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Depletion of androgen receptor low molecular weight isoform reduces bladder tumor cell viability and induces apoptosis

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ABSTRACT

Bladder cancer (BlCa) exhibits a gender disparity where men are three times more likely to develop the malignancy than women suggesting a role for the androgen receptor (AR). Here we report that BlCa cells express low molecular weight (LMW) AR isoforms that are missing the ligand binding domain (LBD). Isoform expression was detected in most BlCa cells, while a few express the full-length AR. Immunofluorescence studies detect AR in the nucleus and cytoplasm, and localization is cell dependent. Cells with nuclear AR expression exhibit reduced viability and increased apoptosis on total AR depletion. A novel AR-LMW variant, AR-v19, that is missing the LBD and contains 15 additional amino acids encoded by intron 3 sequences was detected in most BlCa malignancies. AR-v19 localizes to the nucleus and can transactivate AR-dependent transcription in a dose dependent manner. AR-v19 depletion impairs cell viability and promotes apoptosis in cells that express this variant. Thus, AR splice variant expression is common in BlCa and instrumental in ensuring cell survival. This suggests that targeting AR or AR downstream effectors may be a therapeutic strategy for the treatment of this malignancy.

1. Introduction

BlCa in the US is estimated to account for ~81,400 new cases of cancer and ~17,980 deaths in 2020 [1]. While non-muscle invasive disease is more prevalent and treatable, tumor recurrence is common and can lead to muscle invasive cancer [2]. Muscle invasive BlCa has a far higher likelihood of metastasis that ultimately leads to death [3]. One outstanding feature of BlCa is a gender disparity where men are 3–4 times more likely to develop BlCa than women [1]. Reasons proposed for this disparity include environmental as well as lifestyle factors such as smoking [4]. However, the gender disparity is apparent even when studies controlled for these variables [4]. Although BlCa is not traditionally thought of as an endocrine-related cancer, evidence is accumulating that AR signaling contributes to this disease.

The AR belongs to the steroid receptor family of transcription factors, and is essential for mediating androgen (testosterone and dihydrotestosterone) signaling [5,6]. Most AR studies have been conducted in prostate cancers, where the ligand regulated transcription factor is pivotal to the development and progression of these malignancies. Studies show that in prostate cells, the AR has a role in proliferation, motility, DNA repair and cell death [7]. Mechanistic studies reveal that when androgen interacts with the heat shock protein-bound AR, the receptor undergoes a conformational change resulting in AR release from the heat shock proteins, and subsequent phosphorylation, dimerization and translocation into the nucleus [6,8]. Once in the nucleus the AR, along with co-factors, bind to androgen response elements to regulate gene expression [8].

AR activity can be limited by androgen deprivation therapy for the treatment of prostate cancer. These therapies are initially successful, but eventually tumors recur, often with restored AR signaling. Restoration of AR singling may occur via several mechanisms. The AR consists of an N-terminal domain, a DNA-binding domain, a hinge region and a C-

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terminal ligand-binding domain (LBD) [6]. Alternately spliced variants of the AR may arise that leads to low molecular weight (LMW) isoforms [6], which are missing the LBD and can regulate gene expression in the absence of hormone [9]. LMW AR isoforms, particularly the AR-V7 splice variant, have been linked to fatal androgen independent forms (called castrate resistant) of prostate cancer [10–14] and are thought to be a key mechanism by which tumors restore AR function post androgen deprivation therapy.

Multiple reports argue that the AR has a role in malignancies that are not considered endocrine cancers including kidney, lung, breast, liver and bladder [5,15–17]. Early studies showed that in bladder cancers AR expression is higher in tumor than in non-tumor bladder tissue and AR levels are higher in bladder tumors from males than from females [18], and recent meta-analysis of immunohistochemical studies concluded that AR positivity correlated with gender [19]. Animal models have supported the notion that androgens contribute to bladder carcinogenesis. Supplementation of female rats with testosterone promotes N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN) induced bladder tumors [20] and urothelium targeted AR knockout mice have a reduced incidence of BBN induced tumors [21]. In the β-catenin driven model of bladder tumorigenesis, males develop tumors at a rate of 45% compared with 3% for females [22]. Moreover, an immunohistochemical assessment of human tumor tissue revealed that AR staining is more intense and localizes to the nucleus in tumors from males than females [22]. Together, these studies argue that the AR is a likely participant in bladder tumorigenesis and that AR signaling may be a key factor in the predilection of males to bladder cancer.

Here we report that most bladder tumor-derived cells express varying levels of AR LMW isoforms while a few express the full-length AR (FL-AR). siRNA mediated depletion of all AR isoforms reduces cell viability of bladder tumor cells and induces apoptosis in cells with nuclear AR expression. A newly identified splice variant AR-v19, is expressed in most tumor-derived cell lines and primary malignancies. AR-v19 localizes to the nucleus and transactivates transcription from an AR-dependent promoter in a dose dependent manner. AR-v19 siRNA mediated depletion reduces cell viability and induces apoptosis. Our studies show that LMW AR isoforms are commonly expressed in bladder tumor cells and have a role in bladder tumor cell viability.

2. Materials and methods

2.1. Cell culture, plasmids, siRNA, and transfections

UM-UC-3, TCCSUP, J82, T24, HT1197, HT1377, RT4, SW780, 5637, LNCaP, and 22Rv1 cells, were obtained from ATCC (Manassas, VA). All cell lines were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), mmol/liter glutamine and 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen/Thermo Fisher Scientific; Waltham, MA) at 37 °C and 5% CO2. Cells used in studies were under passage 20 from the time they were received from ATCC. siRNA transfections were carried out using Lipofectamine RNAiMAX (Invitrogen) or PolyPlus (at an oligonucleotide concentration of 50 nM unless otherwise specified). The following oligonucleotides from Dharmacon (Thermo Scientific) were used for transfection; siAR-smart pool (L003400-00), control non-targeting oligonucleotide (D-001210-01), LBD targeting (J-003400-06) [11], and AR-v19 specific siRNAs designed by us A (GGGAUGACUCUGGGAGGGUUUUU); B (GAUGACUCUGGGAGGGUC CUU). Enzalutamide was obtained from Selleckchem (Houston, TX).

2.2. RNA extraction and PCR analysis

Snap frozen human bladder tissue was used for the preparation of RNA. RNA was extracted using the RNeasy kit (Qiagen, 74106) and cDNA was generated using QuantiTect (Qiagen; Germantown, MD) reverse transcription based on the manufacturer's protocol. RT PCR was performed using the following primers from Invitrogen: total AR (F: TGGATAGCTACTCCGGACCTT, R: CCCAGAAGCTTCATCTCCAC), AR-LBD (F: GCCTTGCTCTCTAGCCTCAA, R:GTCGTCCACGTGTAAGTTGC), V7 (F: CCATCTTGTCGTCTTCGGAAATGTTATGAAGC, R: TTTGAATGAGGCAAGTCAGCCTTTCT), AR-v19 (F: TGAAGCAGGGATGACT CTGG, R:CCAAATCGAAGTGGATTAATGA) and HPRT (F: GCCAGACTTTGTTGGATTTG, R: CTCTCATCTTAGGCTTTGTATTTTG). The standard curves were generated for each primer set using serial dilutions of cDNA to standardize variations in PCR reactions. In addition, after each run, melting curves were used to verify the melting temperature of the amplicon. All qPCR reactions were performed in triplicates. HPRT levels served as a quantification control. cDNAs were in ddH2O and added to QuantiTech SYBR Green PCR master mix (Qiagen) and 200 nM of each primer. PCR conditions had an initial denaturation step at 95 C for 20 min 30 s, 40 cycles at 95 C for 13 s. Data were collected by the Mastercycler ep Realplex (Eppendorf AG, Hamburg, Germany). For direct visualization PCR amplified products were electrophoresed on 3% agarose gels and the DNA was visualized by staining with GelRed (Biotium; Fremont, CA).

2.3. Viability assays

Cells were plated at 10,000–20,000 cells/well in a 12 well plate and 24 h later treated with 10uM ENZ or DMSO control for 2 or 4 days. Viability was assessed using Cell Counting Kit – 8 (CCK-8) following manufacturer's recommendations (Dojindo; Rockville, MD). All experiments were performed in triplicate. Data is displayed as the mean ± standard deviation.

For experiments assessing the growth of cells transfected with total AR siRNA, cells were plated in a 24 well plate at 10,000–20,000 cells/well and transfected 24 h later with a control oligonucleotide or AR targeting siRNA. Proliferation was assessed two, four, or six days later using CCK-8. All experiments were performed in triplicate. Data is displayed as the mean ± standard deviation. For quantitative studies using ENZ in conjunction with total AR targeting siRNA, cells were plated 24 h prior to transfection at 12,000 cells/well on a 12 well plate. 24 h post transfection, cells were treated with either ENZ or vehicle. Proliferation was assessed using CCK-8. All studies were performed in triplicate. Data are displayed as the mean ± standard deviation.

2.4. Colony formation assays

Cells were plated in 6-well plates at 15,000 cells/well. The following day, cells were transfected with control or AR specific siRNA and were propagated for 10–12 days post transfection. Colonies were then stained using crystal violet and photographed. Image J was used to quantify the images.

2.5. Cell lysates and western blot studies

Cells were placed in a 4 °C RIPA lysis buffer containing a protease inhibitor mixture (Sigma). Thirty to fifty micrograms of protein were separated on 8%, 10%, or 12% SDS-PAGE gels, transferred to 0.45 μm nitrocellulose membranes (Thermo Scientific), and blocked with 5% nonfat dry milk in phosphate-buffered saline and 0.1% Tween 20 (PBS-T) or OneBlock Western-CL blocking buffer (Genesee Scientific; El Cajon, CA). Membranes were incubated with primary antibody overnight at 4C. The following antibodies were used: AR (N20) (Santa Cruz Biotechnology; Santa Cruz, CA) or A303 (Bethyl; Montgomery, TX), cleaved PARP (Cell Signaling; Danvers, MA), tubulin (Thermo Scientific), GAPDH (Santa Cruz Biotechnology). The following day, membranes were incubated with secondary antibody conjugated to HRP and development was carried out using SuperSignal West Femto chemoluminescence (Thermo Scientific) or were imaged using LI-COR near-infrared western blot detection. Loading was assessed either by GAPDH or tubulin.

2.6. 3' RACE and AR-v19 cloning

The 3′ Race system for rapid amplification of cDNA ends was purchased from ThermoFisher. RNA was isolated from cells using RNeasy plus kit (Qiagen). The following AR exon 3 primer was used for the forward reaction 5′ CCATCTTGTCGTCTTCGGAAATGTTATGAAGC 3'. The reaction was carried out following manufacturer's instructions. The PCR products was cloned into the TOPO plasmid vector and introduced into DH5α competent cells. individual colonies were isolated and sequenced.

The following primers were used to generate full length AR-v19 (F: TGTGTCTTCTTCTGCACGAG, R: CTACAGTTGCAGGCACTCAGAA). The PCR products was cloned into the TOPO vector, introduced into competent cells. Plasmids harboring the expected size cDNA were first identified by analyses on 1% agarose gels and the sequence was verified by sequencing. The AR-v19 cDNA was cloned into the pcDNA3 expression plasmid into the BamH1 and Xho1 sites.

2.7. Caspase 3/7 assays

Cells were plated at 20,000 cells per well in 12 well plates, 3 wells per treatment. Cells were transfected using Lipofectamine 2000 or jetPRIME (Polyplus) and the manufacturers associated protocol as described above. 3 Days after transfection CellEvent Caspase 3/7 reagent (Invitrogen) was applied, per manufacturers protocol, post transfection and imaged using an EVOS FL 2 Auto Imaging System.

2.8. Luciferase assays

The AR-luciferase plasmid along with AR-v19 expression or parent plasmid along with the B-gal expression plasmid were transfected into UM-UC-3 cells. 48 h following transfection cells were harvested and subject to luciferase and β-galactosidase assays. Each sample's luciferase value was normalized to its respective β-galactosidase control. Relative luciferase units (RLU) were then calculated for cells transfected with differing amounts of the AR-v19 expression plasmid or parent control. RLU for vector control was then set to "1".

2.9. Immunofluorescence studies

Cells were plated at 60,00 cells per well in a 2 chambered glass slide. The following day, the chambers were removed and the slides were rinsed with pre-warmed 1x PBS, followed by 4% paraformaldehyde fixation, washed in PBS + .3% Triton-X100 (PBS-TX) and incubated in PBS-TX + 1% BSA for 1 h at room temperature. Primary antibodies (AR N-20, Santa Cruz Biotechnology #sc-816 and Tubulin, Cell Signaling #3873) were diluted in PBS-TX +1% BSA overnight at 4C. Slides were washed in PBS-TX, followed by the secondary antibody (Alexa Fluor 488 #A11008 and Alexa Fluor 647 #A21235, Invitrogen) for 1 h at room temperature (in the dark). Vectashield with DAPI was applied and allowed to dry before visualization using an EVOS FL2 Auto Imaging System.

2.10. Bladder cancer patient derived xenografts

4-5-week old NOD. Cg-Prkdcscid Il2rgtm1Wjl/SzJ (aka, NSG) female mice were obtained from Jackson Laboratories (Sacramento, CA) and implanted subcutaneously in the flank with ~1 mm3 cancerous tissue from previously established PDX tissue. Xenografts were propagated and harvested when they reached the allowable size of ~1.5 cm in any direction. After mice were euthanized the tumors were resected and flash frozen in liquid nitrogen until RNA preparation. The study was conducted under an approved IACUC protocol.

2.11. Urothelial carcinoma tissue

Urothelial carcinoma tissue samples were obtained from the UC Davis Cancer Center Biorepository (Director- Dr. Regina Gandour-Edwards) under a CC approved IRB protocol. The samples were stored in liquid nitrogen until RNA preparation.

2.12. Statistics

For all assays, *a* two tailed two sample equal variance student's t-test was used to assess differences between samples. For RT-qPCR experiments, the delta-delta Ct method was used to calculate fold change in gene expression as described before (https://www.sciencedirect.com/science/article/pii/S1046202301912629). A $p < 0.05$ was considered as statistically significant.

3. Results

3.1. AR low molecular weight isoforms are expressed in most bladder tumor-derived cells

To gain a broader understanding of AR signaling in BlCa, we embarked on a robust study utilizing nine different bladder tumor-derived cell lines (Table 1) and evaluated AR transcript and protein expression, and AR subcellular localization. Most of the cells were derived from high grade malignancies from males and females. To define AR mRNA transcript expression we used primers to sequences that encode the N-terminal segment of the AR protein (exons 1 and 2) which is common to FL AR and splice variants (Fig. 1A left panel), and primers to the LBD which detect AR transcripts encoding a domain which is present in FL AR, but not in the commonly detected AR alternatively spliced isoforms (Fig. 1A right panel). While most cells express varying levels of AR N-terminal domain encoding transcripts, some have either very low or undetectable levels of transcripts encoding the AR LBD. The RNA studies were confirmed by western immunoblot analysis of AR protein expression (Fig. 1B) which show that cells with very low levels or no LBD containing transcripts, have very low amounts of FL-AR protein. Prostate tumor-derived 22Rv1 cells which express FL and LMW AR isoforms were used as a control. As expected, AR protein is far more abundant in prostate than BlCa cells. Since AR transcriptional activity is reliant on its translocation to the nucleus, we used immunofluorescence to define AR subcellular localization in BlCa derived cells (Fig. 1C). Asynchronously proliferating cells were fixed and stained with AR antibodies. DAPI staining was used to detect the nucleus, and tubulin staining defined the cytoplasmic compartment. In UM-UC-3, TCCSUP, J82 and T24 cells, AR expression is more nuclear than cytoplasmic. In contrast SW780, HT1376, and HT1197 cells, AR expression is more pronounced in the cytoplasm. In RT4 cells, AR is present equally in both compartments. Since AR protein levels in 5637 were below detection, these cells were not used in the analysis. Notably, some cells with nuclear AR predominantly express LMW isoforms, suggesting that these isoforms can translocate into the nucleus regardless of FL-AR.

3.2. AR depletion reduces viability and promotes apoptosis

To define the importance of AR in bladder tumor cell viability, cells were treated with a pool of AR targeting siRNAs to deplete all AR isoforms. Non-targeting siRNA served as a control. Following AR depletion there was a time dependent decrease in cell viability (Fig. 2A). TCCSUP and UM-UC-3 cells, which express full length AR responded to this treatment as previously reported [23]. However, we also saw a robust response to AR depletion in cells that express predominantly LMW isoforms and this effect was most pronounced in T24 cells (Fig. 2A), suggesting that these forms are functional. Efficacy of AR depletion was verified by western immunoblot analysis (Fig. 2B). HT1376 and HT1197 cells with predominantly cytoplasmic AR were not affected by

Table 1
Characteristics of bladder cancer cell lines derived from human tumors and used in the current study (available through ATCC).

Cell Line	Morphology	Organ	Tumor Stage/ Grade	Disease	Age	Gender	Ethnicity	History	Metastasis
UM-UC-3	Epithelial	urinary bladder	n/a	TCC		Male	Caucasian		
J82	Epithelial	urinary bladder	T3	TCC	58	Male	Caucasian	None	None
T24	Epithelial	urinary bladder	Grade 3	TCC	81	Female	Caucasian	Electro-coagulation	
TCCSUP	Epithelial	urinary bladder	Grade 4	TCC	67	Female	Unknown	4-month pre-operative hematuria	Metastases to bone Marrow
RT4	Epithelial	urinary bladder	Grade 1	Traditional cell papilloma	63	Male	Caucasian	n/a	unknown
SW780	Epithelial	urinary bladder	Grade 1	TCC	80	female	Caucasian	preoperative chemotherapy (Thiotepa)	
HT1197		urinary bladder	n/a	carcinoma	44	Male	Caucasian		
HT1376	Epithelial	urinary bladder	Grade 3	carcinoma	58	Female	Caucasian		
5637	Epithelial	urinary bladder	Grade 2	carcinoma	68	Male	Unknown		

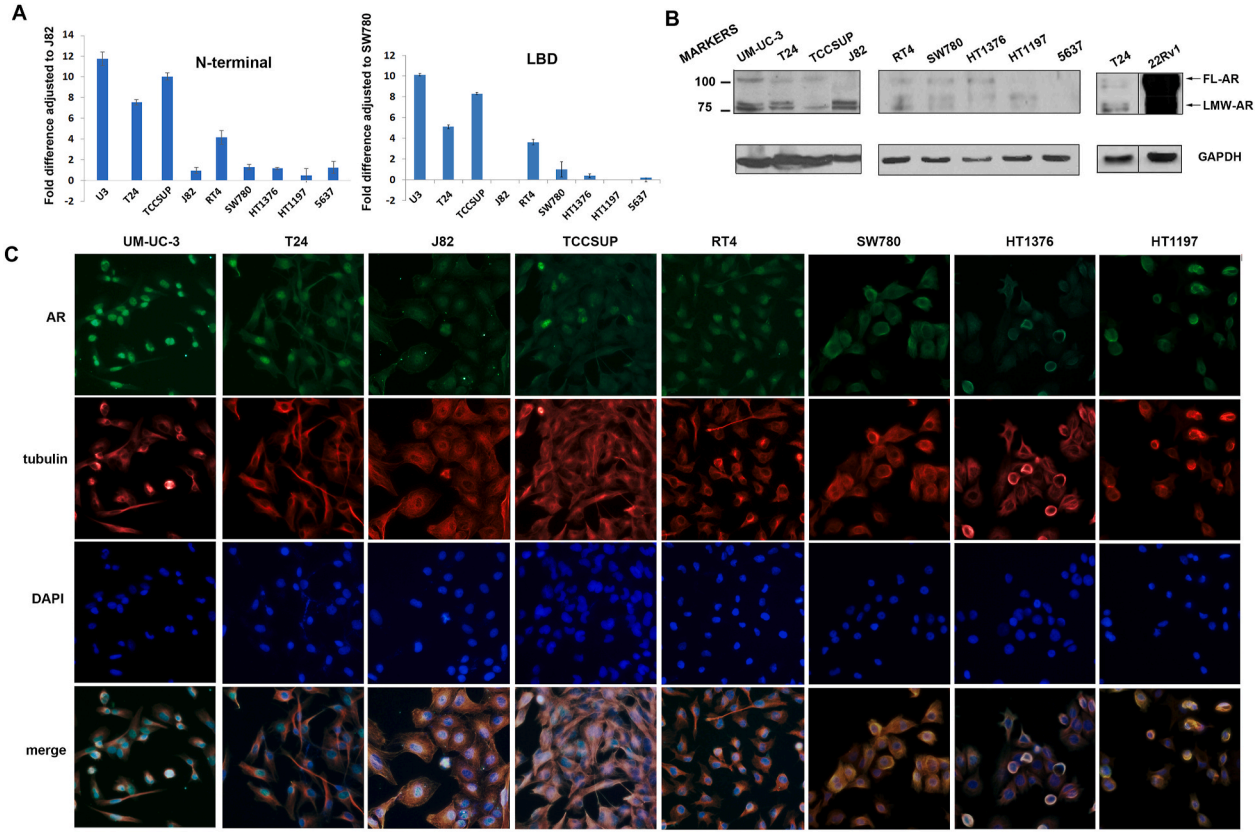

Fig. 1. AR expression in nine bladder tumor derived cell lines. A. RT PCR was used to detect all AR isoforms (left panel) and transcripts encoding the FL-AR (right panel). B. Western blot analysis of bladder tumor-derived cells. Prostate AR expressing 22Rv1 cells were included as a positive control. GAPDH served as a gel loading control. C. Immunofluorescence was used to define AR (green) subcellular localization. DAPI staining (blue) was used to detect nuclei.

AR depletion, indicating that AR localization is an important parameter of AR dependence. Unexpectedly, AR depletion in SW780 cells repeatedly resulted in a significant increase in the number of viable cells, hence in this cellular context AR may be constraining rather than promoting proliferation. Collectively, these results argue that in cells with nuclear AR expression, AR is necessary for optimal cell viability.

AR sensitive cells subjected to AR depletion exhibited physiological characteristics of programmed cell death. To determine if AR depletion promoted an apoptotic response, we performed caspase 3/7 assays. UM-UC-3, TCCSUP, T24, J82 and RT4 cells show a significant increase in caspase 3/7 activity, consistent with an induction of apoptosis (Fig. 2C). In confirmation of the caspase 3/7 results, western immunoblot analysis revealed a significant increase in cleaved PARP, a marker of apoptosis (Fig. 2D), suggesting that the decrease in cell viability was at least in part due to an induction of apoptosis.

3.3. Novel AR-v19 AR splice variant is expressed in most bladder tumor-derived cells

Studies of prostate cancer cells have identified many AR splice

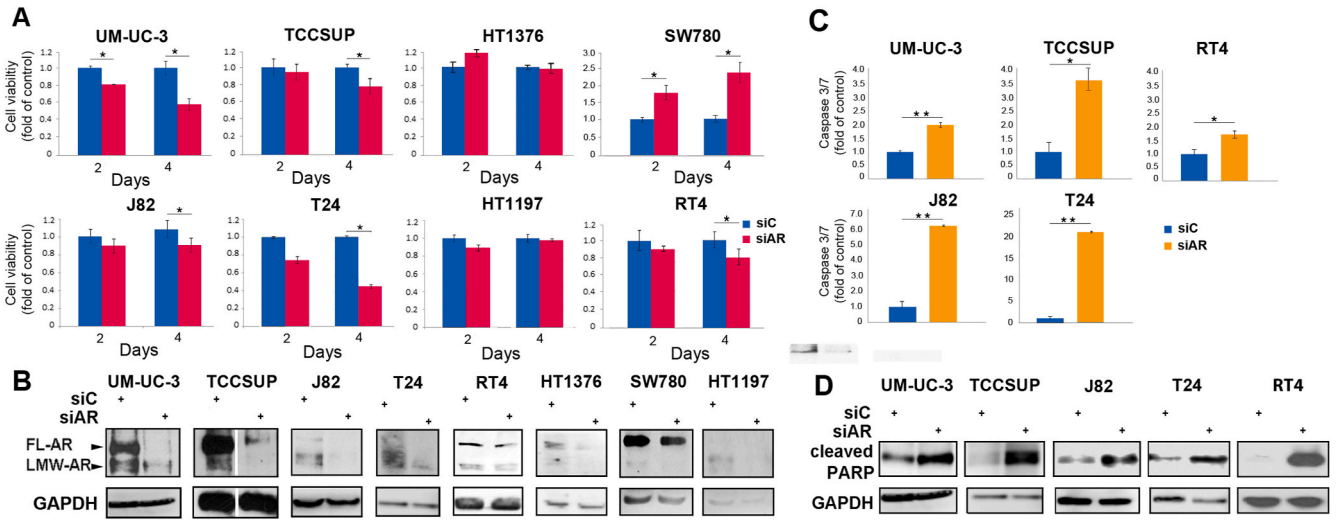

Fig. 2. siRNA mediated decrease of all AR isoforms effectively reduces cell viability in multiple cell lines. A. CCK-8 assays conducted at 2- and 4-days following siRNA cell transfection shows a reduction in cell viability. Data is shown as fold changes of control treatment. B. Western blot analysis of cells treated with AR targeting all AR isoforms (siAR) or with non-targeting control siRNA (siC) confirms AR depletion. GAPDH served as a loading control. C. Caspase 3/7 assays of cells treated with siC or siAR for 4 days indicates that AR depletion induces apoptosis. Data is shown as fold change of control treatment. Western blot analysis shows an increase in PARP cleavage due to AR depletion. GAPDH served as a loading control. The viability assays were conducted in triplicate and the error bars denote standard deviation. * = $P \leq 0.05$, ** = $P \leq 0.01$.

variants, and some have been shown to be prominent in castrate resistant prostate tumors [11,12,14,24]. In particular, the AR3/AR V7 (v7) splice form is commonly expressed not only in castrate-resistant prostate tumors [25], but also in mammary and liver tumor cells [15,26]. Primers specific to v7 were used in RT-PCR analyses to detect v7 expression in nine bladder tumor-derived cell lines. Our studies reveal that v7 is present in UM-UC-3, TCCSUP, HT1376 and at low levels in T24 cells (Fig. 3A). PCR analysis shows that the amplified product has the correct predicted size (Fig. 3B upper panel).

However, some cells that are reliant on AR signaling and express LMW AR, do not express v7. Therefore, we reasoned that they must have different splice variants. We used 3′RACE to identify additional AR LMW forms that contain the N-terminal region but not the LBD. We identified a novel splice variant that we named AR-v19, since the highest AR-v designation reported to date is AR-v18 [15]. The full-length AR-v19 PCR product was cloned and sequenced. This splice variant contains an N-terminal activation domain encoded by exon 1, and the DNA binding domain encoded by exons 2 and 3. The novel C-terminal sequence maps to intron 3 and encodes an additional 15 amino acids (Fig. 3C, full DNA and protein sequence in Supplementary Fig. 1). We found variable expression of AR-v19 in different BlCa cell lines (Fig. 3A). Interestingly, BlCa cells that express v7 also express AR-v19 suggesting that, as in the prostate context, BlCa cells can express multiple AR splice variants. AR-v19 expression was also assessed in primary bladder tumor samples

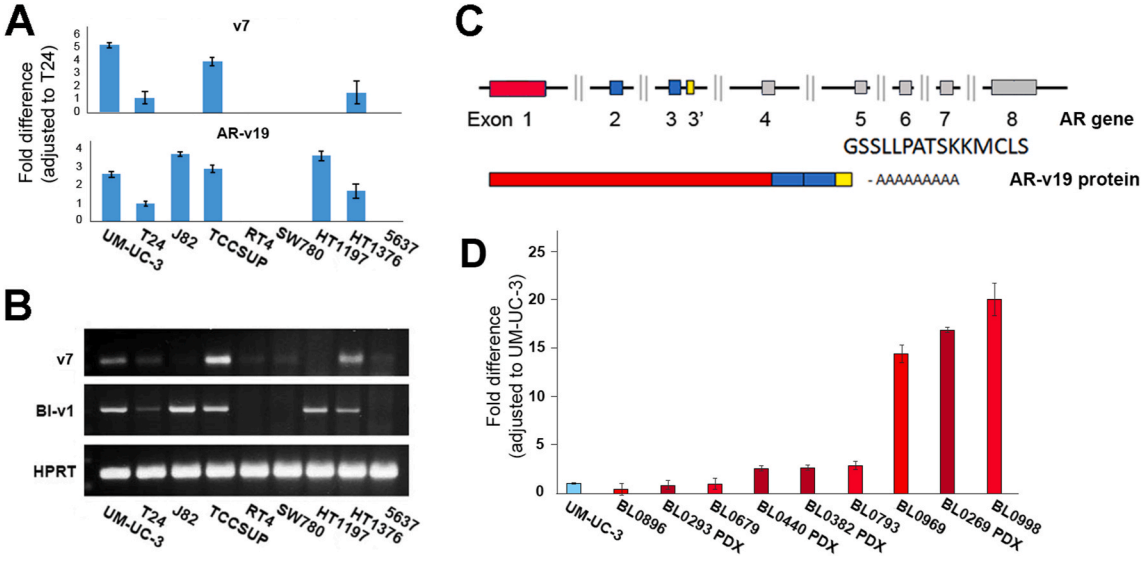

Fig. 3. Identification of a novel AR splice variants in bladder tumor cells. A. Realtime PCR detected v7 and AR-v19 AR isoforms in multiple bladder-tumor derived cells. Expression levels are expressed as fold difference with levels in T24 cells set as 1. The analysis was conducted in quadruplicate. HPRT served as an RNA control. B. Agarose gels showing PCR amplification product. C. Schematic of the AR gene and AR-v19 transcript and protein. Exon 1 (red) encodes the activation domain, exons 2 and 3 (blue) encode the DNA binding domain, while the exons encoding the hinge and LBD are in grey. The novel 3′ exon encoding the additional novel 15 aa (yellow) maps to sequences in intron 3. D. AR-v19 expression in primary bladder tumors and in BlCa PDX tumors where expression is shown as fold of UM-UC-3 AR-v19 (blue) levels.

and in four bladder PDX tumors (Fig. 3D). RT-PCR analysis indicates that most BlCa tumors expressed the AR-v19 isoform, although expression is low in some and highly elevated in others. An analysis of available tumor and patient characteristics (Supplementary Table 1) did not reveal a correlation between AR-v19 and known tumor features. Overall, our analysis suggests that varying levels of AR-v19 splice variant expression is common in bladder malignancies.

To determine if AR-v19 was functional, sequences encoding this isoform were cloned into an expression plasmid and were transfected into UM-UC-3 cells where AR can localize to the nucleus. Following selection, multiple AR-v19 overexpressing clones were isolated and expanded (Fig. 4A). For comparison, cells were transfected with the FL-AR encoding plasmid or the parental vector and individual clones were isolated and expanded. Western immunoblot analysis confirmed elevated FL or AR-v19 AR expression. Since we were able to select multiple clones overexpressing parental, AR-v19 or FL-AR plasmids, elevated expression of the FL-AR or the splice variant is not detrimental to BlCa cell proliferation. We detected small variation in the proliferation rates of AR-v19 expressing cells, but overall rates did not differ significantly from cells transfected with the parental vector. (Supplementary Fig. 2). RT-PCR analysis confirmed expression levels of AR-v19 and FL-AR at the transcriptional level (Fig. 4B). Immunofluorescence studies demonstrate FL- AR and AR-v19 overexpressing cells have highly elevated nuclear AR levels when compared with parental UM-UC-3 cells, hence AR-v19, like FL-AR localizes predominantly to the nucleus in these cells (Fig. 4C). Overexpression of AR-v19 (UM-UC-3-9 clone) elevated expression of LBD containing FL-AR transcripts (Fig. 4D, left panel) while AR-v19 depletion in UM-UC-3 cells reduced FL-AR transcript levels (Fig. 4D, right panel), indicating that AR-v19 positive autoregulates the AR gene. To determine if AR-v19 can drive AR-dependent transcription, an AR-v19 expression plasmid was co-transfected into UM-UC-3 cells along with an AR-dependent promoter-luciferase construct. Inclusion of the β-galactosidase plasmid controlled for transfection efficiency. AR-v19 transactivated transcription from an AR-dependent promoter in a dose-dependent manner (Fig. 4E), demonstrating that it can function as a transcriptional regulator.

3.4. AR-v19 depletion reduces bladder cell viability and induces apoptosis

To define the effect of AR-v19 depletion we designed two siRNAs that reduced AR-v19 levels (Supplementary Fig. 3). Both were equally effective, therefore the siRNA designated as A was used in subsequent studies. Cells that were sensitive to AR modulation and expressed AR-v19 were subjected to further studies. In all four cell lines AR-v19 depletion impeded cell viability (Fig. 5A), but the decrease was apparent on day six rather than day four as seen on total AR reduction. Depletion of AR-v19 transcript was verified by RT-PCR (Fig. 5B). Colony formation assays confirmed a reduction of cell viability upon AR-v19 depletion (Fig. 5C). Caspase 3/7 assays and cleaved PARP analysis showed that AR-v19 depletion promotes apoptosis (Fig. 5D and E). Hence, in four different cellular contexts, AR-v19 is instrumental in maintaining cell viability by inhibiting apoptosis.

3.5. AR-v19 depletion is more effective than enzalutamide in reducing bladder tumor cell viability

Enzalutamide (ENZ), a second generation therapeutic that targets the AR ligand binding domain, reduces AR-dependent signaling in prostate cells and previous studies found that ENZ was effectively in inhibiting viability of UM-UC-3 and TCCSUP, cells that express FL- AR [27,28]. We assessed the efficacy of ENZ in attenuating cell viability in nine BlCa cell lines, where prostate LNCaP and 22Rv1 cells served as positive controls (Supplementary Fig. 4A). ENZ effectivity reduced UM-UC-3 and TCCSUP cell viability as previously reported, and the magnitude of inhibition was similar to the effect on prostate 22Rv1 cells but more limited than the effect on LNCaP cells. This is consistent with previous reports that ENZ greatly reduced LNCaP cell viability, while the effect on 22Rv1 was more modest [29]. As expected, cells that do not

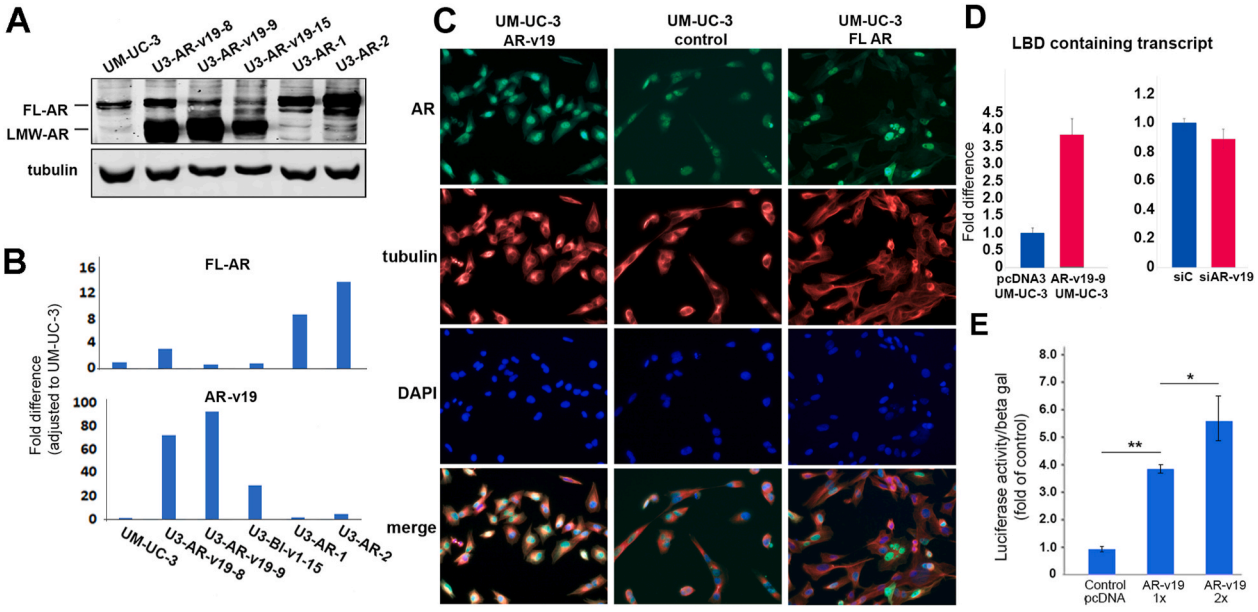

Fig. 4. AR-v19 localizes to the nucleus and transactivates AR-dependent gene expression in a dose dependent manner. A. Western blot analysis of UM-UC-3 clones expressing AR-v19 or FL-AR under control of the CMV promoter. Cells harboring the parental plasmid were included for comparison and tubulin served as a gel loading control. B. Quantification of FL-AR protein expression (top panel) and LMW AR forms used Image J software. Intensity was adjusted to tubulin and expressed as fold difference to UM-UC-3. C. Immunofluorescent (green) staining indicates that cells overexpressing cells AR-v19 or FL AR have highly elevated AR protein levels which localizes primarily to the nucleus. DAPI staining (blue) defined the nucleus, while tubulin (red) stained the cytoplasm. D. The AR-v19 overexpressing UM-UC-3-9 cells have elevated expression of LBD containing FL-AR (left panel) while AR-v19 depletion in UM-UC-3 cells reduced LBD containing FL-AR transcript levels (right panel). E. Increasing amounts of AR-v19 expression plasmid were co-transfected with an AR-dependent promoter-reporter construct and β-galactosidase expressing plasmid was included as a transfection control. 2 days following transfection cells were harvested and subjected to luciferase assays, where β-galactosidase served as a control. Studies were conducted in triplicate and repeated twice. * = $P \leq 0.05$, ** = $P \leq 0.01$.

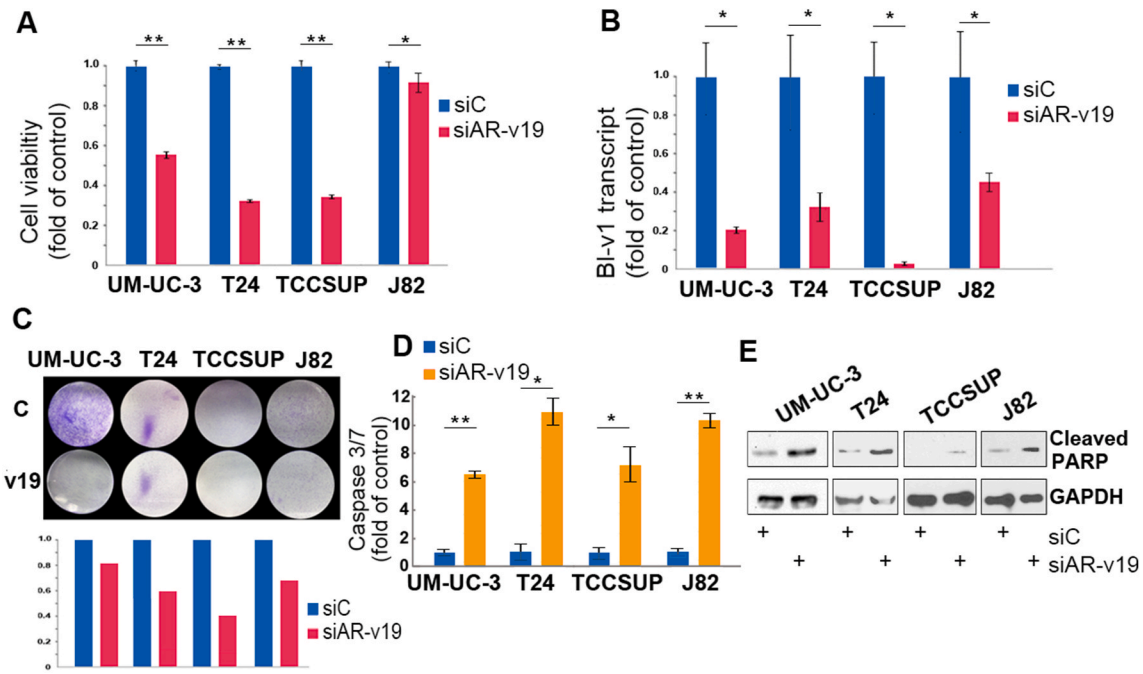

Fig. 5. AR-v19 depletion reduces cells viability and promotes apoptosis. A. siRNA mediated decrease of the AR-v19 variant (red) reduces UM-UC-3, T24, TCCSUP, and J82 cell viability. Non-targeting siRNA served as a control (blue). The data shown as fold of control. Cells were treated AR-v19 targeting and non-targeting siRNA for 6 days. B. AR-v19 depletion was documented by RT-PCR. C. Upper panel- Colony formation assays of cells transfected with control or AR-v19 targeting siRNA show a decrease in cells treated with AR-v19-targeting siRNA. Cells were cultured for 10–12 days following siRNA treatment. Lower panel- The images were quantified with image J where the control was set to 100 percent. D. Caspase 3/7 assays were used to detect apoptosis in control (blue) and AR-v19 (orange) siRNA treated cells. E. Western blot analysis shows an increase in PARP cleavage with AR depletion. GAPDH served as a loading control. The viability and caspase 3/7 assays were conducted in triplicate and the error bars denote standard deviation. * $\leq$0.05, **$\leq$ 0.01.

express FL-AR and cells that do not express FL-AR in the nucleus are refractory to ENZ. Treatment of UM-UC-3 cells with ENZ following AR-v19 depletion indicates that AR-v19 is more effective in limiting cell viability and the combination is slightly more effective (Supplementary Figs. 4B and C). Resistance of prostate malignancies to ENZ therapy is associated with splice variant expression [30], therefore we tested if increased splice variant expression dampened cell response to ENZ. Unexpectedly, two independent AR-v19 overexpressing UM-UC-3 cells remained sensitive to ENZ (Supplementary Fig. 4D) arguing that splice variant overexpression does not confer resistance in this cellular context. Cells overexpressing the FL-AR were also sensitive to ENZ suggesting that sensitivity to this treatment is dependent on the presence of FL-AR and on localization of AR to the nuclear compartment rather than absolute FL-AR levels.

4. Discussion

In this study we found that most BlCa cells express AR splice variants and splice variant depletion promotes apoptosis. An alteration of splicing expands the repertoire of proteins that have acquired, or lost properties present in the canonical protein. Many AR variants retain the N-terminal activation domain and the DNA binding domain and acquire novel sequences encoded by cryptic exons within intron 3. This renders many splice variants constitutively active and independent of ligand. The ability of LMW AR isoforms to solely drive cellular proliferation of AR-dependent cells was confirmed by studies where CRISPR engineering was used to remove FL-AR [31].

The noted greater incidence of BlCa in males strongly argues that androgen signaling may contribute to tumorigenesis. To uncover the importance of AR in high grade BlCa we conducted an analysis utilizing nine cell lines derived from muscle invasive malignancies. Most cells express FL-AR, LMW AR splice variants, or both. Notably, LMW isoform expression is common and present in some cells that have almost no FL-AR. However, the expression levels of AR in BlCa cells is significantly lower than in prostate cancer cells, a result that is similar to what was noted for AR expression in breast cancer malignancies [32]. Immunofluorescence studies found that the subcellular distribution of AR varies where some cells have abundant nuclear AR while others have AR confined mostly to the cytoplasm. The difference in AR expression or localization does not differ between cells derived from males and females. Cells with nuclear AR, regardless of total AR levels, are affected by total AR depletion even if they expressed predominantly LMW isoforms. Cells where AR is mostly cytoplasmic are unaffected by AR modulation, underscoring the importance of AR subcellular location. The causes of differential subcellular AR distribution are unknown. Different complements of AR co-interactors and co-regulators present in cells or differential AR post translational modification such as phosphorylation may promote or hinder AR localization, and these regulatory mechanisms may be exploitable to limit AR signaling.

The well-studied v7 AR splice variant discovered in prostate malignancies where expression correlates with resistance to ENZ [30], has also been detected in breast and liver tumor derived cells and in primary malignancies as well as in non-tumor tissue [15,26]. We found that v7 was expressed in ~50% of the surveyed cells, confirming that v7 is commonly expressed. Prostate tumor studies also revealed that tumor cells express many splice variants, but not all drive cell proliferation or enhance cell survival [33]. The new splice variant AR-v19 is expressed in six of nine bladder tumor derived cell lines, arguing that in the bladder cancer context its expression is common. The importance of AR-v19 was demonstrated by AR-v19 depletion studies, where in four different cellular contexts, a reduction of this splice variant promoted an apoptotic response. UM-UC-3 cells overexpressing AR-v19 had elevated FL-AR levels and cells where AR-v19 was depleted had reduced FL AR overexpression indicative of positive autoregulation.

An analysis of primary BlCa tumors and PDX tumors confirmed that BlCa cells express varying AR-v19 levels. It is unclear if this variant is

commonly expressed in other malignancies such as breast and liver, but we found that 22Rv1 prostate cancer cells express this variant as well. The BL0269 PDX tumor with highly elevated AR-v19 expression has two features that are different from the other PDX tumors; 1) this tumor was refractory to all tested treatments where the other tumors were responsive to either cisplatin, gemcitabine or combination treatment [34,35], and 2) this tumor had a higher number of mutated DNA repair genes. Expanded studies will be needed to determine if splice variant expression is linked to these tumor properties.

Depletion of total AR and the AR-v19 isoform promotes an apoptotic response, but unexpectedly overexpression of AR-v19 in UM-UC-3 cells neither accelerates cell proliferation nor hinders cell viability. AR activity is dependent on, and altered by, co-regulators. Therefore, if co-regulators are limiting, an increase of AR would be insufficient to alter cell physiology. Moreover, elevated AR expression may alter properties, such as response to certain therapeutics, interaction of the cells with matrix, or survival of cells under stressful conditions such as hypoxia. The biphasic response to AR signaling noted in prostate malignancies, where super elevation of AR signaling reduces cell proliferation, was not evident in BlCa cells [36–38]. The induction of apoptosis on total AR depletion argues that one role of AR signaling is protecting cells from programmed cell death. As expected, cells that express the FL-AR, UM-UC-3 and TCCSUP, were also sensitive to ENZ. The extent of ENZ-mediated decrease in viability was comparable to what is observed with prostate 22Rv1 cells which express an abundant amount of LMW variants. The effect of depleting AR-v19 isoform on UM-UC-3 cells is greater than the effect of ENZ, and there was a small enhancement in limiting cell viability by the inclusion of ENZ. The unexpected effect of ENZ on cells with elevated levels of AR-v19 suggests that the FL-AR is functional even when AR-v19 is abundant.

Since a significant percent of muscle invasive cells appear to depend on AR for optimal viability, limiting AR signaling is an attractive therapeutic strategy. Men with BlCa who were treated with 5α-reductase inhibitors, drugs that prevent the conversion of testosterone to the more potent dihydrotestosterone, exhibited a lower risk of BlCa related death than men not treated with these agents [39] suggesting that this strategy may be effective in a sub population of patients. Thus, the use of abiraterone for treatment of advanced BlCa (NCT02788201), or the combination of enzalutamide with gemcitabine and cisplatin in urothelial cancer (NCT02300610) has been initiated. Targeting AR downstream effectors could also prove to be promising. The Bl-Ca AR-driven transcriptome is not well defined, but recent studies show that AR-dependent alteration of cirRNA FNTA expression results in repression of the RNA editing ADAR2 gene. This serves to activate KRAS signaling and affect BlCa cell invasiveness and sensitivity to cisplatin-based chemotherapy [40]. Thus, this analysis links AR to KRAS, a known BlCa oncogene.

While the current study established a role for AR splice variants in high grade BlCa cells, additional studies are needed to determine if AR isoforms are expressed in non-muscle invasive cancers and if expression is prognostic. Moreover, it is unknown how expression of AR splice variants affect treatment susceptibility and if splice variant expression can be utilized to stratify which patients would benefit from specific treatments. Additional studies are required to address these issues.

In summary, our studies support the emerging view that in malignancies not traditionally considered hormone-dependent, the AR, and particularly AR splice variants, have a role in tumor cell survival. FL-AR and the newly identified AR-v19 splice variant are commonly expressed in Bl-Ca cell lines as well as in primary and PDX tumors. AR-v19 is expressed in the nucleus and can promote AR-dependent transcription. AR depletion limits cell viability and promotes apoptosis in cells with nuclear AR expression. AR-v19 depletion increases apoptosis indicating it has a role in maintaining cell viability. Further studies are needed to identify bladder specific AR-mediated transcriptomes to uncover networks and pathways that are appropriated by AR signaling to enhance cell proliferation and viability. This may uncover vulnerabilities that could be exploited to design novel therapeutic strategies for the treatment of bladder malignancies.

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Author contribution

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Declaration of competing interest

The authors declare no conflict interest. The work reported here does not represent the views or opinions of the Department of Veteran Affairs or the United States Government.

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Appendix A. Supplementary data

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References

[1] R.L. Siegel, K.D. Miller, A. Jemal, Cancer statistics, CA A Cancer J. Clin. 70 (2020) 7–30.
[2] B.A. Carneiro, J.J. Meeks, T.M. Kuzel, M. Scaranti, S.A. Abdulkadir, F.J. Giles, Emerging therapeutic targets in bladder cancer, Canc. Treat Rev. 41 (2015) 170–178.
[3] M.A. Knowles, C.D. Hurst, Molecular biology of bladder cancer: new insights into pathogenesis and clinical diversity, Nat. Rev. Canc. 15 (2015) 25–41.
[4] P. Hartge, E.B. Harvey, W.M. Linehan, D.T. Silverman, J.W. Sullivan, R.N. Hoover, J.F. Fraumeni Jr., Unexplained excess risk of bladder cancer in men, J. Natl. Cancer Inst. 82 (1990) 1636–1640.
[5] A.P. Lombard, M. Mudryj, The emerging role of the androgen receptor in bladder cancer, Endocr. Relat. Canc. 22 (2015) R265–R277.
[6] E.A. Messner, T.M. Steele, M.M. Tsamouri, N. Hejazi, A.C. Gao, M. Mudryj, P. M. Ghosh, The androgen receptor in prostate cancer: effect of structure, ligands and spliced variants on therapy, Biomedicines 8 (2020).
[7] S. Wen, Y. Niu, S.O. Lee, C. Chang, Androgen receptor (AR) positive vs negative roles in prostate cancer cell deaths including apoptosis, anoikis, entosis, necrosis and autophagic cell death, Canc. Treat Rev. 40 (2014) 31–40.
[8] M.M. Centenera, L.A. Selth, E. Ebrahimie, L.M. Butler, W.D. Tilley, New opportunities for targeting the androgen receptor in prostate cancer, Cold Spring Harb. Perspect. Med. 8 (2018).
[9] M. Mudryj, C.G. Tepper, On the origins of the androgen receptor low molecular weight species, Horm Canc. 4 (2013) 259–269.
[10] M. Nakazawa, E.S. Antonarakis, J. Luo, Androgen receptor splice variants in the era of enzalutamide and abiraterone, Horm Canc. 5 (2014) 265–273.
[11] R. Hu, T.A. Dunn, S. Wei, S. Isharwal, R.W. Veltri, E. Humphreys, M. Han, A. W. Partin, R.L. Vessella, W.B. Isaacs, G.S. Bova, J. Luo, Ligand-independent androgen receptor variants derived from splicing of cryptic exons signify hormone-refractory prostate cancer, Canc. Res. 69 (2009) 16–22.
[12] Z. Guo, X. Yang, F. Sun, R. Jiang, D.E. Linn, H. Chen, H. Chen, X. Kong, J. Melamed, C.G. Tepper, H.J. Kung, A.M. Brodie, J. Edwards, Y. Qiu, A novel

androgen receptor splice variant is up-regulated during prostate cancer progression and promotes androgen depletion-resistant growth, Canc. Res. 69 (2009) 2305–2313.
[13] S.M. Dehm, L.J. Schmidt, H.V. Heemers, R.L. Vessella, D.J. Tindall, Splicing of a novel androgen receptor exon generates a constitutively active androgen receptor that mediates prostate cancer therapy resistance, Canc. Res. 68 (2008) 5469–5477.
[14] S. Sun, C.C. Sprenger, R.L. Vessella, K. Haugk, K. Soriano, E.A. Mostaghel, S. T. Page, I.M. Coleman, H.M. Nguyen, H. Sun, P.S. Nelson, S.R. Plymate, Castration resistance in human prostate cancer is conferred by a frequently occurring androgen receptor splice variant, J. Clin. Invest. 120 (2010) 2715–2730.
[15] D.G. Hu, T.E. Hickey, C. Irvine, D.D. Wijayakumara, L. Lu, W.D. Tilley, L.A. Selth, P.I. Mackenzie, Identification of androgen receptor splice variant transcripts in breast cancer cell lines and human tissues, Horm Canc. 5 (2014) 61–71.
[16] L. Stone, Kidney cancer: androgen receptor–a new target in renal cell carcinoma? Nat. Rev. Urol. 11 (2014) 425.
[17] R. Berardi, F. Morgese, A. Santinelli, A. Onofri, T. Biscotti, A. Brunelli, M. Caramanti, A. Savini, M. De Lisa, Z. Ballatore, C. Pompili, M. Salati, P. Mazzanti, M. Torniai, S. Cascinu, Hormonal receptors in lung adenocarcinoma: expression and difference in outcome by sex, Oncotarget 7 (2016) 82648–82657.
[18] E. Laor, Z.J. Schiffman, J.D. Braunstein, R.E. Reid, B.M. Tolia, L.G. Koss, S.Z. Freed, Androgen receptors in bladder tumors, Urology 25 (1985) 161–163.
[19] H. Ide, S. Inoue, H. Miyamoto, Histopathological and prognostic significance of the expression of sex hormone receptors in bladder cancer: a meta-analysis of immunohistochemical studies, PLoS One 12 (2017) e0174746.
[20] S. Terada, N. Suzuki, K. Uchide, K. Akasofu, E. Nishida, Effect of testosterone on the development of bladder tumors and calculi in female rats, Gynecol. Obstet. Invest. 34 (1992) 105–110.
[21] J.W. Hsu, I. Hsu, D. Xu, H. Miyamoto, L. Liang, X.R. Wu, C.R. Shyr, C. Chang, Decreased tumorigenesis and mortality from bladder cancer in mice lacking urothelial androgen receptor, Am. J. Pathol. 182 (2013) 1811–1820.
[22] C. Lin, Y. Yin, K. Stemler, P. Humphrey, A.S. Kibel, I.U. Mysorekar, L. Ma, Constitutive beta-catenin activation induces male-specific tumorigenesis in the bladder urothelium, Canc. Res. 73 (2013) 5914–5925.
[23] S.A. Boorjian, H.V. Heemers, I. Frank, S.A. Farmer, L.J. Schmidt, T.J. Sebo, D. J. Tindall, Expression and significance of androgen receptor coactivators in urothelial carcinoma of the bladder, Endocr. Relat. Canc. 16 (2009) 123–137.
[24] E.S. Antonarakis, C. Lu, H. Wang, B. Luber, M. Nakazawa, J.C. Roeser, Y. Chen, T. A. Mohammad, Y. Chen, H.L. Fedor, T.L. Lotan, Q. Zheng, A.M. De Marzo, J. T. Isaacs, W.B. Isaacs, R. Nadal, C.J. Paller, S.R. Denmeade, M.A. Carducci, M. A. Eisenberger, J. Luo, AR-V7 and resistance to enzalutamide and abiraterone in prostate cancer, N. Engl. J. Med. 371 (2014) 1028–1038.
[25] E.S. Antonarakis, C. Lu, H. Wang, B. Luber, M. Nakazawa, J.C. Roeser, Y. Chen, T. A. Mohammad, H.L. Fedor, T.L. Lotan, Q. Zheng, A.M. De Marzo, J.T. Isaacs, W. B. Isaacs, R. Nadal, C.J. Paller, S.R. Denmeade, M.A. Carducci, M.A. Eisenberger, J. Luo, AR-V7 and resistance to enzalutamide and abiraterone in prostate cancer, N. Engl. J. Med. 371 (2014) 1028–1038.
[26] A.M. Dauki, J.S. Blachly, E.A. Kautto, S. Ezzat, M.H. Abdel-Rahman, C.C. Coss, Transcriptionally active androgen receptor splice variants promote hepatocellular carcinoma progression, Canc. Res. 80 (2020) 561–575.
[27] T. Kawahara, H. Ide, E. Kashiwagi, K.A. El-Shishtawy, Y. Li, L.O. Reis, Y. Zheng, H. Miyamoto, Enzalutamide inhibits androgen receptor-positive bladder cancer cell growth, Urol. Oncol. 34 (2016) 432, e415-423.
[28] A. Tyagi, B. Chandrasekaran, V. Kolluru, S. Rai, A.C. Jordan, A. Houda, J. Messer, M. Ankem, C. Damodaran, A. Haddad, Combination of androgen receptor inhibitor and cisplatin, an effective treatment strategy for urothelial carcinoma of the bladder, Urol. Oncol. 37 (2019) 492–502.
[29] N. Nadiminty, R. Tummala, C. Liu, J. Yang, W. Lou, C.P. Evans, A.C. Gao, NF-kappaB2/p52 induces resistance to enzalutamide in prostate cancer: role of androgen receptor and its variants, Mol. Canc. Therapeut. 12 (2013) 1629–1637.
[30] Y. Zhu, S.L. Dalrymple, I. Coleman, S.L. Zheng, J. Xu, J.E. Hooper, E. S. Antonarakis, A.M. De Marzo, A.K. Meeker, P.S. Nelson, W.B. Isaacs, S. R. Denmeade, J. Luo, W.N. Brennen, J.T. Isaacs, Role of androgen receptor splice variant-7 (AR-V7) in prostate cancer resistance to 2nd-generation androgen receptor signaling inhibitors, Oncogene (2020).
[31] E. Kounatidou, S. Nakjang, S.R.C. McCracken, S.M. Dehm, C.N. Robson, D. Jones, L. Gaughan, A novel CRISPR-engineered prostate cancer cell line defines the AR-V transcriptome and identifies PARP inhibitor sensitivities, Nucleic Acids Res. 47 (2019) 5634–5647.
[32] T.E. Hickey, C.M. Irvine, H. Dvinge, G.A. Tarulli, A.R. Hanson, N.K. Ryan, M. A. Pickering, S.N. Birrell, D.G. Hu, P.I. Mackenzie, R. Russell, C. Caldas, G.V. Raj, S. M. Dehm, S.R. Plymate, R.K. Bradley, W.D. Tilley, L.A. Selth, Expression of androgen receptor splice variants in clinical breast cancers, Oncotarget 6 (2015) 44728–44744.
[33] R. Hu, W.B. Isaacs, J. Luo, A snapshot of the expression signature of androgen receptor splicing variants and their distinctive transcriptional activities, Prostate 71 (2011) 1656–1667.
[34] M. Zimmermann, S.S. Wang, H. Zhang, T.Y. Lin, M. Malfatti, K. Haack, T. Ognibene, H. Yang, S. Airhart, K.W. Turteltaub, G.D. Cimino, C.G. Tepper, A. Drakaki, K. Chamie, R. de Vere White, C.X. Pan, P.T. Henderson, Microdose-induced drug-DNA adducts as biomarkers of chemotherapy resistance in humans and mice, Mol. Canc. Therapeut. 16 (2017) 376–387.
[35] C.X. Pan, H. Zhang, C.G. Tepper, T.Y. Lin, R.R. Davis, J. Keck, P.M. Ghosh, P. Gill, S. Airhart, C. Bult, D.R. Gandara, E. Liu, R.W. de Vere White, Development and characterization of bladder cancer patient-derived xenografts for molecularly guided targeted therapy, PLoS One 10 (2015) e0134346.
[36] S.J. Libertini, C.G. Tepper, M. Guadalupe, Y. Lu, D.M. Asmuth, M. Mudryj, E2F1 expression in LNCaP prostate cancer cells deregulates androgen dependent growth, suppresses differentiation, and enhances apoptosis, Prostate 66 (2006) 70–81.
[37] P. Chatterjee, M.T. Schweizer, J.M. Lucas, I. Coleman, M.D. Nyquist, S.B. Frank, R. Tharakan, E. Mostaghel, J. Luo, C.C. Pritchard, H.M. Lam, E. Corey, E. S. Antonarakis, S.R. Denmeade, P.S. Nelson, Supraphysiological androgens suppress prostate cancer growth through androgen receptor-mediated DNA damage, J. Clin. Invest. 129 (2019) 4245–4260.
[38] J. Simard, R. Veilleux, Y. de Launoit, D.E. Haagensen, F. Labrie, Stimulation of apolipoprotein D secretion by steroids coincides with inhibition of cell proliferation in human LNCaP prostate cancer cells, Canc. Res. 51 (1991) 4336–4341.
[39] C.S. Wang, C.C. Li, Y.S. Juan, W.J. Wu, H.Y. Lee, 5alpha-reductase inhibitors impact prognosis of urothelial carcinoma, BMC Canc. 20 (2020) 872.
[40] J. Chen, Y. Sun, Z. Ou, S. Yeh, C.P. Huang, B. You, Y.C. Tsai, T.J. Sheu, X. Zu, C. Chang, Androgen receptor-regulated circFNTA activates KRAS signaling to promote bladder cancer invasion, EMBO Rep. 21 (2020) e48467.