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Urologic Oncology: Seminars and Original Investigations 37 (2019) 492–502

UROLOGIC ONCOLOGY

Combination of androgen receptor inhibitor and cisplatin, an effective treatment strategy for urothelial carcinoma of the bladder

Ashish Tyagi, PhD[a], Balaji Chandrasekaran, PhD[a], Venkatesh Kolluru, PhD[a], Samarpit Rai, MD[a], Adrienne C. Jordan, MD[b], Alatassi Houda, MD[b], Jamie Messer, MD[a], Murali Ankem, MD[a], Chendil Damodaran, PhD[a], Ahmed Haddad, MD PhD[a,*]

[a] *Department of Urology, University of Louisville, Louisville, KY*
[b] *Department of Pathology, University of Louisville, Louisville, KY*

Received 3 December 2018; received in revised form 5 February 2019; accepted 10 March 2019

Abstract

Purpose: The role of androgen receptor (AR) signaling in bladder cancer (BCa) is not fully characterized. This study aimed to delineate the role of AR signaling in BCa and to determine whether the combination of AR inhibitor, Enzalutamide (Enz), and Cisplatin (Cis) efficiently inhibit the growth of BCa cells.

Methods: AR expression was determined in 89 human urothelial BCa specimens by immunohistochemistry. A panel of BCa cell lines was treated with Cis, Enz, or a combination of both (Enz + Cis). We determined the expression of AR, changes in apoptotic signaling, DNA damage, and analyzed effect on epithelial mesenchymal transformation markers.

Result: AR expression was detected in 61.4% of tumors from male BCa patients. Inhibition of AR signaling by Enz effectively inhibited the growth of AR^+ BCa cells by inducing apoptosis (26%) in AR^+ TCCSUP ($P = 0.0201$) and J82 (15%, $P = 0.0386$) cells. Interestingly, Enz + Cis synergistically inhibited the proliferation of BCa cells even at low concentrations by inducing proapoptotic signaling in AR^+ BCa cells. Invasive and migratory potential of TCCSUP and J82 cells were reduced with Enz + Cis treatment, and associated with down-regulation of mesenchymal markers.

Conclusions: A high percentage of the bladder tumors from male patients in our cohort expressed AR. The combination of Enz and Cis synergistically inhibited growth of BCa cells more efficiently than single agent alone. This supports the rationale for future investigation of AR antagonists in combination with standard chemotherapy in MIBC. Published by Elsevier Inc.

Keywords: Urothelial carcinoma; Androgen receptor; Combination therapy; Muscle invasive bladder cancer

1. Introduction

Bladder cancer (BCa) is one of the primary causes of cancer death in the US and worldwide [1]. In 2017, approximately 79,030 new cases of BCa were expected with an estimated 16,870 people dying of BCa in the US [2]. BCa, which is mostly urothelial carcinoma, is categorized according to invasiveness of tumor into nonmuscle invasive and muscle invasive bladder cancer (MIBC). Nonmuscle invasive bladder cancer has good prognosis, although is associated with frequent recurrence following local therapy. MIBC constitutes approximately 30% of all BCa cases. Radical cystectomy with pelvic lymph node dissection and neoadjuvant cisplatin (Cis) based chemotherapy is the gold standard for treatment. Cis based regimens such as MVAC (methotrexate, vinblastine, doxorubicin, and Cis) are also employed in the treatment of patients with metastatic BCa [3–5]. Response to Cis treatment is variable, and prognosis of patients with advanced BCa remains poor.

BCa is about 4 times more common in men than in women, even after adjusting for carcinogen exposure such as smoking which is higher in men [6]. Accumulating preclinical evidence suggests a critical role for androgen receptor (AR) signaling in urothelial carcinogenesis, progression, and also the gender-specific occurrence [7]. AR inactivation

*Corresponding author. Tel.: 502-852-5229; fax: 502-852-2123.
E-mail address: ahmed.haddad@louisville.edu (A. Haddad).

https://doi.org/10.1016/j.urolonc.2019.03.008
1078-1439/Published by Elsevier Inc.

by AR antagonists such as Bicalutamide (Bical), Flutamide (Flut), or AR knockdown has resulted in tumor regression in BCa [8–10]. Enzalutamide (Enz) is a synthetic AR signaling inhibitor that not only blocks androgen binding to the AR with a 5 to 8-fold higher affinity compared with Bical, but also prevents AR nuclear translocation, DNA binding, and coactivator recruitment [11]. The efficacy of Enz in reducing the growth of BCa cells remains poorly characterized, and the effect of AR antagonists in combination with Cis based chemotherapy has not been studied in BCa. Based on the preclinical evidence supporting a role for AR in bladder carcinogenesis, we anticipated that targeting AR signaling with Enz in combination with Cis could lead to the development of an effective treatment regimen for BCa patients.

In the current study, we demonstrate that AR is expressed in over half of patients with MIBC, and that the combination of Enz and Cis synergistically inhibit the growth of BCa cells more efficiently than single agent alone.

2. Materials and methods

2.1. Patients and tissue samples

After Institution Review Board approval at our institution, we included 39 consecutive patients for whom bladder tissue was available from cystectomy or transurethral resection of bladder tumor at our institution from 2013 to 2017. Among these, 28 were diagnosed with BCa and 11 with benign inflammatory conditions of the bladder (included as controls). Of the 28 BCa patients at our institution, formalin fixed paraffin embedded tissue was obtained by transurethral resection of bladder tumor in 3 patients and from cystectomy specimen in 25 patients. Five patients had received prior BCG therapy, 6 had received neoadjuvant chemotherapy and 3 patients received mitomycin.

We also utilized a commercial tissue microarray that consists of tissue obtained from 50 BCa patients including 6 controls (US Biomax, Inc.). Pathologic details for the samples collected are shown in Table 1. Tumor pathologic staging was confirmed by an experienced uro-pathologist (H.A.).

2.2. Immunohistochemistry analysis of human bladder tissue

Bladder tissues from the above patients were subjected to Immunohistochemistry for AR expression and the staining location was recorded according to nuclear staining intensity as weak, moderate, or strong as indicated in our previous publications [12,13]. Briefly, the slides of the sections were incubated with primary antibody against AR, followed by incubation with HRP-conjugated secondary antibody at room temperature for 1 hour. Diaminobenzidine (DAB Substrate Kit, Vector Laboratories, Vernon Hills, IL) was used for coloration, and a dark brown color was considered to be positive staining. Two different pathologists calculated the composite score for each tissue core.

2.3. Cell culture and chemicals

RT4, J82, TCCSUP, T24, UMUC3, PC3, and C42B cell lines were obtained from the American Type Culture Collection (ATCC). The UROsta cell line, was provided by Dr. Donald Sens (University of North Dakota, Grand Forks, ND) and grown in DMEM medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/ml), and streptomycin (100 units/ml). T24 and RT4 cells were maintained in McCoy's 5A modified medium, TCCSUP, J82, and UMUC3 in Eagle's Minimum Essential Medium (ATCC 30-2008) supplemented with 10% FBS, penicillin (100 units/ml), and streptomycin (100 units/ml) at 37°C in a humidified atmosphere of 5% CO_2, as mentioned in the ATCC manual. Dihydrotestosterone (DHT) and Flut were

Table 1
Immunohistochemical analysis of bladder cancer tissue specimens showing correlation between AR expression and gender, tumor grade as well as clinicopathological features, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, # not significant.

	Gender					
	Male			Female		
Median age (years)	63			51		
Clinicopathological feature	Tumor grade	AR^+	AR^-	Tumor grade	AR^+	AR^-
Nonmuscle invasive	Ta	1	–	Ta	1	4
	Tis	5	5	Tis	1	2
	T1	7	5	T1	2	5
Muscle invasive	T2	11	5	T2	–	–
	T3	8	7	T3	–	–
	T4	3	–	T4	–	–
Total		35	22		4	11
Normal/inflammation only/−ve for carcinoma		3	6		2	6
Total no. of specimens		66			23	

procured from Sigma, and Bical from Santa Cruz Biotechnology. Enz was obtained from Selleckchem.com and Cis from EMD Millipore Sigma.

2.4. Cell proliferation and soft agar colony formation assays

BCa cells were treated with various concentrations of Enz, Bical, Flut, or Cis and incubated at 37°C for 24 hours. Cell viability was then measured using MTT assays (Cell Signaling, Danvers, MA), as previously described [14–16]. TCCSUP and J82 were chosen for DHT and further experiments based on their high sensitivity to Enz and suitability for combination experiments. For DHT experiments, cells cultured in phenol red-free medium supplemented with 5% charcoal-stripped FBS for 18 hours were cocultured with DHT (10 nm) and Enz (IC_{50} concentration) for 24 hours to avoid any unknown quantity of androgen. A combination experiment was performed using different concentrations of Cis with a constant Enz dose; similarly, different concentrations of Enz were combined with a constant dose of Cis on both TCCSUP and J82 cells for isobologram analysis.

Anchorage-independent growth was monitored by colony formation assay using the 3–2,5-diphenyltetrazolium bromide (MTT) based CytoSelect 96-well in vitro tumor sensitivity assay kit (Cell Biolabs, Inc., San Diego, CA), as previously described [17].

2.5. Apoptosis

Apoptosis assays were performed using Annexin V/FITC kit as per manufacturer's instructions (BD Biosciences, San Jose, CA). Briefly, the TCCSUP and J82 cells (0.3×10^6) were seeded in 6-well plates, grown at 37°C for 24 hours and treated with Enz or Cis or in combination for 24 hours. The harvested cells were analyzed as described previously [18].

2.6. qRT-PCR

Total RNA was isolated from cultured cells using TRIzol (Invitrogen). c-DNA was synthesized with a reverse transcription kit (applied biosystems) using SYBR Green qPCR superMix (Qiagen). The Primers used were: AR (forward, 5′-TCAGACAGTCAAGAATTTCAGAGC-3′; reverse, 5′-CGCACAGGTACTTCTGTTTCC-3′), prostate specific antigen (PSA) (forward, 5′-CCCACTGCATCAGGAACAA-3′; reverse, 5′-ATATCGTAGAGCGGGTGTGG-3′). Actin (forward, 5′-CTCCTCCACCTTTGACGCTG-3′; reverse, 5′-CATACCAGGAAATGAGCTTGACAA-3′) was used as an internal control.

2.7. Protein extraction and western blotting

J82 and TCCSSUP cells were treated with Enz or Cis or in combination for 24 hours. The total protein extracts from both cells were prepared with Mammalian Protein Extraction Reagent (Thermo Scientific, Rockford, IL) according to the manufacturer's protocol. For DHT treatment, cells seeded in 6-well plates were cultured in phenol red-free medium supplemented with 5% charcoal-stripped FBS for 18 hours and treated with DHT (10 nM) and Enz separately and in combination for 24 hours. For Enz and Cis combination experiments, the cells grown in 6-well plates were treated with Enz and Cis IC50 dosages separately and Enz + Cis combination dose (isobologram analysis; Section 2.9). Western blotting was performed using specific antibodies against pATM, pATR, phosphorylated checkpoint kinase-1, pHis (DNA damage sampler kit #4445, cell signaling, Danvers, MA), BAX, BCL-2, Cleaved Caspase-3, Cleaved PARP, slug, β-catenin, E-cadherin, N-cadherin and Vimentin (cell signaling), AR (SC-816), PSA (SC-7638), β-actin, and GAPDH (Santa Cruz biotechnology, Dallas, TX). Protein-antibody complexes were visualized using enhanced chemiluminescence (Invitrogen).

2.8. Isobologram analysis

The isobologram method was used for graphical representation of the pharmacologic interaction of 2 individual drugs, generated by selecting a desired fractional cell kill (Fa) and plotting the individual drug doses required to generate that Fa on their respective *x*- and *y*-axis. The combination index (CI) method is a mathematical and quantitative representation of a 2-drug pharmacologic interaction. Using data from the growth-inhibitory experiments and computerized software (CompuSyn, Combo Syn, Inc., Paramus, NJ), CI values were generated over a range of Fa levels from Enz and Cis treatment. CI values 0.1 to 0.90, synergism; 0.91 to 1.11 additive; 1.11 to 3.30, antagonism; 3.31 to 10, strong antagonism.

2.9. Statistical analysis

The data were presented as the mean ± standard error of the mean. We determined significant differences between groups using the unpaired Student's *t* test and one-way ANOVA. These differences were established at $P < 0.05$. All of the statistical analyses were performed using Prism 7 software (GraphPad Software Inc., La Jolla, CA). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{\#}$ not significant.

3. Results

3.1. AR expression in human BCa specimens and panel of BCa cell lines

AR was expressed in 61.4% of bladder tumors from male patients compared to 26.6% of tumors from female patients (Table 1). In males, AR expression was demonstrated in 56.5% of nonmuscle invasive tumors compared to 65% of muscle invasive tumors and 33% of benign urothelium samples (Table 1, and Fig. 1A).

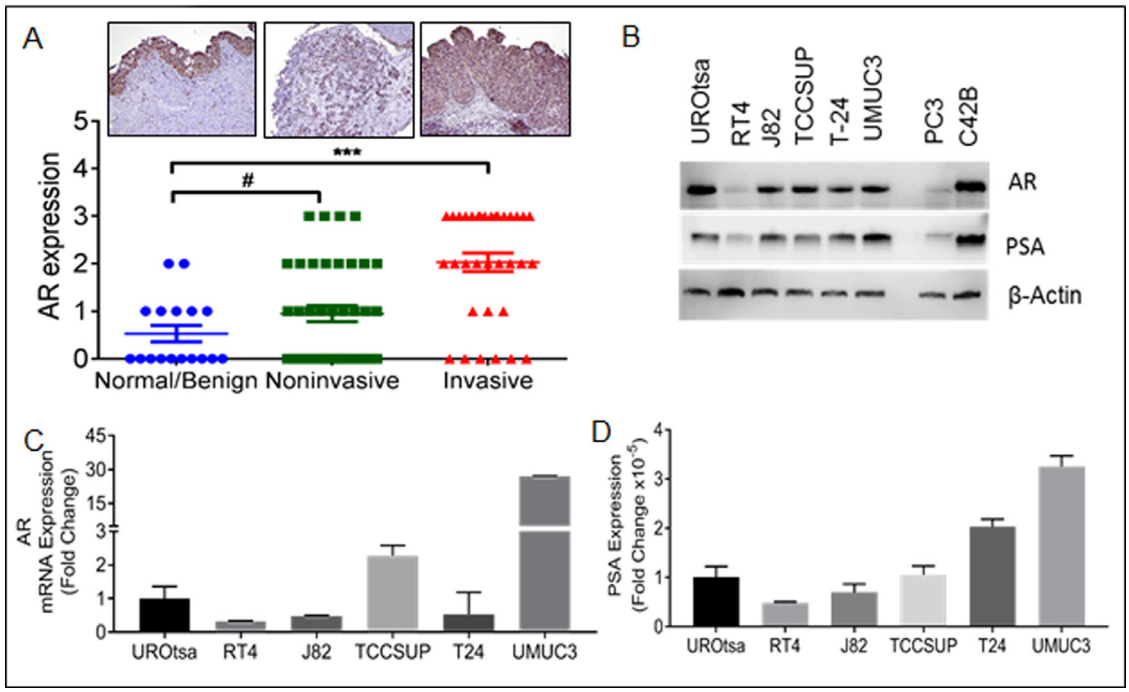

Fig. 1. (A) Immunohistochemical analysis showing nuclear AR expression (brown stained cells) in different pathological grades of human BCa patient specimens. (B) AR and PSA Immunoblot expression in panel of BCa cell lines. (C, D) mRNA expression of AR and PSA in panel of BCa cell lines.

Cell lines screened for AR expression included an immortalized normal human urothelium cell line (UROtsa), and cell lines derived from urothelial papilloma (RT4) and highly metastatic urothelial carcinoma (J82, TCCSUP, T24, and UMUC3) cell lines. PC3 and C42b were used as negative (PC3) and positive control (C42b) for AR and PSA expression. AR protein and mRNA was detected in all BCa cell lines except RT4 (Fig. 1B and C and Supplementary Fig. 1). To understand the functional status of AR, we analyzed expression of PSA, which is a down-stream AR target (Fig. 1B and D, and Supplementary Fig. 1). PSA protein expression was highest in UMUC3 cell line. PSA mRNA levels were detected in all cell lines and highest in UMUC3 (Fig. 1D). Thus, all BCa cell lines tested expressed AR and PSA at the protein and transcript levels except RT4 which had very low levels of AR and PSA expression.

3.2. Enz inhibits the growth of AR^+ BCa cell lines

To determine the inhibitory effect of Enz human BCa cell lines were cultured in respective growth medium and treated with different concentrations of Enz for 24 hours. AR null RT4 cell line was resistant to Enz treatment, and minimal growth inhibition was also seen for AR^+ UROtsa, UMUC3, and T24 (Supplementary Fig. 2). Significant growth inhibition was seen in AR^+ cell lines TCCSUP (IC_{50}: 4.4 μM; $P < 0.001$) and J82 (IC_{50}: 6.6 μM; $P < 0.001$) (Fig. 2A).

To further confirm AR function, we treated TCCSUP and J82 cells with Bical and Flut. Similar dose-dependent decrease in viability was observed (Fig. 2B and C). The IC_{50} concentrations for Bical were 5 μM for TCCSUP ($P < 0.001$) and 4.6 μM for J82 cell lines ($P < 0.001$) (Fig. 2B). Similarly, the IC_{50} for Flut for TCCSUP (5 μM; $P < 0.001$) and J82 (5μM; $P < 0.001$) (Fig. 2C) was comparable to Enz inhibition ($P < 0.001$) (Fig. 2A). Since TCCSUP and J82 cells showed significant inhibition of proliferation with antiandrogen, and at lower concentrations compared to other BCa cell lines, we selected these 2 cell lines for further studies.

3.3. AR inhibitors induce apoptosis in AR^+ BCa cells

After confirming the inhibitory effect of AR inhibitors, we investigated whether inhibition of growth may be due to induction of apoptosis. We performed AnnexinV/FITC analysis after treating with Enz in both TCCSUP and J82 cells. Significant induction of apoptosis was seen in both TCCSUP (26%; $P < 0.01$) (Fig. 2D) and J82 (15%; $P < 0.05$) with Enz treatment (Fig. 2E). Similarly, Bical and Flut induced apoptosis in both TCCSUP (26% and 29% respectively, $P < 0.01$) and J82 (18.4 and 11% respectively, $P < 0.05$) (Fig. 2D and E). To further validate the induction of apoptosis, we analyzed expression of proapoptotic markers in both TCCSUP and J82. The expression of proapoptotic markers BAX, Cleaved Caspase 3, and Cleaved PARP were significantly upregulated as compared to vehicle-treated TCCSUP and J82 cells (Fig. 2F

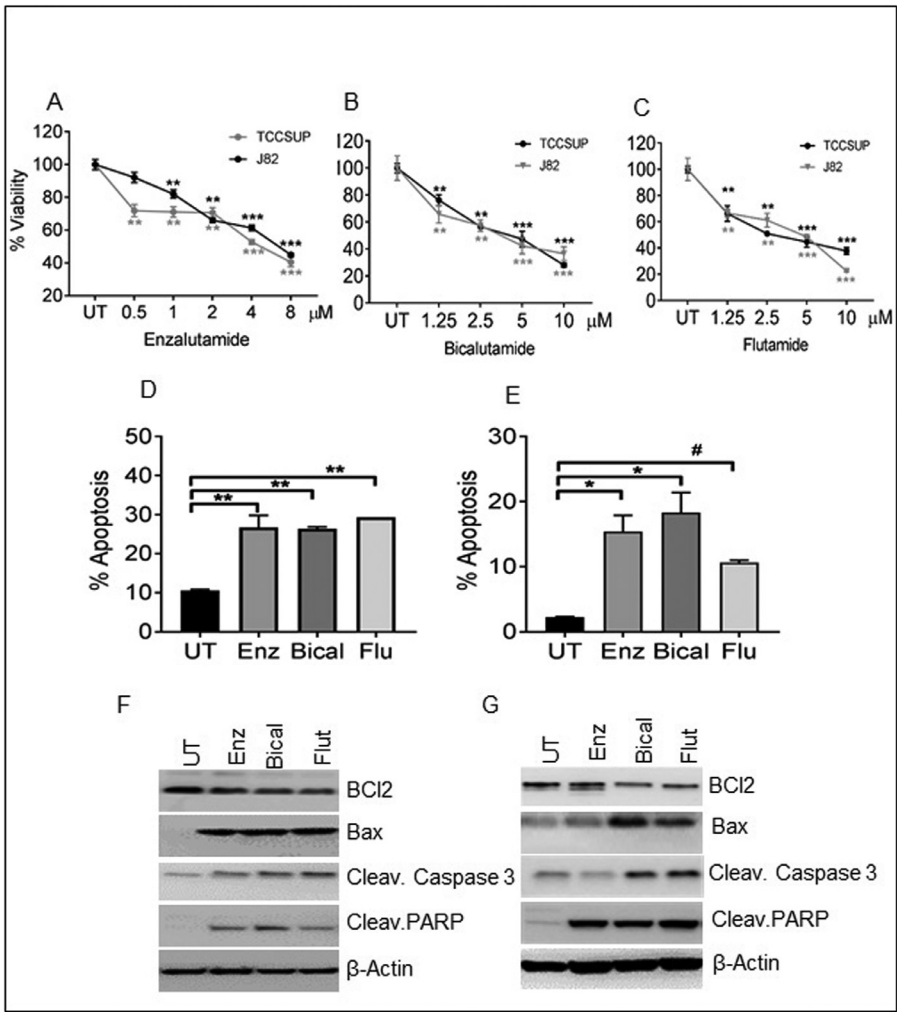

Fig. 2. MTT cell proliferation assay of TCCSUP and J82 cells treated with 0.1% DMSO (as control) or indicated concentration of Enz (A) Bical (B) Flut (C) for 24 hours. (D and E) TCCSUP and J82 were either treated with IC_{50} concentrations of Enz, Bical, and Flut, or vehicle and apoptosis assay was performed. The percentage of apoptotic TCCSUP and J82 cells was counted from 2 independent experiments. (F and G) western blotting of proapoptotic/antiapoptotic proteins. Student's *t* test and one way Annona was used to calculate statistical significance between vehicle control and each treatment. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{\#}$ not significant.

and G, respectively). On the other hand, downregulation of BCl-2 was observed in both cell lines (Fig. 2F and G and Supplementary Fig. 3). These results suggest that AR inhibitors effectively induce apoptosis in BCa cells.

3.4. Enz inhibits androgen-induced cell proliferation in BCa

Next, to understand whether AR is functional in BCa cell lines, both TCCSUP and J82 cells were treated with DHT and then by Enz for 24 hours, and cell viability was assessed. DHT treatment significantly ($P < 0.05$) increased cell proliferation of AR^{+} TCCSUP (33.3%) and J82 (17.28%) cell lines (Fig. 3A and B, respectively). Enz inhibited DHT-induced cell proliferation significantly ($P < 0.01$) in both TCCSUP (43.8%) and J82 (28.92%) (Fig. 3A and B, respectively).

DHT treatment increased AR mRNA expression in TCCSUP to a greater degree than J82 cell line and no significant change in AR expression was seen with Enz treatment in either cell line (Fig. 3E and F). Significant downregulation of PSA expression (Protein and mRNA) was seen in Enz treated J82 and TCCSUP cell lines (Fig. 3C–H and Supplementary Fig. 4). AR translocation to the nucleus is one of the primary mechanisms by which Enz executes its function. To understand whether this process was also contributing to the inhibition of BCa cell viability, we analyzed AR nuclear translocation status in Enz treated cells through confocal imaging. AR translocation to nucleus was significantly inhibited in Enz treated MIBC cells (Fig. 3I). The cells with translocated AR were quantified and the results suggest that Enz significantly ($P < 0.05$) prevents nuclear shuttling of AR (Fig. 3I).

3.5. Enz chemosensitizes Cis's therapeutic effect in BCa

Cis treatment in TCCSUP (IC_{50}-8.2 μM) and J82 (IC_{50}-13.02 μM) demonstrated dose dependent growth inhibition

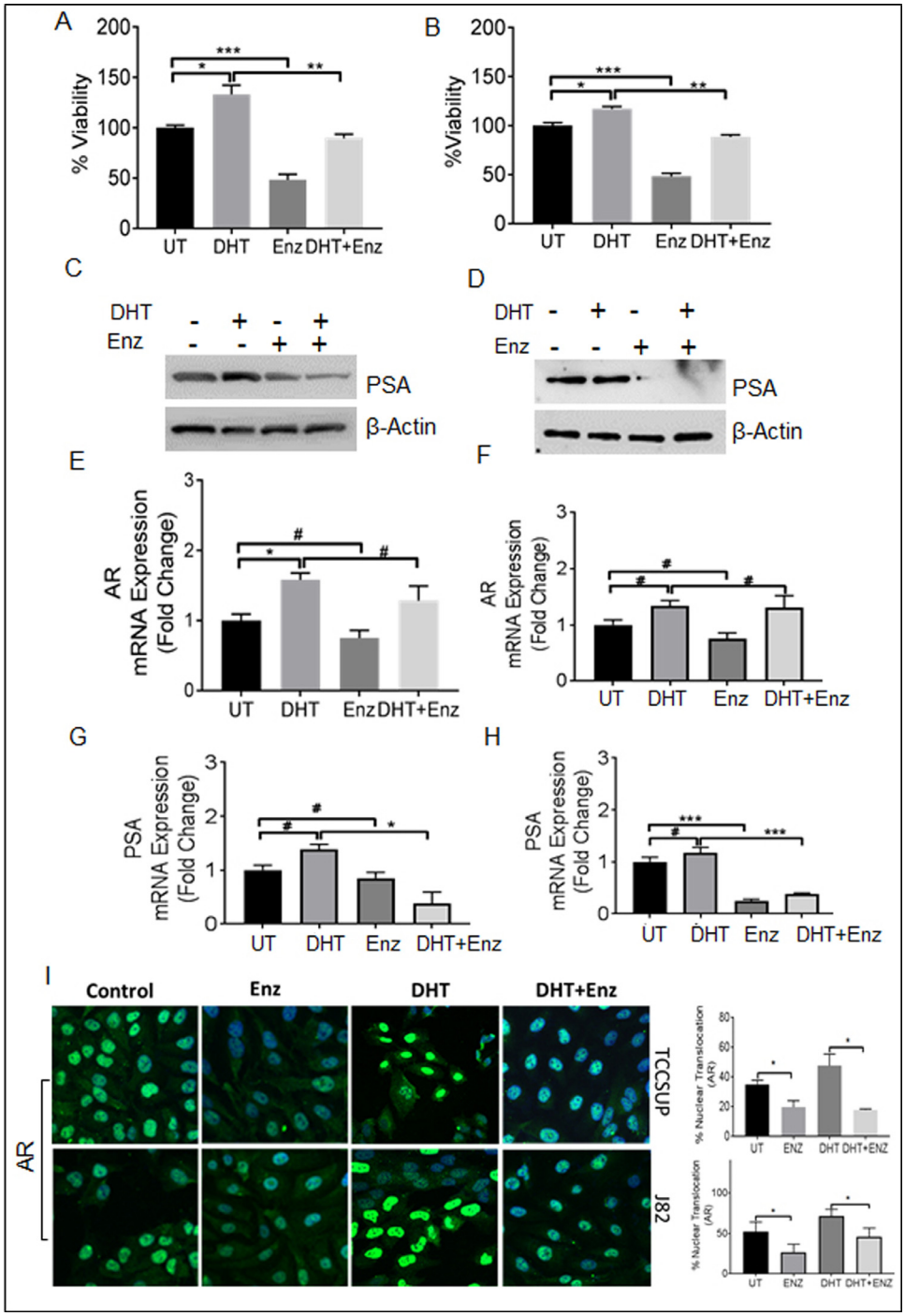

Fig. 3. (A and B) MTT assay was performed in TCCSUP and J82 cell lines, each value represents the mean + SD from at least 3 independent experiments. (C and D) western blot analysis for effect on AR and PSA expression. (E and F, G and H) qPCR analysis with AR and PSA specific primers. (I) BCa cells were treated as indicated above and stained with AR antibody for confocal imaging. The percentage of cells stained green were counted from 2 independent experiments. Student's *t* test was used to calculate statistical significance between vehicle and treatment at each concentration. $^{*}P < 0.05$ and $^{**}P < 0.01$, $^{***}P < 0.001$, $^{\#}$ not significant.

(Supplement Fig. 5). These data were used to determine combination index (CI) values through an isobologram analysis. A CI value of 0.35 was obtained with 5 μM of Cis (IC_{30}) and 1.25 μM of Enz (IC_{15}) for TCCSUP and CI of 0.48 in J82 cells (5 μM of Cis (IC_{20}) and 2.5 μM of Enz (IC_{15}). Based on the isobologram analysis, we selected the same doses for molecular studies of combination therapy in both TSSCUP and J82. The Enz + Cis combination demonstrated significant inhibition of BCa cell growth (Fig. 4A and B). Earlier we confirmed that Enz treatment induces apoptosis in TCCSUP (26%) and J82 (15%). To analyze whether lower dosages of Enz + Cis (when used in

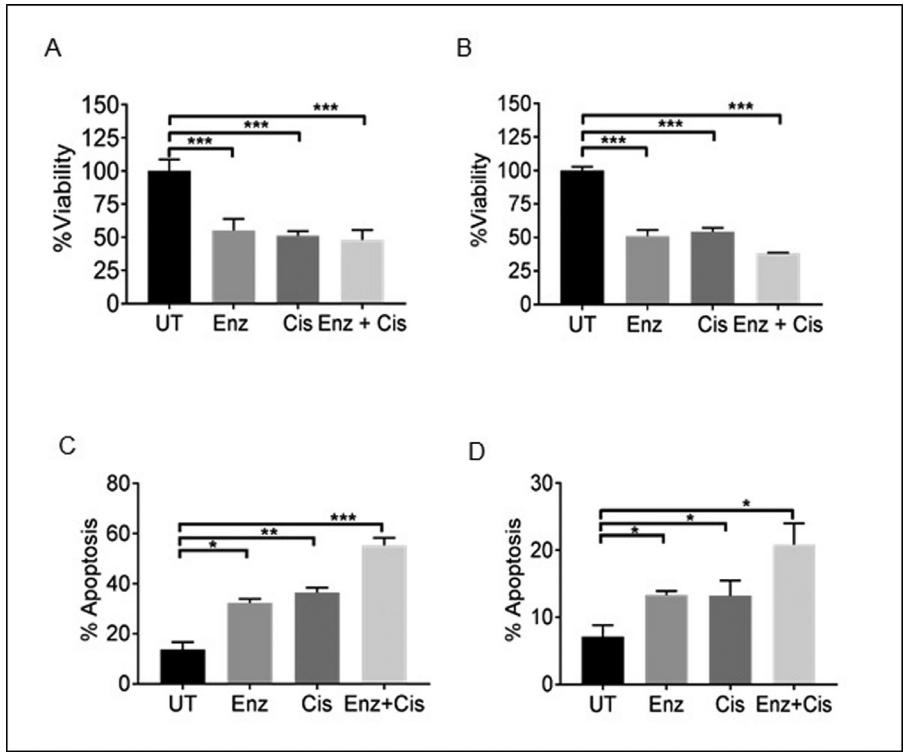

Fig. 4. (A) MTT assay was performed in TCCSUP cultured with either DMSO (UT), Enz/Cis alone, or the Enz + Cis combination (5 μM of Cis and 1.25 μM of Enz). (B) J82 cells were treated with DMSO, Enz/Cis, or Enz + Cis combination (5 μM of Cis and 2.5 μM of Enz) for 24 hours. (C and D) Flow cytometry-based apoptosis assay was performed using Annexin V−FITC and propidium iodide staining. The percentages of apoptotic TCCSUP and J82 cells were counted from 2 independent experiments Student's *t* test was used to calculate statistical significance between vehicle control and treatment at each concentration. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{\#}$ not significant.

combination) can initiate apoptotic signaling in BCa cells, we treated both cell lines with Enz, Cis, and Enz + Cis combination. Interestingly, the drug combination induced apoptosis significantly more than the monotherapies. Apoptosis induced in TCCSUP by Enz + Cis combination (58.29%; $P < 0.001$) was significantly higher compared to Enz alone (26%; $P < 0.01$) and Cis alone (38%; $P < 0.05$) (Fig. 4C). In J82, Enz + Cis combination induced higher apoptosis (21%; $P < 0.05$) as compared to Enz alone (13.3%; $P < 0.05$) and Cis alone (13.2%; $P < 0.05$) (Fig. 4D).

3.6. Enz potentiates Cis induced DNA damage in BCa cells

Cis-based therapies work by inducing DNA damage or inhibiting DNA damage repair mechanisms in BCa. To understand whether Enz has the ability to potentiate Cis induced DNA damage in BCa, TCCSUP and J82 cells were treated with Enz and Cis alone or the combination Enz + Cis derived from isobologram analysis. Cis treatment increased DNA damage response (DDR) regulators ATM, ATR, phos-Histone1, and phosphorylated checkpoint kinase-1 expression in both cell lines (Fig. 5A, Supplementary Fig. 6A). In combination, Enz + Cis induced a similar effect on DDR and checkpoint kinase expression as single agent Cis in both cell lines.

AR transcript level were downregulated in Enz + Cis treated TCCSUP ($P < 0.05$) and J82 ($P < 0.05$) cells in contrast to standalone Enz/Cis treatments (Fig. 5B and C). Interestingly, Enz + Cis treatment significantly reduced PSA expression in TCCSUP ($P < 0.05$) and J82 ($P < 0.001$) (Fig. 5D and E). Further, western blot analysis also suggested increased expression of BAX, Cleaved Caspase3, and Cleaved PARP in both cell lines when treated with the combination Enz + Cis (Fig. 5F and G). On the other hand, BCl-2 expression was inhibited in both cell lines (Fig. 5F and G). The densitometry analysis results are represented in Supplementary Fig. 7. These results substantiate our preliminary findings of the effective potency of the Enz + Cis combination even at lower dosages.

3.7. Enz and Cis in combination efficiently suppress the epithelial and mesenchymal transition (EMT) in AR$^+$ BCa

Cell migration and invasion are prominent markers of tumor progression and metastasis. A scratch wound-healing assay and a transwell invasion assay were performed to assess the effects of Enz + Cis combination on migration and invasion in both BCa lines.

Enz + Cis combination significantly reduced the migratory potential of TCCSUP (65.9%; $P < 0.01$) and J82 (55.9%, $P < 0.01$) cells (Fig. 6A and B). The invasive

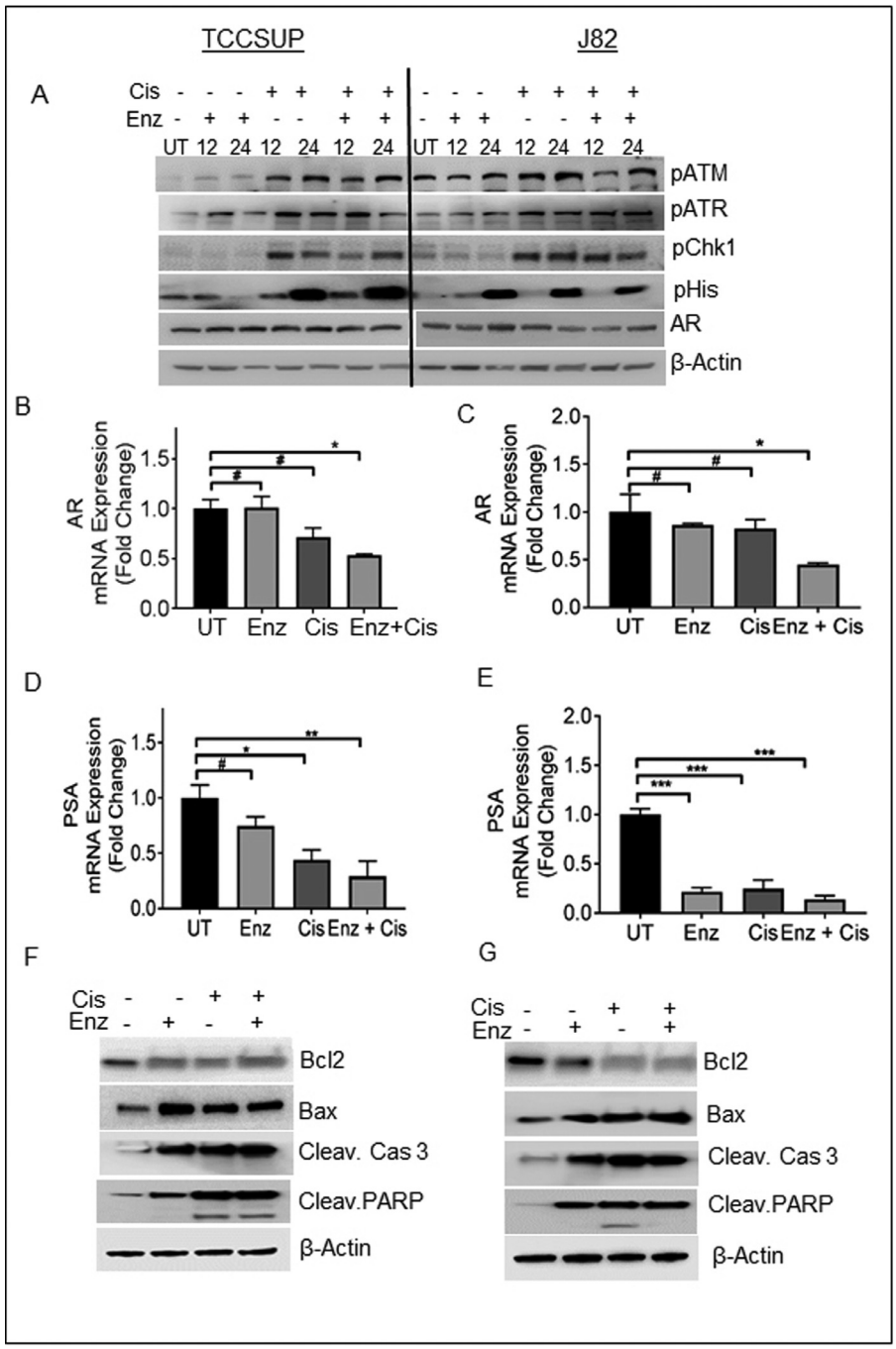

Fig. 5. (A) TCCSUP and J82 cells treated as mentioned above, for 12 and 24 hours and cell lysates used for western blot analysis for effect on DDR markers. (B and C) TCCSUP and J82 cells were treated as mentioned above for 24 hours and extracted mRNA was used for qPCR analysis with AR specific primers. (D and E) TCCSUP and J82 cells with ENZ, CIS, and ENZ+CIS for 24 hours and mRNA were subjected to qPCR analysis with PSA specific primers. (F and G) TCCSUP and J82 cells were treated with Enz, Cis, and Enz + Cis combination, cell lysates were used for western blot analysis for apoptosis markers. Student's *t* test was used to calculate statistical significance between vehicle control and treatment at each concentration. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$., $^{\#}$ not significant.

capacity was also remarkably suppressed in cells treated with Enz + Cis combination i.e. 87% ($P < 0.01$) in TCCSUP and 83% ($P < 0.01$) in J82 (Fig. 6C and D) as compared to monotherapies.

At the molecular level, Enz + Cis combined treatment upregulated E-cadherin expression and downregulated N-cadherin, β-catenin, Vimentin, and Slug expression in both TCCSUP and J82 cell lines (Fig. 6E and F, Supplementary Fig. 8). Based on these results, it is evident that Enz and Cis, when combined, not only inhibit cell viability but also affect epithelial to mesenchymal transition capabilities of BCa cells.

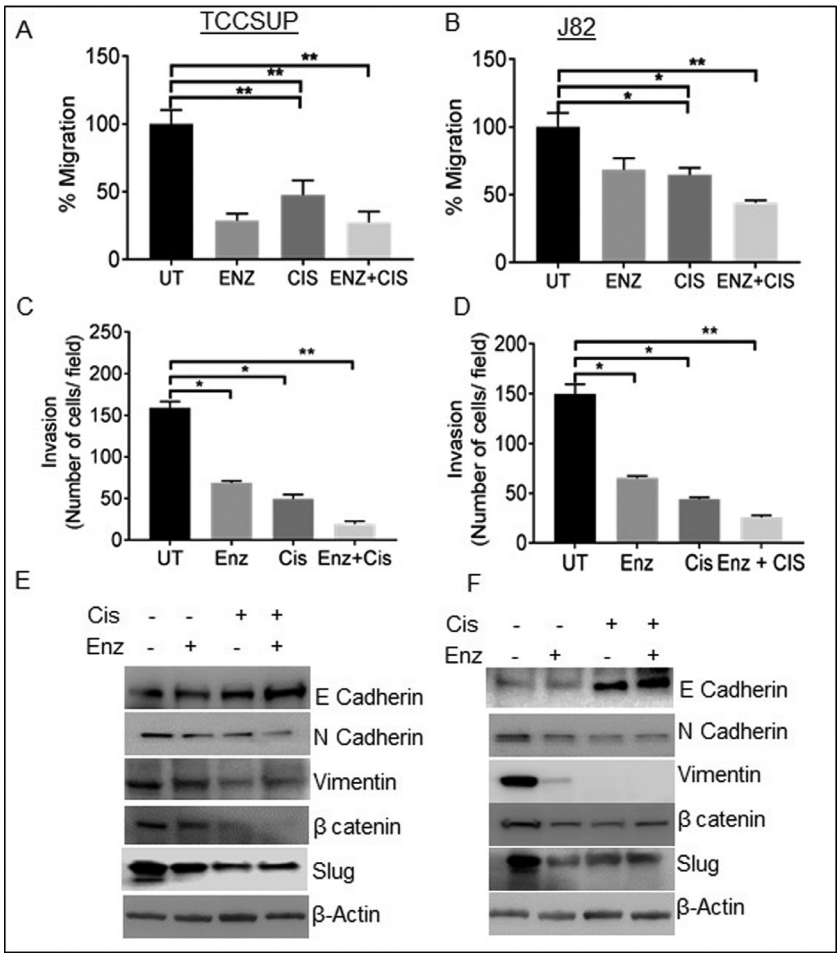

Fig. 6. (A and B) TCCSUP and J82 cells were plated, and a linear wound across the monolayer was created. The cells were then treated with an individual drug or combination as mentioned previously. The wound gap was photographed for 0 hour and 24 hours. The gap width was measured (μm) using ImageJ software. (C and D) Transwell invasion assay for TCCSUP and J82 cells was performed using Boyden chambers. Invaded cells were stained with crystal violet and counted. (E and F) Western blot analysis was performed to determine the expression of EMT markers in TCCSUP and J82 cells treated for 24 hours. Student's *t* test was used to calculate statistical significance between treated and untreated cells $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$., $^{\#}$ not significant.

4. Discussion

Despite recent advances in therapies for BCa, survival for patients with MIBC remains poor. Cis based chemotherapy is considered standard of care in the neoadjuvant setting prior to cystectomy, and is also employed in the treatment of metastatic disease. Unfortunately, many patients are not eligible for Cis due to comorbidities or impaired renal function. In this study, we evaluated the potent AR antagonist Enz as a potential chemo-sensitizing agent in combination with Cis. We demonstrated that the combination doses, although lower than IC_{50} dosages for the individual drugs, induce significant apoptosis and inhibit migratory as well as invasive potential of BCa cells.

The role of the AR in BCa has been reported in several recent studies [19–20]. In our results, 61.4% of male BCa patients expressed AR, which positively correlated with tumor stage. Reports regarding AR expression in tumors from patients with BCa are contradictory. While some studies have demonstrated a significant decrease in AR expression with high tumor stage and grade [21,22], others have shown that muscle invasive tumors have higher (15%) AR expression compared to nonmuscle invasive (9%) tumors [23]. Another study correlated higher tumor recurrence risk in patients with tumors expressing more AR [24]. In our studies, the expression of AR is high in male BCa patients and only 26.6% female patients expressed AR. We also observed AR expression in the majority of BCa cell lines, which is consistent with the findings of other investigators [10,24–26]. In our study, the AR^{+} cell lines TCCSUP and J82 were considerably more sensitive to the effects of Enz, as compared to AR^{+} UMUC3 and UROtsa cell lines. We did not evaluate for AR splice variants such as AR-V7, which have been implicated in resistance to Enz in prostate cancer [27]. Resistance to Enz can also be consequent to mutations at ligand binding domain of AR [28]. Interestingly, the AR^{+} prostate cancer cell line, LNCaP, which has a mutation at the ligand binding domain of AR demonstrates growth inhibition at higher concentrations Enz than the AR^{+} BCa cell lines tested in our study (TCCSUP

and J82) [29]. The reason for higher sensitivity of some AR^+ BCa cell lines compared to others needs to be further investigated.

Activation of AR has been correlated with BCa progression [26,30] and resistance to Cis-based therapies [31]. Enz is a preferred choice over Flut and Bical, owing to its multipronged action against AR signaling. It has a higher binding affinity to AR, prevents AR translocation to the nucleus, and is detrimental to AR binding to DNA as well as coactivators. Interestingly, in our studies, Enz inhibited cell proliferation by inducing apoptosis in AR^+ BCa cell lines. We anticipated that by blocking androgen-induced AR signaling and inducing DNA damage with Cis we would efficiently inhibit BCa cell proliferation. In our studies, we showed synergy between Enz and Cis in inhibiting cancer cell proliferation in AR^+ BCa cell lines. Interestingly, Enz treatment either alone or in combination seemed to have little additional effect on DDR expression (ATR, ATM, p-Chk1, and p-His) compared to Cis treatment alone (although combination was employed at IC_{20} concentration compared to IC_{50} concentration for single agent treatment). Despite the lack of induction of DDR by combination treatment, the combination of both agents induced significant induction of apoptosis in BCa cells. The same combination significantly inhibited invasive and migratory potential of both the BCa cell lines, and was associated with decreased EMT as evidenced by reduced β-catenin and increase in E-cadherin expression. AR and β-catenin have been positively correlated i.e. increased in high-grade prostate cancer [32], their interaction results in increase of transcriptional activity of AR in response to androgen [33]. β-catenin degradation and increase in E-cadherin expression were able to block EMT in prostate cancer [34]. Similar results were observed in the present study. The initiation of EMT is a primary attribute that leads to cancer metastasis and thus contributes to the poor prognosis of cancer patient. Targeting EMT is considered as a hallmark for improving overall survival of a patient [35]. In this respect Enz and Cis combination can be of significance.

Our study is not without its limitations. TCCSUP and J82 BCa cell lines were chosen based on effective inhibition by Enz (<10 μM) treatment, however other AR^+ cell lines T24, UMUC3 were sensitive at higher concentrations (>19 μM). We did not evaluate the reason, however, a splice variation or AR mutations could be the reason of Enz resistance in T24 and UMUC3. Further, some of the patients in the cohort received prior BCG, mitomycin, or neoadjuvant chemotherapy and this may have affected expression of AR or PSA in tumor samples from these patients. Despite the limitations, it is worth mentioning here that as proof of principle, this is the first study wherein the combination of Enz and Cis has been shown to inhibit growth of BCa cells in Enz sensitive AR^+ BCa cell lines.

Overall, this study demonstrates that AR is expressed in over half of patients with MIBC, and that the combination of Enz and Cis synergistically inhibited growth of BCa cells more efficiently than single agent alone. This supports the rationale for future investigation of AR antagonists in combination with standard chemotherapy in MIBC. Utilizing the combination therapy could potentially allow for use of lower Cis doses which is desirable given that patients with advanced BCa are often elderly, with multiple comorbidities and impaired renal function. This study further highlights the importance of AR in BCa and supports the rationale for future investigation of AR antagonists in combination with standard chemotherapy in MIBC.

Acknowledgments

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.urolonc.2019.03.008.

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