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Original Article

Mineralocorticoid receptor signaling inhibits bladder cancer progression

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Abstract: The biological or clinical significance of mineralocorticoid receptor (MR) in urothelial cancer remains largely unknown. The present study aimed to determine the functional role of MR in bladder cancer progression. In two of the human bladder cancer lines expressing MR, treatment with a natural MR ligand, aldosterone, significantly reduced cell proliferation and migration, which was restored by three MR antagonists clinically used, spironolactone (except colony formation of androgen receptor-positive cells cultured in the presence of androgens), eplerenone, and esaxerenone. Similarly, MR knockdown via shRNA virus infection resulted in significant increases in cell viability/migration, as well as colony formation, compared with control sublines. In addition, MR knockdown augmented the expression of β-catenin, c-fos, and N-cadherin, and lowered that of E-cadherin and p53, indicating the induction of the cadherin switching. Immunohistochemistry in surgical specimens detected MR signals in 58 (92.1%; 36.5% weakly-positive/1+, 44.4% moderately-positive/2+, and 11.1% strongly-positive/3+) of 63 muscle-invasive bladder cancers, which was significantly lower than in adjacent non-neoplastic urothelial tissues (100%; 15.7% 1+, 37.3% 2+, and 47.1% 3+). Moreover, patients with MR-high (3+) tumor had a significantly lower risk of cancer-specific mortality (P=0.039). Multivariable analysis further showed that strong MR expression was an independent predictor of cancer-specific survival in patients with muscle-invasive bladder cancer (hazard ratio 0.117, P=0.039). These findings suggest that MR signaling functions as a tumor suppressor in urothelial carcinoma and prevents tumor growth. Accordingly, there is a possibility that the concurrent use of anti-mineralocorticoids, particularly eplerenone and esaxerenone, in patients with bladder cancer rather contributes to the promotion of disease progression.

Keywords: Aldosterone, anti-mineralocorticoid, bladder cancer, eplerenone, esaxerenone, mineralocorticoid receptor, spironolactone, tumor progression, urothelial carcinoma

Introduction

Urinary bladder tumor, mostly urothelial carcinoma, has been one of the most commonly diagnosed malignancies, especially in men [1, 2]. Moreover, the numbers of new bladder cancer cases and cancer related deaths throughout the world have increased from 429,800 and 165,100 in 2012 [1] to 573,278 and 212,536 in 2020 [2], respectively. In particular, muscle-invasive bladder cancer is often associated with metastatic disease where the overall 5-year survival rate remains low (*e.g.* 8.3% [3]). Further identification of key molecules or signaling pathways responsible for the progression of urothelial carcinoma may thus be required to ultimately provide novel targeted therapy options for advanced bladder cancer.

Steroid hormone receptors are ligand-inducible transcription factors and are known to involve a wide variety of physiological and pathological processes. Of these, androgen receptor (AR) [4, 5], estrogen receptors (*e.g.* ERα, ERβ) [5, 6], and glucocorticoid receptor [5, 7, 8] have been implicated in the pathogenesis and progression of urothelial cancer. By contrast, the biological or clinical significance of mineralocorticoid

receptor (MR) in bladder cancer remains poorly understood [9].

As a member of the nuclear receptor superfamily, MR physiologically functions via binding of its ligands, such as aldosterone, the major endogenous mineralocorticoid, in the kidney and several other organs and thereby plays a vital role in regulating ionic and water transports, leading to, for example, the reabsorption of sodium [10]. Meanwhile, anti-mineralocorticoids, such as spironolactone, eplerenone, and a novel non-steroidal selective MR antagonist esaxerenone, have been widely prescribed as diuretic agents for the treatment of various pathologic conditions, including hypertension, heart failure, chronic kidney disease, and primary aldosteronism [11]. Interestingly, it has been documented that some of glucocorticoids (primarily as agonists), as well as other steroids, including progesterone (as an antagonist), bind to the MR [11, 12]. In addition, spironolactone has been shown to possess anti-androgenic and progestational activities, while other anti-mineralocorticoids, including eplerenone and esaxerenone, specifically bind to the MR [11].

We have recently demonstrated that MR activation is associated with the suppression of urothelial tumorigenesis [13], which is believed to be a distinct process from tumor progression. In the present study, we aimed to determine the functional role of MR signaling in urothelial cancer outgrowth.

Materials and methods

Cell culture and chemicals

Human bladder cancer lines, UMUC3, TCCSUP, J82, and 5637, as well as an immortalized human normal urothelial line SVHUC and a human embryonic kidney cell line 293T, were originally obtained from the American Type Culture Collection. A histiocytic lymphoma line U937 was a kind gift from Dr. Michael W. Becker (University of Rochester Medical Center, Rochester, NY, USA). In TCCSUP and J82, MR-short hairpin RNA (shRNA) (sc-38836-V, Santa Cruz Biotechnology), a pool of concentrated, transduction-ready lentiviral particles containing three target-specific constructs encoding 19-25 nt shRNA (*i.e.* TCCSUP-MR-shRNA, J82-MR-shRNA), or non-silencing control shRNA (sc-108080, Santa Cruz Biotechnology) (*i.e.* TCCSUP-control-shRNA, J82-control-shRNA) was stably expressed, as we described previously [13, 14]. UMUC3/TCCSUP/J82/5637/293T (and TCCSUP/J82 sublines), U937, and SVHUC were maintained in Dulbecco's modified Eagle's medium (Gibco), RPMI-1640 (Mediatech), and Ham's F-12K (Mediatech), respectively, supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/mL), and streptomycin (100 μg/mL) at 37°C in a humidified atmosphere of 5% CO_2. TCCSUP, J82, and their sublines were cultured in phenol red-free medium supplemented with 10% FBS or 5% charcoal-stripped FBS (CS-FBS) at least 24 hours before actual assays. We obtained aldosterone, spironolactone, and eplerenone from Cayman Chemical, and esaxerenone from ChemScene.

Cell viability

The MTT assay was used to assess the cell viability. Cells (3-5 × 10^3/well) seeded in 96-well tissue culture plates were incubated for 96 hours, and at the end of the culture 10 μL MTT stock solution (5 mg/mL; Sigma-Aldrich) was added to each well for 3 hours at 37°C. The medium was replaced with 100 μL dimethyl sulfoxide and incubated for 5 minutes at room temperature. The absorbance was then measured at a wavelength of 570 nm with background subtraction at 630 nm.

Colony formation

The clonogenic assay was used to assess the clonogenic potential. Cells (1 × 10^3/well) seeded in 6-well tissue culture plates were allowed to grow until colonies in the control well were certainly detectable. The cells/colonies were then fixed with methanol and stained with 0.1% crystal violet. The number of colonies in photographed pictures was quantitated, using ImageJ software (National Institutes of Health).

Cell migration

A scratch wound-healing assay was used to assess the ability of cell migration. Cells at a density of 90-100% confluence in 6-well tissue culture plates were scratched manually with a sterile 200 μL plastic pipette tip. The cells were incubated in serum-free medium for 24 hours, fixed with methanol, and stained with 0.1%

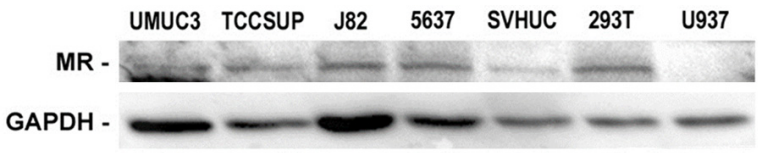

Figure 1. MR expression in bladder cancer cell lines. Western blotting of MR in human bladder urothelial carcinoma cells (UMUC3, TCCSUP, J82 and 5637) and human normal urothelial SVHUC cells, as well as non-urothelial positive (293T) and negative (U937) control cells. GAPDH served as a loading control.

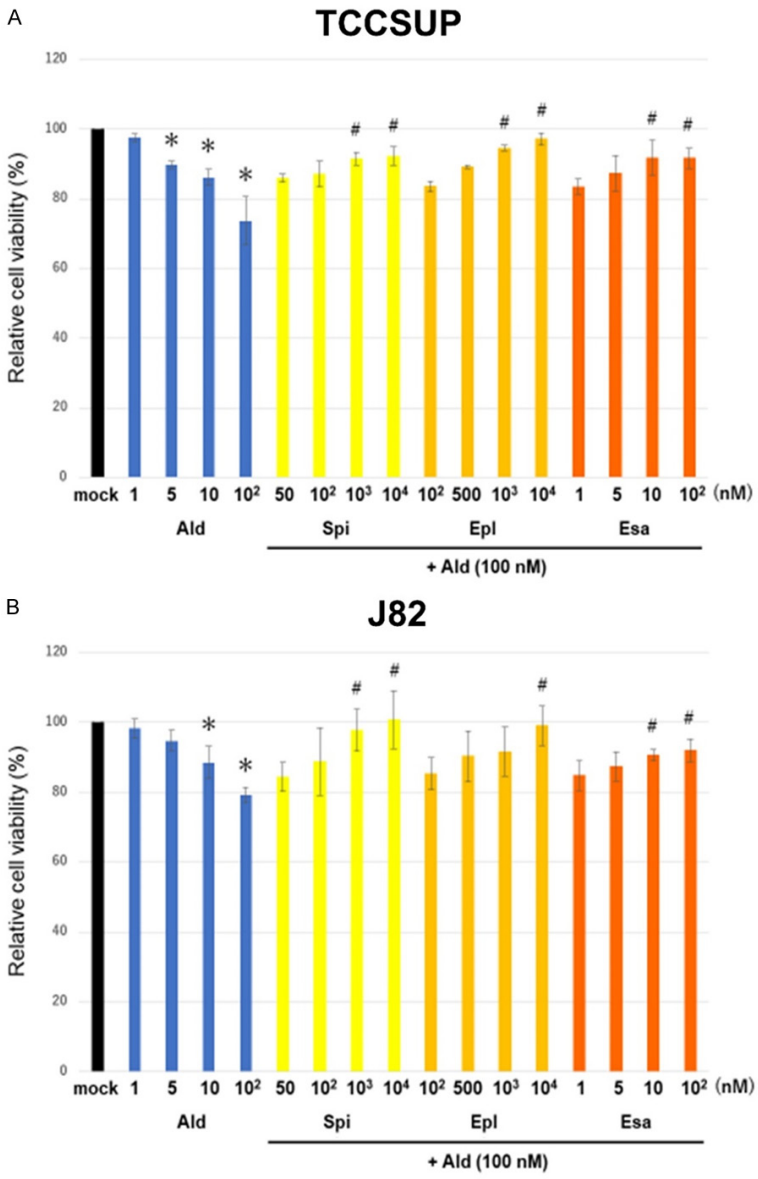

Figure 2. Effects of MR ligands on the proliferation of bladder cancer cells. MTT assay in TCCSUP (A) and J82 (B) cells cultured for 96 hours in medium containing 5% CS-FBS as well as ethanol (mock), aldosterone (Ald; 1-100 nM) alone, or aldosterone (100 nM) plus spironolactone (Spi; 50-10,000 nM), eplerenone (Epl; 100-10,000 nM), or esaxerenone (Esa; 1-100 nM). Cell viability presented relative to that of mock-treated cells represents the mean (± SD) from three independent experiments. **P*<0.05 (vs. mock treatment). #*P*<0.05 (vs. aldosterone 100 nM alone).

crystal violet. The width of the wound area was then quantitated, using the ImageJ.

Cell invasion

Cell invasiveness was determined using 24-well transwell inserts (6.5 mm diameter polyethylene terephthalate filter with 8 μm pore size, Corning) coated with BD Matrigel™ (BD Biosciences). Cells (2×10^4) in 100 μL serum-free medium were added to the upper compartment of invasion chamber, whereas 600 μL medium supplemented with 10% FBS was added to the lower chamber. After 24 hours, invaded cells were washed with PBS, removed with a cotton swab on the upper surface of the membrane, fixed with methanol, stained with 0.1% crystal violet, and counted.

Western blotting

Cell pellets from 6-well tissue culture plates were lysed in RIPA buffer supplemented with a protease and phosphatase inhibitor cocktail (Halt Protease and Phosphatase Inhibitor cocktail; Thermo Scientific). Equal amounts (30 μg) of proteins extracted from the cells were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10%). After transferring onto a polyvinylidene difluoride membrane electronically, the membrane was blocked with 3% bovine serum albumin (for MR) or 3% milk (for GAPDH) and incubated with a primary antibody against MR (clone H10E4C9F, diluted 1:250, Abcam) or GAPDH (clone 6C5, diluted 1:2000, Santa Cruz Biotechnology) at 4°C overnight, followed by 1-hour incubation with an

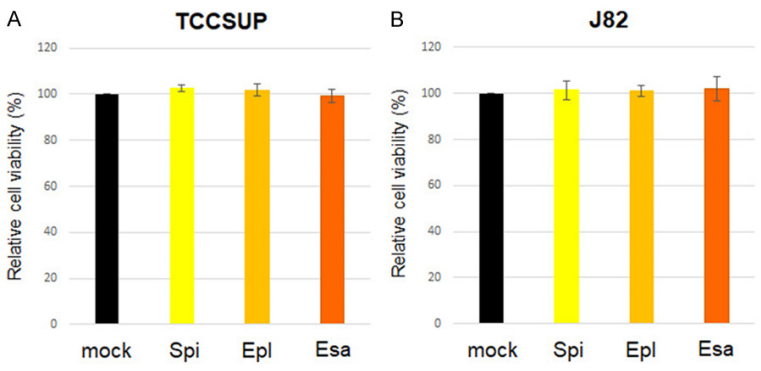

Figure 3. Effects of MR antagonists on the proliferation of bladder cancer cells cultured in a hormone-depleted condition. MTT assay in TCCSUP (A) and J82 (B) cells cultured for 96 hours in medium containing 5% CS-FBS as well as ethanol (mock), spironolactone (Spi; 10 μM), eplerenone (Epl; 10 μM), or esaxerenone (Esa; 100 nM). Cell viability presented relative to that of mock-treated cells represents the mean (± SD) from three independent experiments.

HRP-conjugated secondary antibody (Cell Signaling Technology) at room temperature. Chemiluminescent signals were then detected using the ChemiDOC™ MP (Bio-Rad) imaging system.

We also used Simple Western™ system [15, 16]. Proteins (4 μg) were separated by size (12-230 kDa) and the signals were visualized on a Wes system with the Compass software (version 6.0.0) (ProteinSimple). The primary antibody against MR (clone H10E4C9F, diluted 1:50), β-catenin (clone E-5, diluted 1:50, Santa Cruz Biotechnology), c-fos (sc-52, diluted 1:50, Santa Cruz Biotechnology), E-cadherin (clone G-10, diluted 1:50, Santa Cruz Biotechnology), N-cadherin (ab12221, diluted 1:250, Abcam), p53 (clone B-P3, diluted 1:50, Santa Cruz Biotechnology), or GAPDH (clone G-9, diluted 1:250, Santa Cruz Biotechnology) was used.

Immunohistochemistry

A set of bladder tissue microarray (TMA) consisting of transurethral resection specimens of muscle-invasive urothelial carcinoma, along with adjacent normal-appearing urothelial tissues, were constructed, upon approval by the institutional review boards, as previously described [17]. None of the patients had received therapy with radiation or anti-cancer drugs prior to the collection of the tissues included in the TMA. Immunohistochemical staining was performed on the sections (5 μm thick), using a primary antibody against MR (clone H10-E4C9F, dilution 1:100), as we described previously [13, 14, 17]. Positive controls for the stain (*e.g.* kidney, colon) and negative tissue elements were both evaluated and were adequate for the interpretation in the bladder TMA. All stains were manually quantified by a single pathologist (H.M.) who was blinded to sample identify. The immunoreactive scores (range: 0-12) calculated by multiplying the percentage of immunoreactive cells (0% = 0; 1-10% = 1; 11-50% = 2; 51-80% = 3; 81-100% = 4) by staining intensity (negative = 0; weak = 1; moderate = 2; strong = 3) were considered negative (0; score 0-1), weakly positive (1+; score 2-4), moderately positive (2+; score 6-8), and strongly positive (3+; score 9-12).

Statistical analysis

The Fisher's exact test or chi-square test was used to evaluate the associations between categorized variables, while the Student's *t*-test was used to compare continuous data. Survival rates in patients were calculated by the Kaplan-Meier method and comparison was made by log-rank test, using GraphPad Prism version 10.0.2. The Cox proportional hazards model was used to determine statistical significance of predictors in a multivariable setting. All statistical analyses (except log-rank test) were performed, using EZR software (Jichi Medical University Saitama Medical Center). *P* values of <0.05 were considered statistically significant.

Results

Expression of MR in bladder cancer lines

We first examined the status of MR expression in bladder urothelial cancer cell lines, using western blotting, along with adequate positive (*i.e.* 293T) and negative (*i.e.* U937) control cell lines [18]. All the four bladder cancer lines examined, including AR-positive UMUC3 and TCCSUP, and AR-negative J82 and 5637 [19],

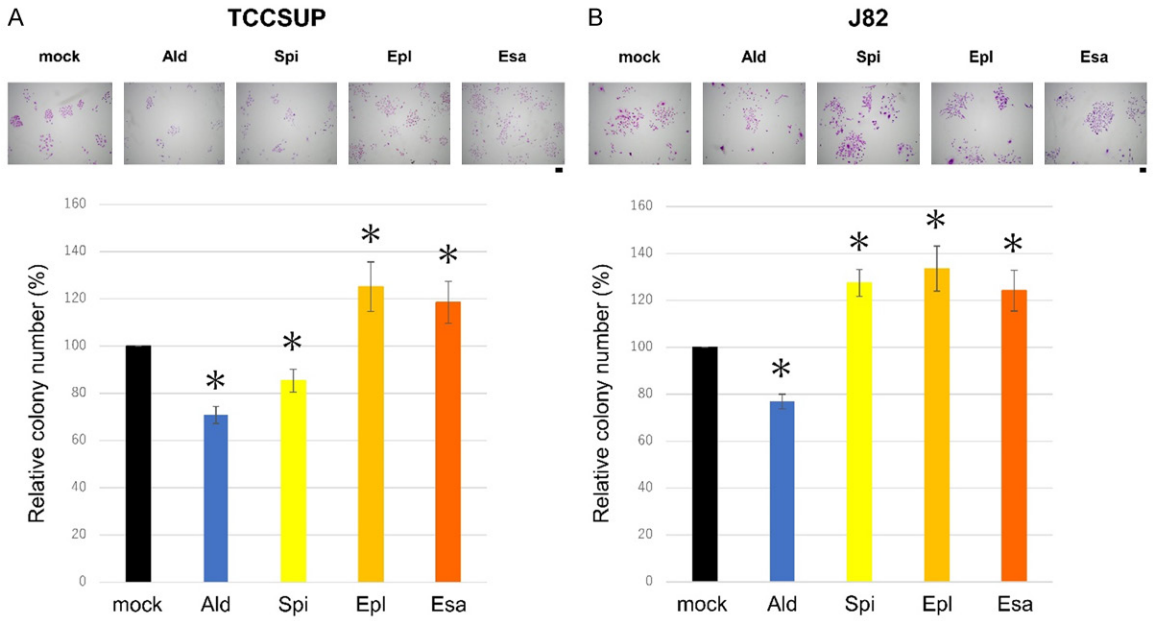

Figure 4. Effects of MR ligands on the colony formation of bladder cancer cells. Clonogenic assay in TCCSUP (A) and J82 (B) cells cultured for 10 days in medium containing 10% FBS as well as ethanol (mock), aldosterone (Ald; 100 nM), spironolactone (Spi; 10 μM), eplerenone (Epl; 10 μM), or esaxerenone (Esa; 100 nM). The number of colonies (≥20 cells) presented relative to that of mock-treated cells represents the mean (± SD) from three independent experiments. The scale bars under the images indicate 200 μm. *P<0.05 (vs. mock treatment).

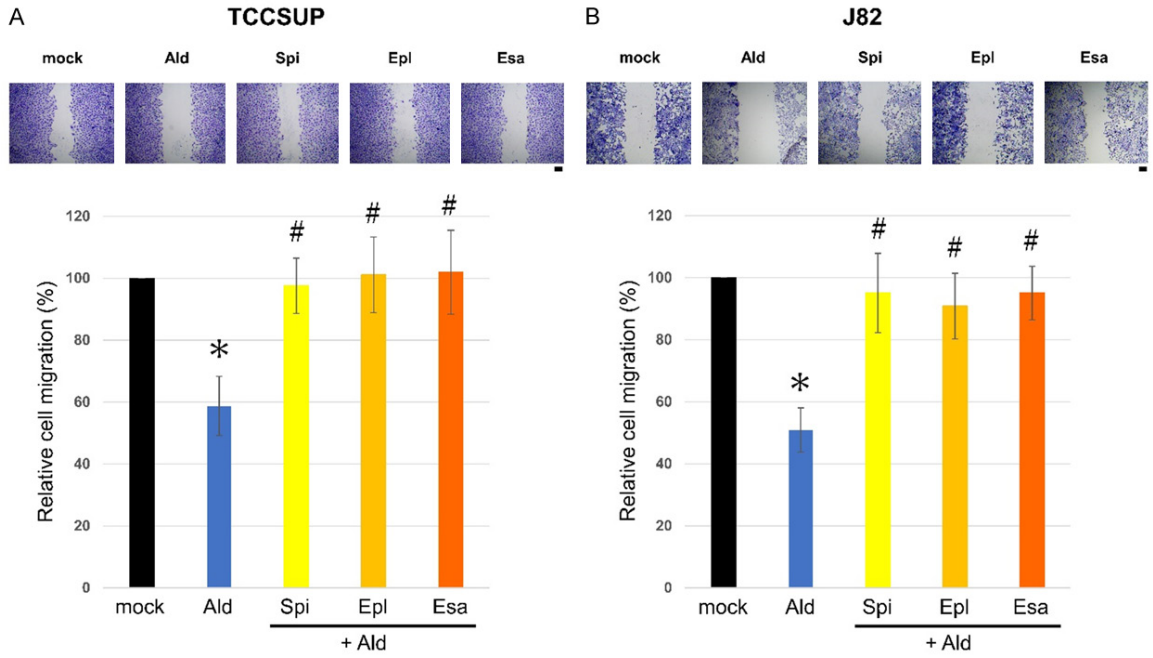

Figure 5. Effects of MR ligands on the migration of bladder cancer cells. Scratch wound-healing assay in TCCSUP (A) and J82 (B) cells. The cells grown to confluence were gently scratched, and the wound area was measured after 24-hour culture in serum-free medium containing ethanol (mock), aldosterone (Ald; 100 nM) alone, or aldosterone (100 nM) plus spironolactone (Spi; 10 μM), eplerenone (Epl; 10 μM), or esaxerenone (Esa; 100 nM). Width of the wound area presented relative to that of mock-treated cells represents the mean (± SD) from three independent experiments. The scale bars under the images indicate 200 μm. *P<0.05 (vs. mock treatment). #P<0.05 (vs. aldosterone alone).

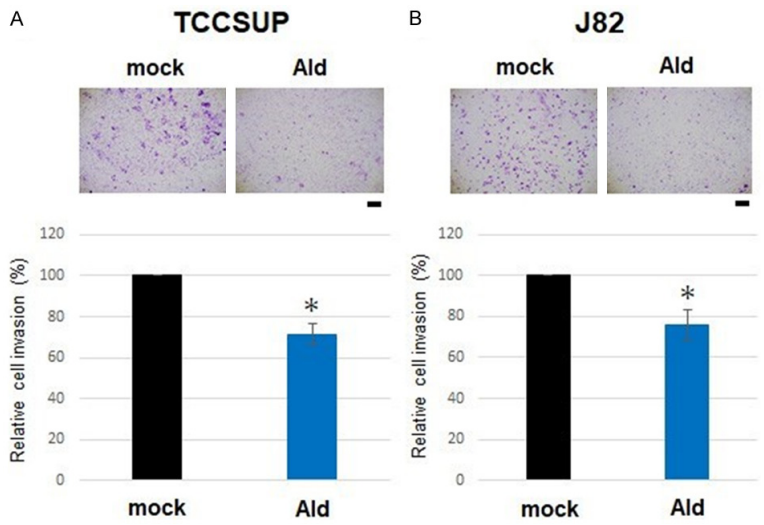

Figure 6. Effects of aldosterone on the invasion of bladder cancer cells. Transwell invasion assay in TCCSUP (A) and J82 (B) cells. The cells were cultured in serum-free medium containing ethanol (mock) or aldosterone (Ald; 100 nM) for 24 hours. Cell invasion presented relative to that of mock-treated cells represents the mean (± SD) from three independent experiments. The scale bars under the images indicate 200 μm. *P<0.05 (vs. mock treatment).

as well as a non-neoplastic urothelial line SVHUC, were found to express the MR (**Figure 1**).

Impact of MR activation/inactivation on bladder cancer cell growth

We compared the cell viability (via MTT assay), colony formation (via clonogenic assay), and cell migration (via scratch wound-healing assay) of two bladder cancer lines cultured with a MR agonist, aldosterone, and/or one of MR antagonists, spironolactone, eplerenone, and esaxerenone. The MTT assay in steroid hormone-depleted (*i.e.* CS-FBS) medium showed that aldosterone treatment significantly reduced the cell viability in a dose-dependent manner, which was at least partially restored by each anti-mineralocorticoid (**Figure 2**). However, in this hormone-depleted condition, MR antagonists alone (without aldosterone treatment) did not significantly change the cell viability (**Figure 3**). Similarly, the clonogenic assay in normal FBS medium showed that colony formation was significantly inhibited by aldosterone and induced by eplerenone or esaxerenone (**Figure 4**). Spironolactone significantly reduced and induced the colony formation in AR-positive TCCSUP and AR-negative J82, respectively, implying its inhibitory effect in TCCSUP cells predominantly as an AR antagonist. Additionally, in the scratch wound-healing assay in serum-free medium, aldosterone significantly inhibited the cell migration, whereas anti-mineralocorticoids blocked the effect of aldosterone (**Figure 5**). The transwell assay was also performed to test the impact of the MR agonist on cell invasiveness. Aldosterone treatment in the upper chamber resulted in significant reduction in the invasion of TCCSUP/J82 cells (**Figure 6**).

To further assess the functions of MR in bladder cancer cells, we compared the cell growth in TCCSUP and J82 sublines stably expressing control-shRNA vs. MR-shRNA. We first confirmed considerable reduction in MR protein expression in knockdown sublines (**Figure 7A**). Then, as expected, MR knockdown resulted in significant increases in cell viability (**Figure 7B**), colony formation (**Figure 7C**), and cell migration (**Figure 7D**), compared with the respective controls. Meanwhile, aldosterone treatment did not significantly inhibit the growth of TCCSUP/J82 sublines stably expressing MR-shRNA (**Figure 8**). These findings suggest the inhibitory actions of MR in urothelial cancer progression.

Expression of MR vs. tumor suppressor/oncogenic proteins in bladder cancer cells

We next assessed the impact of MR signaling on the expression of several molecules known to involve urothelial cancer progression, including β-catenin [20, 21], c-fos [22, 23], E-cadherin [24, 25], N-cadherin [25, 26], and p53 [27, 28]. Western blotting in control vs. MR-knockdown sublines consistently demonstrated that the expression levels of MR was inversely or positively correlated with those of β-catenin/c-fos/N-cadherin or E-cadherin/p53, respectively (**Figure 9**).

Expression of MR in bladder cancer tissues and its impact on patient outcomes

Finally, we immunohistochemically determined the expression of MR in a set of TMA containing

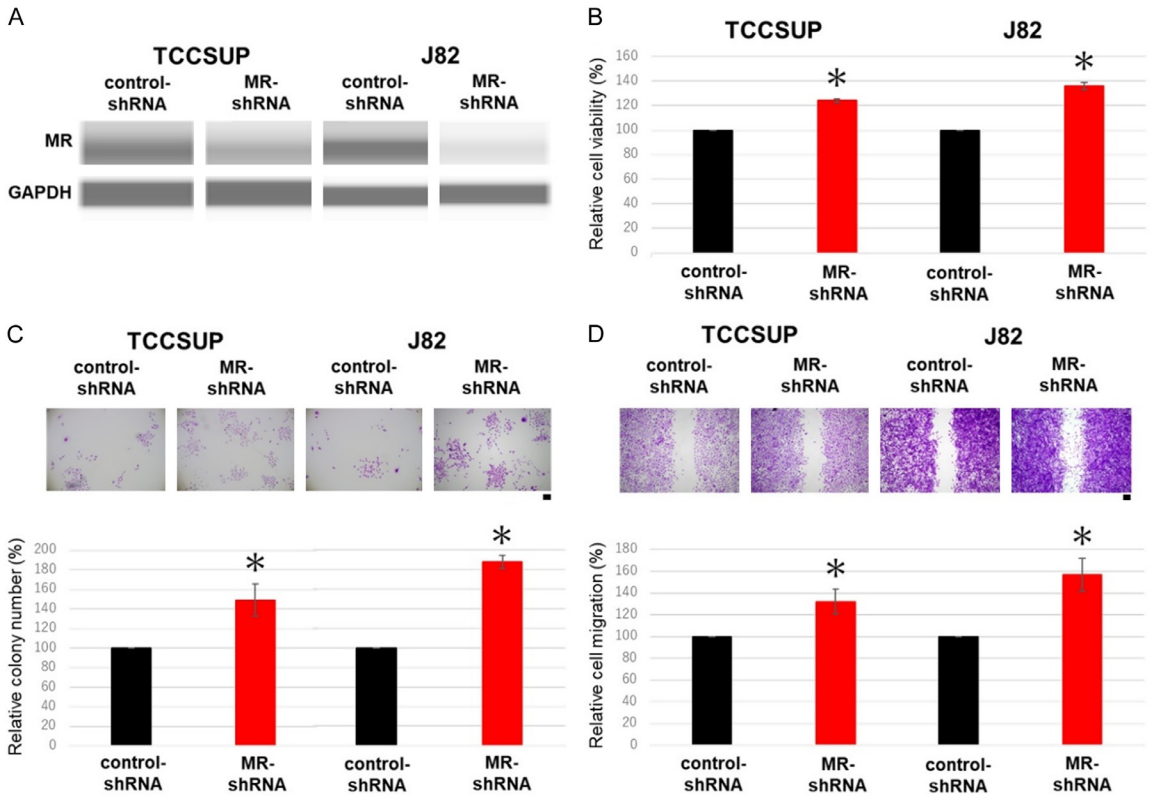

Figure 7. Effects of MR knockdown on the growth of bladder cancer cells. A. Western blotting (Simple Western™ system) of MR in TCCSUP-control-shRNA vs. TCCSUP-MR-shRNA and J82-control-shRNA vs. J82-MR-shRNA. GAPDH served as a loading control. B. MTT assay in TCCSUP-control-shRNA vs. TCCSUP-MR-shRNA and J82-control-shRNA vs. J82-MR-shRNA cultured in medium containing 10% FBS for 96 hours. C. Clonogenic assay in TCCSUP-control-shRNA vs. TCCSUP-MR-shRNA and J82-control-shRNA vs. J82-MR-shRNA cultured in medium containing 10% FBS for 10 days. D. Scratch wound-healing assay in TCCSUP-control-shRNA vs. TCCSUP-MR-shRNA and J82-control-shRNA vs. J82-MR-shRNA cultured in serum-free medium for 24 hours after scratching. Cell viability, colony number (≥20 cells), or width of the wound area presented relative to that of control-shRNA subline represents the mean (± SD) from three independent experiments. The scale bars under the images indicate 200 μm. *P<0.05 (vs. control-shRNA subline).

63 cases of muscle-invasive bladder cancer and corresponding non-neoplastic bladder tissues (**Figure 10A**). Overall, MR was positive in 92.1% (36.5% 1+, 44.4% 2+, and 11.1% 3+) of tumors, which was significantly weaker than in adjacent benign urothelial tissues [100% (15.7% 1+, 37.3% 2+ and 47.1% 3+)] (**Table 1**). However, there were no significant differences in the status of MR expression between sexes, pathologic T stages, or the absence and presence of lymph node metastasis.

We further investigated possible associations between MR expression and patient outcomes. Kaplan-Meier analysis, along with the log-rank test, revealed that the risk of progression after radical cystectomy was marginally (P = 0.067) lower in patients with MR-high (3+) tumor than in those with MR-low (0/1+/2+) tumor (**Figure 10B**). More strikingly, patients with MR-high tumor had a significantly (P = 0.039) lower risk of cancer-specific mortality (**Figure 10C**).

To determine if MR expression status was an independent prognosticator, we performed multivariable analysis, using the Cox model (**Table 2**). In our cohort of patients with muscle-invasive disease, MR expression (hazard ratio 0.117, P = 0.039), as well as pT stage (hazard ratio 2.910, P = 0.032), showed significance for cancer-specific survival.

Discussion

Limited evidence from preclinical studies has suggested that MR signaling contributes to pre-

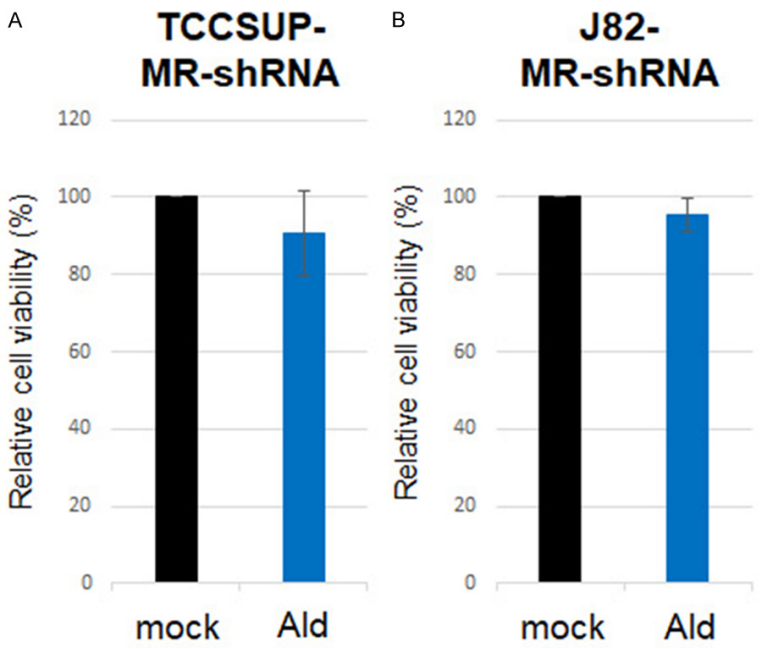

Figure 8. Effects of aldosterone on the proliferation of MR-knockdown bladder cancer cells. MTT assay in TCCSUP-MR-shRNA (A) and J82-MR-shRNA (B) cells cultured for 96 hours in medium containing 5% CS-FBS as well as ethanol (mock) or aldosterone (Ald; 100 nM). Cell viability presented relative to that of mock-treated cells represents the mean (± SD) from three independent experiments.

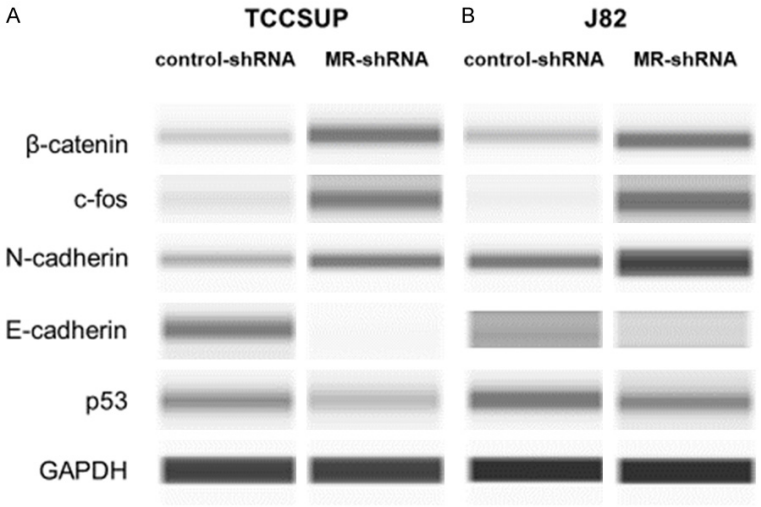

Figure 9. Effects of MR knockdown on the expression of tumor suppressor/oncogenic molecules in bladder cancer cells. Western blotting (Simple Western™ system) of β-catenin, c-fos, N-cadherin, E-cadherin, and p53 in TCCSUP-control-shRNA vs. TCCSUP-MR-shRNA (A) and J82-control-shRNA vs. J82-MR-shRNA (B). Representative images for GAPDH, which serves as a loading control, are shown.

venting the outgrowth of malignancies, such as breast cancer [29], colorectal cancer [30], liver cancer [31], and pancreatic cancer [32]. Meanwhile, a meta-analysis of observational clinical studies has failed to demonstrate a significant impact of spironolactone treatment on the incidence of various malignancies [33], except for prostate cancer whose risk has been shown to rather be significantly lower [33-35], presumably due to its action as an anti-androgen. Instead, the down-regulation of *MR* gene expression in several types of malignancies, including bladder cancer, has been documented [31, 36-38], suggesting the role of MR as a tumor suppressor. Indeed, a case-control study demonstrated that spironolactone use was associated with a significantly lower risk of developing bladder cancer in females (odds ratio 0.81, $P<0.001$) [39], but not in males (odds ratio 1.03, $P>0.05$) where anti-androgenic vs. anti-mineralocorticoid activities of spironolactone might have conflicting effects on tumor development. Then, we have recently demonstrated *in vitro* evidence to indicate that MR activation results in the prevention of urothelial tumorigenesis [13]. In the present study, we further investigated the impact of MR signaling on the growth of bladder cancer cells.

We assessed the effects of MR ligands on the cell viability, colony formation, and cell migration of bladder cancer lines and first found that an agonist (*i.e.* aldosterone) and three antagonists (*i.e.* spironolactone, eplerenone, esaxerenone) inhibited and induced, respectively, the cell growth. Specifically, these clinically used anti-mineralocorticoids at the doses close to their pharmacological concentrations showed similar efficacy.

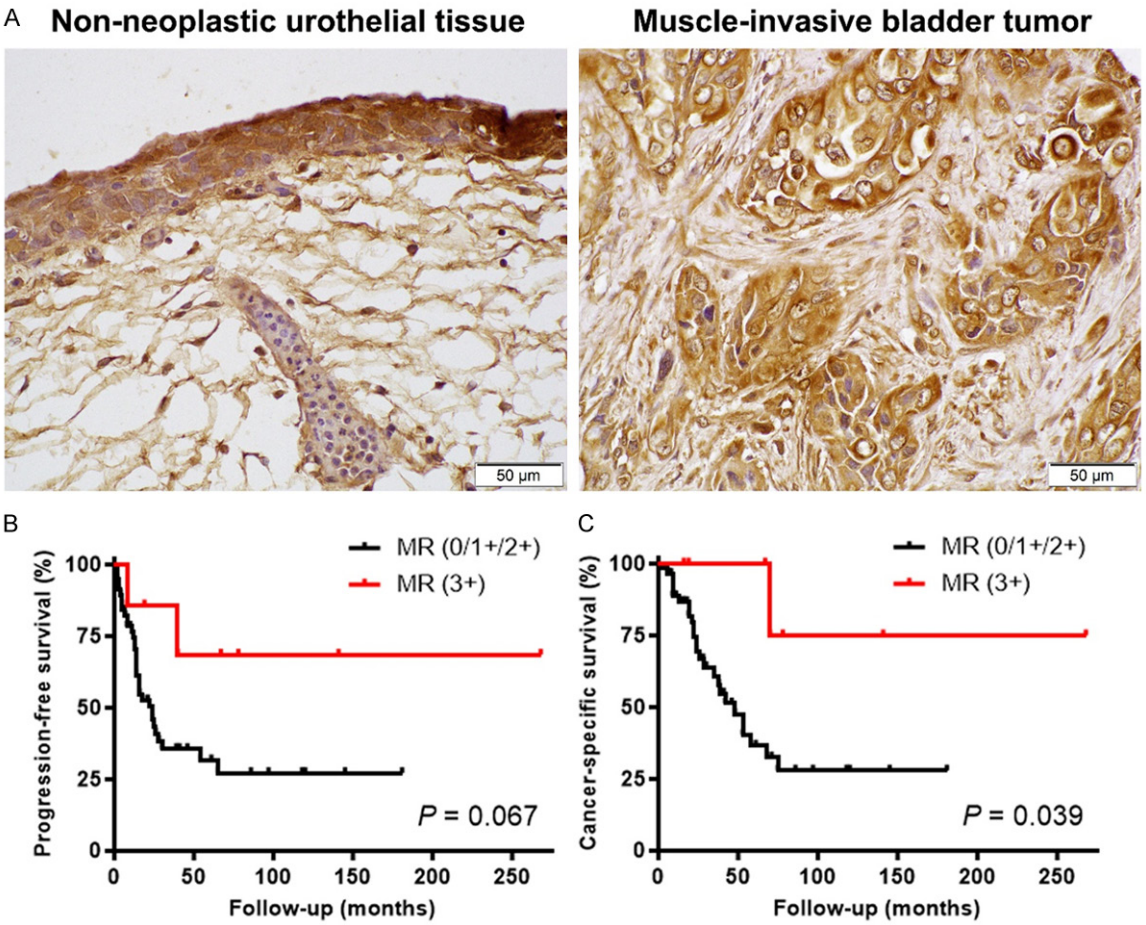

Figure 10. Immunohistochemistry of MR in surgical specimens. (A) Representative images of MR expression in non-neoplastic urothelial tissue and muscle-invasive bladder cancer. Original magnification: 400 ×. Kaplan-Meier curves for progression-free survival (B) or cancer-specific survival (C) in patients with MR-low (n = 56) vs. MR-high (n = 7) muscle-invasive bladder tumor.

Meanwhile, there was no significant impact of anti-mineralocorticoids (in a hormone-depleted condition) and aldosterone (in MR knockdown sublines), which might exclude the off-target effect of the MR ligands on the cell growth. These data thus suggested that MR activity was oppositely associated with the progression of urothelial cancer. This was further confirmed by the promotion of the cell growth by MR knockdown. We then examined the potential downstream targets of MR that had been known to involve urothelial cancer outgrowth [20-28]. We found that the expression levels of MR in bladder cancer cells were positively and inversely associated with those of tumor suppressive E-cadherin and p53, and oncogenic molecules, including β-catenin, c-fos, and N-cadherin, respectively. Of note, epithelial-mesenchymal transition has been implicated in various aspects of tumor progression, including tumor invasion, metastasis, and therapeutic resistance, and cadherin switching, from the expression of E-cadherin to that of N-cadherin, is its hallmark [40]. We herein demonstrated the cadherin switching in bladder cancer cells, which was induced by MR knockdown. Nonetheless, further investigation is required to not only validate our results but also elucidate the molecular mechanisms responsible for MR-mediated suppression of urothelial cancer progression. MR signaling may also need to be investigated in connection with other signals that are known to functionally interact with MR, such as other steroid hormone receptors (*e.g.* glucocorticoid receptor, AR [9, 11, 12, 41]) each of which has been shown to, by itself, modulate the progression of bladder cancer [4, 8, 19, 42].

Table 1. Correlations of MR expression with clinicopathologic profile of the patients with muscle-invasive bladder cancer

Parameter	*n*	MR expression				*P* value	
		Negative	Positive			0/1+ vs. 2+/3+	0/1+/2+ vs. 3+
		0	1+	2+	3+		
Tissue						0.001	<0.001
Non-neoplastic epithelium	51	0 (0%)	8 (15.7%)	19 (37.3%)	24 (47.1%)		
Urothelial neoplasm	63	5 (7.9%)	23 (36.5%)	28 (44.4%)	7 (11.1%)		
Sex						0.537	1.000
Male	50	4 (8.0%)	17 (34.0%)	23 (46.0%)	6 (12.0%)		
Female	13	1 (7.7%)	6 (46.2%)	5 (38.5%)	1 (7.7%)		
Pathologic T stage						0.334	0.405
pT2	22	2 (9.1%)	5 (22.7%)	11 (50.0%)	4 (18.2%)		
pT3	31	2 (6.5%)	14 (45.2%)	13 (41.9%)	2 (6.5%)		
pT4	10	1 (10.0%)	4 (40.0%)	4 (40.0%)	1 (10.0%)		
Lymph node involvement						0.964	0.695
pN0	38	3 (7.9%)	14 (36.8%)	17 (44.7%)	4 (10.5%)		
pN+	21	1 (4.8%)	8 (38.1%)	9 (42.9%)	3 (14.3%)		
pNX	4	1 (25.0%)	1 (25.0%)	2 (50.0%)	0 (0%)		

Table 2. Univariate and multivariable analyses for cancer-specific survival in patients with muscle-invasive bladder cancer

Parameter	Univariate			Multivariable		
	HR	95% CI	*P* value	HR	95% CI	*P* value
Sex[a]	1.187	0.457-3.082	0.707	0.812	0.316-2.084	0.665
pT stage[b]	2.086	0.655-6.647	0.098	2.910	1.094-7.741	0.032
pN stage[c]	1.704	0.718-4.040	0.165	2.159	0.936-4.981	0.071
MR[d]	0.166	0.063-0.436	0.039	0.117	0.015-0.896	0.039

HR = hazard ratio; CI = confidence interval. [a]Male vs. Female; [b]pT2-3 vs. pT4; [c]pN0/pNX vs. pN+; [d]0/1/2+ vs. 3+.

Our *in vitro* data suggesting the inhibitory role of MR signaling in urothelial cancer growth were supported by immunohistochemical studies in surgical specimens. Consistent with our previous observations in non-invasive bladder tumors [13], we here found that MR expression was significantly down-regulated in muscle-invasive bladder cancers, compared with normal-appearing urothelial tissues. There were no significant associations of the levels of MR expression in bladder cancer with pathologic T stage or lymph node metastasis. Meanwhile, the prognostic significance of MR expression in colorectal [43] and liver [31] cancers has been reported. In line with these studies showing an association between MR overexpression and favorable oncologic outcome of colorectal/liver cancer [31, 43], strong MR expression was found to be an independent factor to predict a significantly lower risk of cancer-specific mortality in patients with muscle-invasive bladder cancer following radical cystectomy.

As aforementioned, spironolactone is a unique anti-mineralocorticoid which also binds to the AR [11]. Thus, the preventive effect of spironolactone on the risk of developing prostate cancer [33-35] where AR is critical for tumor outgrowth [44] may likely be derived from its anti-androgenic activity. Similarly, in the present study in bladder cancer whose progression could be hampered by AR antagonists [19, 45], spironolactone rather strongly inhibited the colony formation of AR-positive cells cultured in medium containing a considerable amount of androgens (*i.e.* normal FBS), whereas its effect on the cell growth was similar to that of other anti-mineralocorticoids in AR-negative cells or even AR-positive cells cultured in androgen-depleted conditions (*i.e.* serum-free, CS-FBS). These findings suggest that AR may play a more dominant role in the growth of urothelial cancer cells and that the stimulatory or inhibitory effects of spironolactone are dependent on the functional activity of MR and AR.

In conclusion, the present results suggest that MR acts as a suppressor for urothelial cancer progression. On the other hand, treatment with anti-mineralocorticoids, especially eplerenone and esaxerenone, for example, hypertension and heart failure, may be harmful to those concurrently with urothelial cancer. Validation and further assessment of MR functions, including their mechanistic details, and those in animal models for bladder cancer are required to determine the biological significance of MR signaling in urothelial cancer progression and address the feasibility of future therapeutic intervention, respectively.

Disclosure of conflict of interest

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