerner SP. Expression of estrogen receptor- α and β in bladder cancer cell lines and human bladder tumor tissue. Cancer 2006; 106: 2610-2616.
[35] Rosenfeld MG, Lunyak VV, Glass CK. Sensors and signals: a coactivators/corepressor/epigenetic code for integrating signal-dependent programs of transcriptional response. Genes Dev 2006; 20: 1405-1428.
[36] Karagianni P, Wong J. HDAC3: taking the SMRT-N-CoRrect road to repression. Oncogene 2007; 26: 5439-5449.
[37] Altintas DM, Vlaeminck V, Angelov D, Dimitrov S, Samarut J. Cell cycle regulated expression of NCoR might control cyclic expression of androgen responsive genes in an immortalized prostate cell line. Mol Cell Endocrinol 2011; 332: 149-162.
[38] Qi J, Tripathi M, Mishra R, Sahgal N, Fazli L, Ettinger S, Placzek WJ, Claps G, Chung LW, Bowtell D, Gleave M, Bhowmick N, Ronai ZA. The E3 ubiquitin ligase siah2 contributes to castration resistant prostate cancer by regulation of androgen receptor transcriptional activity. Cancer Cell 2013; 23: 332-346.
[39] Sharma NL, Massie CE, Ramos-Montoya A, Zecchini V, Scott HE, Lamb AD, MacArthur S, Stark R, Warren AY, Mills IG, Neal DE. The androgen receptor induces a distinct transcriptional program in castration resistant prostate cancer in man. Cancer Cell 2013; 23: 35-47.
[40] Hennessy EJ, Parker AE, O'Neill LA. Targeting Toll like receptors: emerging therapeutics? Nat Rev Drug Discov 2010; 9: 293-307.
[41] Goutagny N, Estornes Y, Hasan U, Lebecque S, Caux C. Targeting pattern recognition receptors in cancer immunotherapy. Target Oncol 2012; 7: 29-54.
[42] LaRue H, Ayari C, Bergeron A, Fradet Y. Toll-like receptors in urothelial cells-targets for cancer immunotherapy. Nat Rev Urol 2013; 10: 537-545.
[43] Heldwein KA, Liang MD, Andresen TK, Thomas KE, Marty AM, Cuesta N, Vogel SN, Fenton MJ. TLR2 and TLR4 serve distinct roles in the host immune response against Mycobacterium bovis BCG. J Leukoc Biol 2003; 74: 277-286.
[44] Scanga CA, Bafica A, Feng CG, Cheever AW, Hieny S, Sher A. MyD88-deficient mice display a profound loss in resistance to Mycobacterium tuberculosis associated with partially impaired Th1 cytokine and nitric oxide synthase 2 expression. Infect Immun 2004; 72: 2400-2404.
[45] Huynh KK, Joshi SA, Brown EJ. A delicate dance: host response to mycobacteria. Curr Opin Immunol 2011; 23: 464-472.
[46] Méndez-Samperio P, Belmont L, Miranda E. Mycobacterium bovis BCG Toll-like receptors 2 and 4 cooperation increases the innate epithelial immune response. Arch Med Res 2008; 39: 33-39.
[47] Santiago ME, Neto LS, Alexandre EC, Munari DP, Andrade MM, Somenzari MA, Ciarlini PC, de Lima VM. Improvement in clinical signs and cellular immunity of dogs with visceral leishmaniasis using the immunomodulator P-MAPA. Acta Trop 2013; 127: 174-180.
[48] Park J, Czapla L, Amaro RE. Molecular simulations of aromatase reveal new insights into the mechanism of ligand binding. J Chem Inf Model 2013; 53: 2047-2056.
[49] McFadyen MC, Melvin WT, Murray GI. Cytochrome P450 enzymes: novel options for cancer therapeutics. Mol Cancer Ther 2004; 3: 363-371.
[50] Karlgren M, Gomez A, Stark K, Svärd J, Rodriguez-Antona C, Oliw E, Bernal ML, Ramón y Cajal S, Johansson I, Ingelman-Sundberg M. Tumor-specific expression of the novel cytochrome P450 enzyme, CYP2W1. Biochem Biophys Res Commun 2006; 341: 451-458.