Androgen activates β -catenin signaling in bladder cancer cells

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Abstract

Androgen receptor (AR) signals have been implicated in bladder carcinogenesis and tumor progression. Activation of Wnt/ β -catenin signaling has also been reported to correlate with bladder cancer progression and poor patients' outcomes. However, cross talk between AR and β -catenin pathways in bladder cancer remains uncharacterized. In radical cystectomy specimens, we immunohistochemically confirmed aberrant expression of β -catenin especially in aggressive tumors. There was a strong association between nuclear expressions of AR and β -catenin in bladder tumors ($P=0.0215$). Kaplan–Meier and log-rank tests further revealed that reduced membranous β -catenin expression ($P=0.0276$), nuclear β -catenin expression ($P=0.0802$), and co-expression of nuclear AR and β -catenin ($P=0.0043$) correlated with tumor progression after cystectomy. We then assessed the effects of androgen on β -catenin in AR-positive and AR-negative bladder cancer cell lines. A synthetic androgen R1881 increased the expression of an active form of β -catenin and its downstream target c-myc only in AR-positive lines. R1881 also enhanced the activity of β -catenin-mediated transcription, which was abolished by an AR antagonist hydroxyflutamide. Using western blotting and immunofluorescence, R1881 was found to induce nuclear translocation of β -catenin when co-localized with AR. Finally, co-immunoprecipitation revealed androgen-induced associations of AR with β -catenin or T-cell factor (TCF) in bladder cancer cells. Thus, it was likely that androgen was able to activate β -catenin signaling through the AR pathway in bladder cancer cells. Our results also suggest that activation of β -catenin signaling possibly via formation of AR/ β -catenin/TCF complex contributes to the progression of bladder cancer, which may enhance the feasibility of androgen deprivation as a potential therapeutic approach.

Key Words

- androgen receptor
- antiandrogen
- bladder cancer
- TCF/LEF
- Wnt/ β -catenin

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Introduction

The androgen receptor (AR), a member of the nuclear receptor superfamily, mediates most of its physiological functions through transcriptional activation of downstream genes by binding to androgens (Heinlein & Chang 2004). In the presence of androgens, the AR located in the

cytoplasm dissociates from heat-shock protein and translocates to the nucleus, leading to regulation of the target genes. AR and other nuclear receptors have been detected in the urothelium and/or stromal cells of the urinary bladder, and emerging data suggest that bladder cancer is

an endocrine-related neoplasm (reviewed in Li *et al.* (2012) and Miyamoto *et al.* (2012)). AR signals have been implicated in bladder carcinogenesis and tumor progression. Specially, promising evidence further documents a critical role of AR in bladder cancer cell proliferation (Miyamoto *et al.* 2007, Johnson *et al.* 2008, Boorjian *et al.* 2009, Wu *et al.* 2010, Zheng *et al.* 2011). Nonetheless, the mechanism by which AR signaling modulates bladder cancer progression remains poorly understood.

The canonical Wnt/ β -catenin signaling pathway has been shown to play a pivotal role in normal cell growth and differentiation, embryonic development, and apoptosis (Morin 1999, Polakis 1999, Vlad *et al.* 2008). It is also proposed to be involved in the development of urogenital system (Lako *et al.* 1998). As a key component of the Wnt signaling pathway, β -catenin is a multifunctional protein and has two major pools: a membrane pool, required for cell–cell adhesion, and cytoplasmic/nuclear pool, responsible for Wnt/ β -catenin signal transduction (Miller & Moon 1996). In the absence of a Wnt signal, β -catenin is usually maintained at a low level because of being constitutively degraded via the ubiquitin proteasome pathway. Wnt signaling inhibits this process, leading to cytosolic β -catenin accumulation. Subsequently, it translocates to the nucleus, forms complexes with members of the T-cell factor (TCF)/lymphoid enhancer factor (LEF) family of transcription factors, and thereby activates target genes, such as the proto-oncogene *c-MYC*, the cell cycle activator *cyclin-D1*, and the epidermal growth factor receptor (*EGFR*; Behrens *et al.* 1996, Brabletz *et al.* 2000, Tan *et al.* 2005). Thus, growing evidence suggests an important role of Wnt/ β -catenin signaling in cell proliferation and differentiation in various types of human malignancies (Miller & Moon 1996, Polakis 2000, Lustig & Behrens 2003, Gavert & Ben-Ze'ev 2007).

Using bladder tissue specimens, differential expression of some genes encoding Wnt proteins has been detected in normal bladders, superficial tumors, and invasive tumors (Bui *et al.* 1998). Both downregulation of the Wnt antagonists (Hsieh *et al.* 2004, Stoehr *et al.* 2004) and upregulation of the Wnt target genes (Shiina *et al.* 2002) have been observed in bladder cancer tissues. It has also been shown that nuclear accumulation of β -catenin correlates with worse outcomes in patients with bladder cancer (Kastritis *et al.* 2009). These observations suggest that Wnt signaling is active in advanced urothelial tumors. Of note, the application of various small molecules that target the Wnt/ β -catenin signaling pathway led to inhibition of bladder cancer cell

proliferation (Urakami *et al.* 2006, Tang *et al.* 2009, Hirata *et al.* 2012).

It has been well documented in several cancers that Wnt/ β -catenin and AR signaling pathways are closely related. It has shown, for instance, that the AR can be activated through the Wnt/ β -catenin pathway in castration-resistant prostate cancer (Wang *et al.* 2008). However, the possible convergence between these two pathways in bladder cancer remains largely unknown. In this study, we focus on investigating the effects of androgens on β -catenin signals in AR-positive and AR-negative bladder cancer cells. To the best of our knowledge, this is the first report to show androgens/AR-mediated activation of Wnt/ β -catenin signaling in bladder cancer cells.

Materials and methods

Bladder tissue microarray and immunohistochemistry

Bladder tissue microarray (TMA) was constructed from 24 formalin-fixed paraffin-embedded cystectomy specimens retrieved from the Surgical Pathology archives, as described previously (Zheng *et al.* 2011, Izumi *et al.* 2013). Appropriate approval from the Institutional Review Board of the University of Rochester Medical Center was obtained before construction and use of the TMA. These 24 patients included 19 men and five women, with a mean age at cystectomy of 66.2 years (range: 49–86 years) and a mean follow-up after the surgery of 11.4 months (range: 3–24 months). All the tumors were histologically diagnosed as high-grade urothelial carcinoma. These included 5 $\leq$ pT1, 19 $\geq$ pT2, 12 pN0, and 12 pN+ tumors.

Immunohistochemical staining was performed on the sections (5 μ m thick) from the bladder TMA, as described previously (Zheng *et al.* 2011, Izumi *et al.* 2013), with minor modifications. Briefly, tissues were deparaffinized in xylene, rehydrated in a graded ethanol series, and incubated in 3% hydrogen peroxide to block endogenous peroxidase. Slides were incubated overnight at 4 °C with an anti-AR (clone N20; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or an anti- β -catenin antibody (clone β -catenin-1, Dako, Carpinteria, CA, USA). The samples were then incubated with a broad-spectrum secondary antibody (Invitrogen, Carlsbad, CA, USA). After being rinsed in PBS, the slides were incubated with diaminobenzidine (Invitrogen) and finally counterstained with hematoxylin. These stains were manually quantified by one pathologist (H M) blinded to sample identity. The expression of β -catenin in cancer cells was classified as Hu *et al.* (2011) described: >70% of cell membranes

stained as normal, otherwise as reduced, and >10% of nuclei or cytoplasm stained as positive.

Cell culture and chemicals

Human urothelial carcinoma cell lines, UMUC3, 5637, and J82, obtained from the American Type Culture Collection (Manassas, VA, USA) were cultured in DMEM (Mediatech, Manassas, VA, USA) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 units/ml penicillin and 100 μ g/ml streptomycin) at 37 °C in a humidified atmosphere of 5% CO₂. At least 18 h before experimental treatment, cells were cultured in phenol red-free DMEM (Mediatech) supplemented with 5% charcoal-stripped FBS. Methyltrienolone (R1881) was purchased from PerkinElmer (Waltham, MA, USA), and hydroxyflutamide (HF) was from Schering (Kenilworth, NJ, USA).

Stable cell lines with AR and AR-short hairpin RNA

Cell lines stably expressing a full-length wild-type human AR (5637-AR and J82-AR) or vector only (5637-V and J82-V) were established using a lentivirus vector (pWPI-AR or pWPI-control) with psPAX2 envelope and pMD2.G packaging plasmids, as we described previously (Zheng *et al.* 2011, Izumi *et al.* 2012). Similarly, stable AR knock-down/control cell lines (UMUC3-AR-short hairpin RNA (shRNA)/UMUC3-control-shRNA) were established with a retrovirus vector pMSCV/U6-AR-shRNA or pMSCV/U6-control-shRNA (Miyamoto *et al.* 2007, Zheng *et al.* 2011).

Reporter gene assay

Cells at a density of 50–60% confluence in 24-well plates were co-transfected with 250 ng Topflash reporter plasmid DNA (plasmid 12456 M50 Super 8 $\times$ TOPFlash containing 7 TCF/LEF binding sites, Addgene, Cambridge, MA, USA) or a control Fopflash reporter plasmid DNA (plasmid 12457 M51 Super 8 $\times$ FOPFlash containing six mutated TCF/LEF binding sites, Addgene) along with 2.5 ng pRL-TK renilla luciferase plasmid DNA, using GeneJuice transfection reagent (Novagen, Gibbstown, NJ, USA). After 6 h of transfection, the cells were cultured in the presence or absence of ligands (R1881, HF, or both) for 24 h. Cell lysates were then assayed for luciferase activity determined using a Dual-Luciferase Reporter Assay kit (Promega, Madison, WI, USA) and luminometer (TD-20/20, Turner BioSystems, Sunnyvale, CA, USA). The ratio of Topflash:Fopflash values normalized to renilla was used as an indicator of Wnt/ β -catenin activity.

Western blot

Protein extraction and western blot were performed, as described previously (Izumi *et al.* 2012), with minor modifications. Separate cytoplasmic and nuclear protein fractions were obtained, using NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific, Rockford, IL, USA). Equal amounts of protein obtained from cell extracts were separated by 10–12% SDS-PAGE and transferred to polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA) by electroblotting using a standard protocol. Specific antibody binding was detected using an anti-AR (clone N20; diluted 1:2000), an anti- β -catenin antibody (clone 14/ β -catenin; diluted 1:2000; BD Bioscience, Franklin Lakes, NJ, USA), an anti-active- β -catenin (clone 8E7; diluted 1:1000; Millipore), an anti-c-myc antibody (clone Y69; diluted 1:1000; Epitomics, Burlingame, CA, USA), an anti-Histone H1 antibody (clone FL-219; diluted 1:1000; Santa Cruz Biotechnology), or an anti-GAPDH antibody (clone 6C5; diluted 1:1000; Santa Cruz Biotechnology), with HRP detection system (Super-Signal West Pico Chemiluminescent Substrate; Thermo Scientific).

RT and real-time PCR

Total RNA (1.0 μ g) isolated from cultured cells, using TRIzol (Invitrogen), was reverse transcribed using 1 μ mol/l oligo (dT) primers and four units of Omniscript reverse transcriptase (Qiagen) in a total volume of 20 μ l. Real-time PCR was then performed in 15 μ l system using SYBR GreenER qPCR SuperMix for iCycler (Invitrogen), as described previously (Zheng *et al.* 2011). The primer sequences are given as follows: β -catenin (forward, 5'-AAGTGGGTGGTATAGAGGCTCTTG-3'; reverse, 5'-GATGGCAGGCTCAGTGATGTC-3') and c-myc (forward, 5'-ACCAGATCCCGGAGTTGGAA-3'; reverse, 5'-CGTCGTTTCCGCAACAAGTC-3'). GAPDH (forward, 5'-AAGGTGAAGGTCGGAGTCAAC-3'; reverse, 5'-GGGGTCATTGATGGCAACAATA-3') was used as an internal control.

Co-immunoprecipitation

Cells were treated with ethanol or R1881 for 24 h, and protein (500 μ g) from the cell lysates was incubated with 2 μ g anti-AR rabbit polyclonal antibody (clone N20)/anti- β -catenin mouse MAB (clone 14/ β -catenin) or normal rabbit/mouse IgG (Santa Cruz Biotechnology) overnight at 4 °C with agitation. To each sample, we added 20 μ l protein A/G-agarose beads (Santa Cruz Biotechnology), incubated for 2 h, and washed four to five times with

Figure 1

IHC of β -catenin (A, B and C) and AR (D) in bladder tissues (magnification: $\times 400$) and progression-free survival rates (E, F, G and H) in patients with high-grade urothelial carcinoma. (A) β -Catenin expression in cell membrane of benign urothelium. (B) Reduced membranous β -catenin expression in urothelial carcinoma. Predominant nuclear expression of

β -catenin (C; arrowheads) and AR (D) in the same tumor. Kaplan-Meier analysis was performed according to the expression of membranous β -catenin (E), cytoplasmic β -catenin (F), nuclear β -catenin (G), or nuclear β -catenin and AR (H), and comparisons were made by log-rank test.

radioimmunoprecipitation assay buffer. Then, we resolved the complex on a 10–12% SDS-PAGE, transferred to the membrane, and blotted with an anti-AR antibody (clone N20; diluted 1:2000), an anti- β -catenin antibody (clone 14/ β -catenin; diluted 1:2000), or an anti-TCF4 antibody (clone EP2033Y; diluted 1:2000; Millipore).

Immunofluorescent staining

Cells were plated onto chamber slides (eight-well Thermo Scientific Nunc Lab-Tek) for immunostaining. After 12 h of seeding, the cells were cultured in DMEM with 5% charcoal-stripped FBS containing ethanol or R1881 for 24 h. At the end of drug treatment, culture medium was aspirated from each well, and the adherent cells were rinsed thrice with PBS and then fixed by 4% paraformaldehyde for 15 min at room temperature. After being washed with 0.1 M glycine for 20 min, the slides were kept in 1% Triton X-100 for 20 min at room temperature. Then, the cells were blocked with blocking buffer for 1 h at 37 °C. A primary antibody was incubated at 4 °C overnight, and Alexa 488- or 568-conjugated secondary antibody (diluted 1:200, Invitrogen) was added for 1 h at 37 °C. DAPI was used to visualize nuclei. Fluorescence images were acquired with an Olympus FV1000 confocal microscope. The number of nuclear staining per visual field was quantified in five randomly selected visual fields per chamber (total 900 cells) by a single observer who was unaware of the treatment group for the cells.

Statistical analyses

Student's *t*-test was used to analyze differences in relative Top/Fop luciferase activities and relative numbers of immunofluorescent staining between the two groups. Differences in protein expression between the two groups from human tissue samples were analyzed by Fisher's exact test. All these statistical tests were two sided. Progression-free survival rates in patients were calculated by the Kaplan-Meier method and comparison was made by log-rank test. *P* value <0.05 was considered statistically significant.

Results

Immunoreactivity in bladder cancer tissue samples

We performed immunohistochemical stains for β -catenin in 24 radical cystectomy specimens of high-grade urothelial carcinoma. Coexisting benign urothelium exhibited β -catenin reactivity mainly with a membranous staining pattern (Fig. 1A), whereas positive signals were also detected in the nucleus and/or cytoplasm of some cancer cells. The expression of membranous and nuclear β -catenin in relation to pathological stages is summarized in Table 1. Overall, 15 (67.5%), eight (33.3%), and nine (37.5%) cancer cases showed reduced membranous expression (Fig. 1B), ectopic cytoplasmic expression, and ectopic nuclear expression (Fig. 1C) respectively. Deep

Table 1 Expression of β -catenin in bladder tissue microarrays.

	n	Membrane			Cytoplasm			Nucleus		
		Normal	Reduced ^a	P value ^b	Negative	Positive ^c	P value ^b	Negative	Positive ^c	P value ^b
All cases	24	9 (37.5%)	15 (62.5%)		16 (66%)	8 (33%)		15 (62.5%)	9 (37.5%)	
Stage (pT)				0.0474			1.0000			0.3256
≤pT1	5	4 (80%)	1 (20%)		3 (60%)	2 (40%)		2 (40%)	3 (60%)	
≥pT2	19	5 (26%)	14 (74%)		13 (68%)	6 (32%)		13 (68%)	6 (32%)	
Lymph node metastasis				0.4003			0.6668			1.0000
pN0	12	6 (50%)	6 (50%)		9 (75%)	3 (25%)		8 (67%)	4 (33%)	
pN+	12	3 (25%)	9 (75%)		7 (58%)	5 (42%)		7 (58%)	5 (42%)	
AR expression				0.1782			1.0000			0.0215
Negative	16	8 (50%)	8 (50%)		11 (69%)	5 (31%)		13 (81%)	3 (19%)	
Positive	8	1 (13%)	7 (87%)		5 (62.5%)	3 (37.5%)		2 (25%)	6 (75%)	

^a<30% of cancer cells showed immunoreactivity.^bFisher's exact test.^c>10% of cancer cells showed immunoreactivity.

invasion ($\geq$ pT2) was strongly associated with reduced membranous β -catenin compared with non-muscle-invasive tumors ($P=0.0474$), but not with cytoplasmic ($P=1.0000$) or nuclear ($P=0.3256$) β -catenin. There were no statistically significant differences in the expression pattern of β -catenin between male vs female tumors and between node-negative vs node-positive tumors. AR expression had also been immunohistochemically analyzed in these 24 bladder cancer specimens, including eight (33.3%) AR-positive cases (Fig. 1D; Zheng et al. 2011). Interestingly, there was a strong correlation of nuclear AR positivity with nuclear β -catenin expression ($P=0.0215$), but not with membranous ($P=0.1781$) or cytoplasmic ($P=1.0000$) β -catenin expression.

To assess possible associations between β -catenin staining and disease progression, we then performed the Kaplan–Meier analysis coupled with log-rank test. Of the 24 patients, eight (33.3%) developed local recurrence and/or metastasis after radical surgery. There was a strong association of reduced membranous expression ($P=0.0276$; Fig. 1E), but not of cytoplasmic expression ($P=0.7532$; Fig. 1F), with poorer prognosis. Furthermore, nuclear β -catenin expression alone ($P=0.0802$; Fig. 1G) or co-expression of nuclear AR and β -catenin ($P=0.0043$; Fig. 1H) strongly correlated with tumor progression after cystectomy. Based on these findings, we anticipated that cross talk between AR and β -catenin signals contributed to the progression of bladder cancer.

Androgen upregulates the expression of active- β -catenin

We then investigated the effects of androgens on the expression of β -catenin in bladder cancer cells. Western

blotting was performed in the stable cell lines with or without AR (i.e. UMUC3-control-shRNA vs UMUC3-AR-shRNA, 5637-AR vs 5637-V, and J82-AR vs J82-V) in the presence or absence of a synthetic androgen R1881 and an AR antagonist HF. Each cell line was found to strongly express β -catenin, and no significant differences in total β -catenin expression among the different treatment groups were observed (Fig. 2A). In contrast, R1881 considerably increased the expression of both an active form of β -catenin and its downstream target c-myc only in AR-positive cells. As expected, HF showing marginal or partial agonist activity could, at least partially, abolish the effects of R1881. A quantitative RT-PCR was also performed to determine whether androgen alters β -catenin and c-MYC gene expression in these cell lines. Correlating with the expression of c-MYC protein, R1881 increased its mRNA levels by 59%/57%/38% in UMUC3-control-shRNA/5637-AR/J82-AR respectively but not in AR-negative lines (Fig. 2B). HF significantly antagonized the effects of R1881 on c-MYC expression in AR-positive cells. Additionally, treatment with R1881 and/or HF resulted in marginal changes in total β -catenin mRNA expression. These results suggest that not only androgens may be able to activate β -catenin in bladder cancer cells but also AR is likely necessary for androgenic upregulation of active- β -catenin and c-MYC expression.

Androgen enhances β -catenin/TCF/LEF1 transcriptional activity

To further confirm whether Wnt/ β -catenin signaling is activated by androgens in bladder cancer cells,

Figure 2

Effects of androgen and antiandrogen on β -catenin expression in bladder cancer cells. Bladder cancer lines (UMUC3-control-shRNA/AR-shRNA, 5637-AR/Vector, and J82-AR/Vector) cultured for 24 h in the presence of ethanol, 10 nM R1881, and/or 10 μ M HF were analyzed on western blotting (A) and quantitative RT-PCR (B). (A) Equal amounts of protein (30–50 μ g) extracted from each cell line were immunoblotted for AR (110 kDa), β -catenin (92 kDa), active- β -catenin (ABC, 92 kDa), and c-myc (57 kDa). GAPDH (37 kDa) served as an internal control. Densitometry values for specific bands standardized by GAPDH that are relative to those of mock

treatment (first lanes; set as onefold) from at least three independent experiments are included below the lanes. * P < 0.05 (vs mock treatment). # P < 0.05 (vs R1881 only). (B) RNA extraction and subsequent real-time RT-PCR for β -catenin and c-MYC were performed, and the expression of each gene was normalized to that of GAPDH. Transcription amount is presented relative to that of mock treatment in each cell line (first lanes; set as onefold). Each value represents an average and s.d. from at least three independent experiments. * P < 0.05 (vs mock treatment). ** P < 0.01 (vs mock treatment). # P < 0.05 (vs R1881 only). ## P < 0.01 (vs R1881 only).

luciferase reporter assays were performed in the cell extracts with transfection of a plasmid harboring optimal TCF/LEF binding sites (Topflash) or mutated TCF/LEF binding sites (Fopflash) as a negative control and treatment of R1881 and/or HF. R1881 increased Topflash/Fopflash activity in AR-positive cells (UMUC3-control-shRNA (1.23-fold; P < 0.01), 5637-AR (1.65-fold; P < 0.05), and J82-AR (2.73-fold; P < 0.01)), compared with respective mock treatments (Fig. 3). HF showing marginal agonist activity could block R1881-induced transactivation in these lines (all P < 0.05). Conversely, in AR knockdown or negative lines, R1881 and HF failed to impact the activity of β -catenin/TCF/LEF1 transcription. Thus, these results suggest that androgen upregulates

Wnt/ β -catenin signaling via the AR pathway in bladder cancer cells.

Androgen induces nuclear translocation of β -catenin and its co-localization with AR

To investigate whether AR signals promote nuclear translocation of β -catenin in bladder cancer cells, as shown in prostate cancer and neuronal cells (Mulholland et al. 2002, Pawlowski et al. 2002, Yang et al. 2002), western blotting was first performed using nuclear and cytoplasmic fractions obtained from AR-positive 5637-AR or UMUC3 cells cultured with different concentrations of R1881 for 24 h. Accumulations of nuclear β -catenin were seen upon

Figure 3

Effects of androgen and antiandrogen on β -catenin transactivation in bladder cancer cells. Bladder cancer lines (UMUC3-control-shRNA/AR-shRNA, 5637-AR/Vector, and J82-AR/Vector) co-transfected with a Topflash or Fopflash luciferase reporter plasmid were cultured for 24 h in the presence of ethanol, 10 nM R1881, and/or 10 μ M HF. Luciferase activity

analyzed in a luminometer is presented relative to that of mock treatment in each cell line (first lanes; set as onefold). Each value represents an average and s.d. from at least three independent experiments. * $P < 0.05$ (vs mock treatment). ** $P < 0.01$ (vs mock treatment). # $P < 0.05$ (vs R1881 only). ## $P < 0.01$ (vs R1881 only).

R1881 treatment in a dose-dependent manner, although decreases in cytoplasmic β -catenin were mostly modest (Fig. 4A). HF antagonized the effect of R1881 on nuclear expression of β -catenin in UMUC3 cells (Fig. 4B).

To further assess androgen-induced nuclear translocation of β -catenin and its co-localization with AR, we performed immunofluorescent staining in three bladder cancer cell lines. As is well known in non-bladder cells, non-ligand-bound AR predominantly expressed in the cytoplasm was translocated into the nucleus of 5637-AR, J82-AR, and UMUC3-control-shRNA cells in the presence of R1881 (Fig. 4C). Unlike neuronal cells, β -catenin was predominantly distributed in the membrane and cytoplasm of bladder cancer cells in the absence of ligand, with roughly 10% in the nucleus (Fig. 4D). After R1881 treatment, nuclear β -catenin expression was seen in 20–42% of AR-positive cells, while membranous β -catenin staining was not significantly altered. Importantly, there were slight decreases in cytoplasmic β -catenin staining in R1881-treated cells compared with untreated cells. Consistent with previous studies in non-bladder cells (Mulholland *et al.* 2002, Pawlowski *et al.* 2002, Yang *et al.* 2002), co-localization of β -catenin and AR in the nucleus was observed in 57–72% of the R1881-treated cells with nuclear staining of β -catenin. By contrast, no R1881-induced nuclear β -catenin expression was seen in AR-negative or AR knockdown bladder cancer cells.

Androgen induces AR association with β -catenin and TCF

Having found evidence for co-localization of AR and β -catenin in the nuclei of bladder cancer cells, we finally

performed co-immunoprecipitation to detect the physical interactions between AR and β -catenin or TCF. Protein extracts were immunoprecipitated with AR or β -catenin, and the immunoprecipitates were analyzed by immunoblotting using anti- β -catenin, anti-AR, or anti-TCF4 antibody (Fig. 5). In AR-positive cells, minimal interactions between AR and β -catenin, AR and TCF4, or β -catenin and TCF4 were seen in the absence of androgens, and R1881 treatment significantly enhanced these interactions. β -Catenin–TCF4 interaction was detected in AR-negative cells, but it was not altered by R1881 treatment. Thus, androgens appeared to induce complex formation among AR, β -catenin, and TCF4 in bladder cancer cells.

Discussion

We and others have documented that AR signals have stimulatory effects on bladder cancer cell proliferation (Miyamoto *et al.* 2007, Johnson *et al.* 2008, Boorjian *et al.* 2009, Wu *et al.* 2010, Zheng *et al.* 2011, Izumi *et al.* 2012). Dysregulation of the Wnt/ β -catenin signaling pathway has also been linked to bladder cancer growth (Bui *et al.* 1998, Shiina *et al.* 2002, Hsieh *et al.* 2004, Stoehr *et al.* 2004, Urakami *et al.* 2006, Kastiris *et al.* 2009, Hirata *et al.* 2012). However, cross talk between the AR and Wnt/ β -catenin pathways in bladder cancer cells remains unclear, although it has been well studied in prostate cancer (Chesire & Isaacs 2003, Wang *et al.* 2008). This study demonstrated molecular evidence for the involvement of AR signals in dysregulation of the Wnt/ β -catenin pathway in bladder cancer cells. First, immunohistochemistry (IHC) in bladder

Figure 4

Effects of androgen on nuclear translocation of β -catenin and its co-localization with AR in bladder cancer cells. Cell lysates from 5637-AR or UMUC3 (A) cultured for 24 h in the presence of increasing amounts of R1881 or those from UMUC3 (B) cultured for 24 h in the presence of ethanol, 1 nM R1881, and/or 10 μ M HF were fractionated into cytoplasmic and nuclear components and immunoblotted for β -catenin (92 kDa). GAPDH (37 kDa) and histone-H1 (32–33 kDa) served as internal controls for cytoplasmic and nuclear proteins respectively. Densitometry values for

specific bands standardized by GAPDH or histone-H1 that are relative to those of mock treatment (first lanes; set as onefold) are included below the lanes. (C) Cells (5637-AR/Vector, J82-AR/Vector, UMUC3-control-shRNA/AR-shRNA) treated with ethanol (mock) or 10 nM R1881 for 24 h were analyzed on immunofluorescence, using an antibody to β -catenin or AR. DAPI was used to visualize nuclei. (D) Nuclear expression of β -catenin and AR was quantified. Each value represents an average and s.d. of triplicates. * $P < 0.01$.

cancer tissues suggested a strong association between nuclear expression of AR and β -catenin, and their co-expression precisely predicted tumor progression. Secondly, in AR-positive bladder cancer cells, androgen

likely activated β -catenin via increases in protein expression of its active form or a Wnt target c-myc, β -catenin/TCF/LEF1 transactivation, and nuclear translocation of AR and β -catenin. Finally, we showed androgen-mediated complex

Figure 5

Effects of androgen on AR/ β -catenin/TCF4 associations. Cells (J82-Vector, J82-AR, 5637-AR) were cultured for 24 h in the presence of ethanol (mock) or 10 nM R1881. Cell lysates immunoprecipitated with anti-AR antibody/normal rabbit IgG or anti- β -catenin antibody/normal mouse IgG were then immunoblotted for rabbit anti-AR (110 kDa), mouse anti- β -catenin

(92 kDa), or rabbit anti-TCF4 (66–72 kDa). Standardized densitometry values for specific blots, compared to mock treatment (set as onefold), from three independent experiments are included below the lanes. * $P < 0.05$ (vs mock treatment).

formation involving AR, β -catenin, and TCF4 in bladder cancer cells.

There are dissenting data as to the correlation of β -catenin staining in bladder cancer with tumor aggressiveness (Garcia del Muro *et al.* 2000, Nakopoulou *et al.* 2000, Zhu *et al.* 2000, Stoeher *et al.* 2002, Kastiris *et al.* 2009). The discrepancy may have resulted from the use of different antibodies and methodologies. Consistent with a previous study (Zhu *et al.* 2000), downregulation of membranous β -catenin expression in bladder cancer compared with non-neoplastic urothelium was detected in our cohort. Our current data also corroborated the demonstration that loss or reduced expression of membranous β -catenin was associated with worse outcome (Garcia del Muro *et al.* 2000, Nakopoulou *et al.* 2000). Nuclear accumulation of β -catenin, as a hallmark of Wnt/ β -catenin activation (Chesire & Isaacs 2003), has been shown to correlate with lymph node involvement and poor prognosis (Kastiris *et al.* 2009). Consistent with these findings, our data showed a trend to associate

between nuclear β -catenin expression and a risk of tumor progression after cystectomy, while there was no relationship between lymph node metastases and the status of β -catenin expression in the nucleus as well as the membrane or cytoplasm, possibly due to relatively small number of cases. It was noteworthy that aberrant accumulation of nuclear β -catenin in conjunction with nuclear AR positivity was a more reliable poor prognosticator. AR expression was also closely correlated with the presence of nuclear β -catenin, but not membranous or cytoplasmic β -catenin, in bladder cancer tissues. Further IHC studies including larger patient cohorts with longer follow-up are needed to validate these preliminary findings of co-expression of AR and β -catenin in bladder cancer and its relationship with patients' outcomes. Nevertheless, the current data suggest that cross talk between the Wnt/ β -catenin and AR pathways contributes to bladder cancer progression.

Our data in cultured cell lines may provide convincing evidence of the cross talk in bladder cancer. As seen in

prostate cancer (Chesire & Isaacs 2003, Wang *et al.* 2008), we anticipated that androgens regulated the expression of β -catenin and its nuclear translocation in bladder cancer cells, which could result in modification of β -catenin/TCF/LEF1 signaling and ultimately activate or inactivate target genes. Downstream components of the canonical Wnt/ β -catenin signaling pathway, such as c-myc, cyclin-D1, and EGFR, have been implicated in several human malignancies including bladder cancer (Behrens *et al.* 1996, Brabletz *et al.* 2000, Tan *et al.* 2005), although the possibility remains that some of these are not direct targets *in vivo* and there are direct target genes relevant to bladder cancer. Western blots showed that androgen induced the expression of an active form of β -catenin, but not total β -catenin, only in AR-positive bladder cancer cells. Our β -catenin/TCF/LEF1 luciferase reporter assay then confirmed that androgen/AR enhanced β -catenin-mediated transactivation. Importantly, an antiandrogen HF could antagonize all these androgen effects in AR-positive bladder cancer cells. We also showed enhanced expression of c-myc at both mRNA and protein levels in androgen-treated cells. The *c-MYC* gene has indeed been found to correlate with the proliferation of bladder cancer cells (Lipponen 1995, Schmitz-Drager *et al.* 1997). Androgen/AR-mediated upregulation of other Wnt targets, including cyclin-D1 (Wu *et al.* 2010) and EGFR (Zheng *et al.* 2011), has also been demonstrated in bladder cancer cells. Furthermore, using immunofluorescence and western blotting, we validated AR-induced nuclear translocation of β -catenin, which has been investigated in several other types of cells (Mulholland *et al.* 2002, Pawlowski *et al.* 2002, Yang *et al.* 2002, Singh *et al.* 2006), in bladder cancer cells with endogenous or exogenous AR. Overall, available data suggest that AR activation positively modulates the Wnt/ β -catenin pathway in bladder cancer cells.

It is well known that nuclear β -catenin is able to interact with not only TCF/LEF1 but also AR. Androgens have been shown to inhibit adipogenic differentiation (Singh *et al.* 2006) and promote myogenic differentiation (Singh *et al.* 2009) of mesenchymal multipotent cells through inducing AR association with β -catenin or TCF4. Consistent with these findings in non-bladder cells, our results indicate that AR activation induces nuclear accumulation of β -catenin, leading to interactions among β -catenin, TCF4, and AR. Surprisingly, weak associations of AR/ β -catenin/TCF4 were detected without androgen treatment. There are several possibilities underlying this observation. First, remaining androgens in charcoal-stripped FBS (Sedelaar & Isaacs 2009) used for

our cell culture led to AR activation since as low as 0.1 nM dihydrotestosterone was shown to regulate the growth of prostate cancer cell lines with endogenous or over-expressed AR (Mizokami *et al.* 2004, Waltering *et al.* 2009). Secondly, AR could be activated by non-androgenic compounds, such as growth factors (Culig *et al.* 1994). We recently found that EGF promoted the growth of bladder cancer cells via the AR pathway (Izumi *et al.* 2012). Thirdly, AR/ β -catenin/TCF4/LEF1 might be able to form complexes in the cytoplasm. In some malignancies, substantial amounts of TCF4/LEF1 have been reported to localize to the cytoplasm (Shair *et al.* 2009, Tian *et al.* 2009). Our immunofluorescent staining showed co-localization of AR and β -catenin in the cytoplasm of some bladder cancer cells without adding androgens. Of note in the current study was that androgen further induced interactions of AR- β -catenin, AR-TCF4, and β -catenin-TCF4.

Despite the fact that the mechanism by which AR regulates β -catenin signaling has been elaborated in prostate cancer (Wang *et al.* 2008), some of the findings in AR-positive prostate vs bladder cancers were in disagreement. For instance, we observed upregulation, rather than downregulation, of β -catenin-mediated transcription by AR signals in bladder cancer. Our co-immunoprecipitation assays suggested physical interactions of not only AR- β -catenin but also AR-TCF4, in addition to β -catenin-TCF4 association, in bladder cancer cells, while in prostate cancer, competition for β -catenin could occur between AR and TCF/LEF1 (Mulholland *et al.* 2003). Mapping studies in non-bladder cells have indeed demonstrated that AR and TCF4 have overlapping binding sites on β -catenin and compete for binding (Yumoto *et al.* 2011). Taken together, our results form the basis of the following hypothetical model in bladder cancer cells. In the absence of androgens, AR and a portion of β -catenin are located in the cytoplasm, while β -catenin also resides in the membrane. Androgen-bound AR interacts with β -catenin and induces their nuclear translocation. In the nucleus, β -catenin-bound AR further interacts with TCF/LEF1 and thereby stimulates transcription of various Wnt/ β -catenin target genes, leading to the promotion of bladder cancer cell growth. Mechanistic studies, such as mapping that may identify bladder-specific AR-binding sites, are required to further elucidate the role of β -catenin and TCF/LEF1 in relation to AR signals in bladder cancer progression.

In conclusion, we demonstrate, for the first time, that androgen activates Wnt/ β -catenin signaling through the AR pathway in bladder cancer cells. Our data not only

suggest that androgen-induced β -catenin/TCF/LEF1 activity, possibly via formation of their complex involving AR, contributes to the regulation of bladder cancer progression in a specific manner but also provide further evidence enhancing the feasibility of androgen deprivation that may interfere with the complex formation as a potential therapeutic approach against bladder cancer.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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