The carbonic anhydrase inhibitor acetazolamide inhibits urinary bladder cancers via suppression of β-catenin signaling

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Abstract
Carbonic anhydrases (CAs) play an important role in maintaining pH homeostasis. We previously demonstrated that overexpression of CA2 was associated with invasion and progression of urothelial carcinoma (UC) in humans. The purpose of the present study was to evaluate the effects of the CA inhibitor acetazolamide (Ace) on N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN)-induced bladder carcinogenesis in mice and explore the function of CA2 in muscle invasion by UC. Male mice were treated with 0.025% (experiment 1) or 0.05% BBN (experiment 2) in their drinking water for 10 weeks, then treated with cisplatin (Cis), Ace, or Cis plus Ace for 12 weeks. In experiment 1, the overall incidence of BBN-induced UCs was significantly decreased in the BBN→Ace and BBN→Cis+Ace groups. In experiment 2, the overall incidence of BBN-induced UCs was significantly decreased in the BBN→Cis+Ace group, and the incidence of muscle invasive UC was significantly decreased in both the BBN→Ace and the BBN→Cis+Ace groups. We also show that overexpression of CA2 by human UC cells T24 and UMUC3 significantly increased their migration and invasion capabilities, and that Ace significantly inhibited migration and invasion by CA2-overexpressing T24 and UMUC3 cells. These data demonstrate a functional association of CA2 with UC development and progression, confirming the association of CA2 with UC that we had shown previously by immunohistochemical analysis of human UC specimens and proteome analysis of BBN-induced UC in rats. Our finding that inhibition of CA2 inhibits UC development and muscle invasion also directly confirms that CA2 is a potential therapeutic target for bladder cancers.

Keywords
acetazolamide, bladder cancer, carbonic anhydrase 2 inhibitor, PIP5K1B, Wnt/β-catenin signaling pathway

Abbreviations: Ace, acetazolamide; BBN, N-butyl-N-(4-hydroxybutyl)nitrosamine; BC, bladder cancer; Cis, cisplatin; EMT, epithelial–mesenchymal transition; MIBC, muscle invasive bladder cancers; NMIBC, nonmuscle invasive bladder cancers; PIP5K1B, phosphatidylinositol 4-phosphate 5-kinase 1B; UC, urothelial carcinoma.

Taisuke Matsue and Min Gi contributed equally to this work.

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1 | INTRODUCTION

Bladder cancer is the tenth most common malignancy worldwide. Approximately 75% of BCs are diagnosed as NMIBCs and 25% as MIBC. NMIBCs are less aggressive than MIBCs and are confined to the bladder mucosa and submucosa. Standard treatments for NMIBCs are transurethral resection and intravesical Bacillus Calmette–Guerin (BCG) immunotherapy. However, the recurrence rate of NMIBC after treatment is >50%, and 20% of recurrent tumors will progress to MIBC. The standard treatment for MIBC is total radical cystectomy and perioperative chemotherapy, but half of the patients develop distant metastasis within 2 years. In recent years, immunotherapy after prior chemotherapy has improved the prognosis of patients with metastatic BC, but the overall therapeutic effect still needs to be improved. Identification of invasion-related factors as potential therapeutic targets is a promising approach in the development of novel therapeutic strategies to manage BC progression.

Carbonic anhydrase (CA) is an enzyme that catalyzes the reversible hydration of carbon dioxide and plays an essential role in maintaining intracellular and extracellular pH homeostasis. However, overexpression of CA leads to excess formation of bicarbonate and consequent acidification of the microenvironment. CA has 15 isoforms, some of which have been reported to be overexpressed in various cancers. For example, overexpression of CA9 is correlated with early stage BC and with poor prognosis in lung cancer, breast cancer, glioma, and oral squamous cell carcinoma, and overexpression of CA12 has been reported in estrogen receptor-positive breast cancer and glioma. These findings have made CAs a potential target for cancer therapy. CA9-specific small molecule inhibitors decreased mammary tumor metastasis in mice, and the CA9 and CA12 inhibitor 4-[(3′-3′,5′-dimethylphenyl)ureido]phenyl sulfamate used alone or in combination with cisplatin (Cis) promoted the apoptosis of the lung small cell carcinoma cell lines DMS 79 and COR-L24 cells in vitro and reduced tumor growth in mouse xenograft models using DMS 79 and COR-L24 cells in vivo.

Currently, there are no reports on the effect of CA inhibitors on BC. Recently, we compared invasive UC with noninvasive UC in male human c-Ha-ras proto-oncogene transgenic (Hras12S) rats and identified CA2 as a novel invasion-related factor by proteome analysis. Importantly, we also found that CA2 was associated with the progression and prognosis of NMIBC in humans, and CA2 was overexpressed in human MIBC. These findings indicate that CA2 is a potential therapeutic target for the treatment of invasive UC.

The purpose of the present study was to evaluate the effects of the CA inhibitor Ace used alone and in combination with Cis on BBN-induced bladder carcinogenesis in mice, and to investigate the role of CA2 in muscle invasion of UC.

2 | MATERIALS AND METHODS

2.1 | Chemicals

BBN and Cis were obtained from FUJIFILM Wako Pure Chemical Corporation. Ace was obtained from Selleck Biotech Co., Ltd. Cis was dissolved in 0.9% saline.

2.2 | Animals

Six-week-old male C57BL-6J mice were obtained from Charles River Japan, Inc. The Laboratory Animal Center of Osaka City University Graduate School of Medicine is accredited by the Center for the Accreditation of Laboratory Animal Care and Use (CALAC), Japan Health Sciences Foundation (JHSF). Mice were housed in polycarbonate cages (five per cage) in experimental animal rooms with a targeted temperature of 22±3°C, relative humidity of 55±5%, and a 12 h light/dark cycle. All animals were acclimated for 1 week before being used for experiments.

2.3 | Experimental protocols

All animal studies were approved by the Institutional Animal Care and Use Committee of Osaka City University Graduate School of Medicine and conducted in accordance with the Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan, 2006). CE2 basal pellet diet (CLEA Japan, Inc.) and tap water were available ad libitum throughout the study. Ace was dissolved in vehicle (0.5% methylcellulose solution) at a target dose of 4 mg/kg b.w. with an administration volume of 5 ml/kg b.w. Cis was diluted in vehicle (0.9% saline) at a target dose of 1 mg/kg b.w. with an administration volume of 0.2 ml/mouse. The concentrations of Ace and Cis were adjusted weekly based on the body weights of the treated mice.

2.4 | Experiment 1: 0.025% BBN-induced urinary bladder carcinogenesis in mice

In total, 120 male C57BL-6 J mice were randomized into six groups (Figure S1A): BBN alone, BBN→Cis, BBN→Ace, BBN→Cis+Ace, Ace alone, and control groups. Mice in the BBN groups (25 mice each) were given 0.025% BBN in their drinking water for 10 weeks. Mice in the Ace alone and control (no treatment) groups (10 mice each) were given tap water for the same period. Three days after the cessation of BBN treatment each group was treated as follows: mice in the BBN→Cis group were administered Cis i.p. once a week at a dose of 1 mg/kg b.w.; mice in the BBN→Ace group and Ace alone group were administered Ace by oral gavage five times a week at a dose of 4 mg/kg b.w.; mice in...
the BBN→Cis+Ace group were administered Cis i.p. once a week and Ace by oral gavage five times a week at the same doses; mice in the BBN alone and control groups were given vehicle. At the end of week 22, mice were euthanized by inhalation of an overdose of isoflurane (Abbott Japan Co., Ltd.) using a Small Animal Anesthetizer (Muromachi Kikai Co., Ltd., MK-A110D) coupled with an Anesthetic Gas Scavenging System (Muromachi Kikai Co., Ltd., MK-T100E).

2.5 | Experiment 2: 0.05% BBN-induced urinary bladder carcinogenesis in mice

In total, 120 male C57BL-6J mice were randomized into four groups (Figure S1B): BBN alone, BBN→Cis, BBN→Ace, and BBN→Cis+Ace groups (30 mice each).

All mice were given drinking water containing 0.05% BBN for 10 weeks. After the end of BBN administration, each group was treated as described above in experiment 1. At the end of week 22, mice were euthanized using the same procedure as in experiment 1.

2.6 | Pathological examination

At necropsy, urinary bladders were immediately inflated by injection of 4% phosphate-buffered paraformaldehyde (PFA) solution and then fixed in the same PFA solution at 4°C. The location, number, and size of all suspected neoplastic lesions were recorded. Urinary bladders were then cut into six strips and processed for embedding in paraffin. In experiment 2, urinary bladders were weighed before being cut into strips, and relative organ weight was calculated using the final body weight. Paraffin-embedded tissue sections of urinary bladders were prepared for H&E staining. Based on invasion depth, mouse urinary UCs were classified as follows: pTa: noninvasive papillary carcinoma; pT1: UC has grown into the layer of connective tissue under the bladder epithelium but has not reached the muscle layer; ≥pT2: UC has grown into or through the muscle layer (https://www.cancerresearchuk.org/about-cancer/bladder-cancer/types-stages-grades/stages Accessed January 6, 2022).

2.7 | Immunohistochemical analysis

Serial sections (3-μm thickness) cut from paraffin-embedded mouse bladder specimens were examined for expression of CA2, Ki-67, and β-catenin by immunohistochemical staining using the avidin–biotin–peroxidase complex (ABC) method. Details are provided in Data S1. All specimens with UCs (42 specimens) in experiment 2 and all specimens in the Ace alone group and control group (10 specimens each) in experiment 1 were used for CA2 and β-catenin immunohistochemical staining. All specimens with UCs and five specimens without UCs from each group in experiment 2 were used for Ki-67 immunohistochemical staining. Overexpression of CA2 in UC was determined as positive when cytoplasmic staining was evident in >5% of the cells. Overexpression of β-catenin in UC was determined as positive when nuclear staining was evident in >5% of the cells. For Ki-67 index, at least 500 urothelial cells in morphologically normal appearing urothelium of each mouse were counted.

2.8 | Cell lines and cell culture

The human UC cell line T24 was obtained from the Institute of Physical and Chemical Research. UMUC3 cells were obtained from the American Type Culture Collection. Cells were cultured in DMEM (FUJIFILM Wako Pure Chemical Corporation) containing 10% FBS (GE Healthcare Japan) at 37°C and 5% CO2 atmosphere. As expression of CA2 in all the cell lines was relatively low and not suitable for function analysis of CA2 knockdown, we determined the function of overexpression of CA2 by CA2-plasmid vector transfection of T24 and UMUC3 cells.

2.9 | Western blot analysis

The following antibodies were used for western blot analysis: anti-CA2 (Abcam, #ab124687, 1:1000), anti-β-catenin (6B3) (Cell Signaling Technology, #9582, 1:1000), anti-N-cadherin (Cell Signaling Technology, #4061, 1:1000), anti-vimentin (Cell Signaling Technology, #5741, 1:1000), HRP-conjugated secondary goat anti-rabbit antibody (Santa Cruz Biotechnology, #sc-2004, 1:10,000), and HRP-conjugated anti-β-actin (Santa Cruz Biotechnology, #sc-47,788, 1:100,000). Details are provided in Data S1.

2.10 | Plasmid transfection

pCDNA3.1+/C-(K)-DYK-CA2 and pCDNA3.1+/C-(K)-DYK (empty vector) were obtained from GenScript. T24 (5 × 10^5 cells/well) and UMUC3 (4 × 10^5 cells/well) were seeded into a six-well plate in 2 ml of DMEM containing 10% FBS. Transfection was conducted using plasmids at 2 μg/well. Lipofectamine 3000 (Invitrogen), and Opti-MEM medium (Thermo Fisher Scientific K.K.) according to the manufacturer’s instructions. In the experiment evaluating the effects of Ace on the migration and invasion capability of UMUC3 and T24 cells, 400μM Ace or vehicle (0.2% DMSO) was added to the medium 12h after transfection. This dose of Ace was the maximum nontoxic dose in UMUC3 and T24 cells. Transfected cells were used for various assays 24h after transfection.

2.11 | Cell proliferation assay

Transfected T24 (1.0 × 10^4 cells/well) and UMUC3 (5.0 × 10^3 cells/well) were seeded in a 96-well plate. After 72h, cell proliferation was measured using a Cell Counting Kit-8 (Dojindo Molecular Technologies) according to the manufacturer’s instructions.
Absorbance was measured with a microplate reader (Thermo Fisher Scientific K.K., MultiSkan FC) with a 450nm wavelength.

2.12 Cell migration and invasion assay

The cell migration assay was performed using a Falcon® Cell Culture Insert with an 8.0-µm pore size polyethylene terephthalate (PET) filter (Corning, Inc.). The cell invasion assay was performed using a Corning® BioCoat™ Matrigel® Invasion Chamber with an 8.0-µm pore size PET filter (Corning, Inc.). Details are provided in Data S1.

2.13 RNA extraction and real-time PCR

Primers and probes (TaqMan Gene Expression Assay) for CA2 (Hs00163869_m1), PIP5K1B (Hs00185914_m1), and RPLP2 (Hs01115130_g1) were purchased from Thermo Fisher Scientific. Details are provided in Data S1.

2.14 Immunofluorescence

Expression levels of CA2 and β-catenin were examined by immunofluorescence staining. CA2-transfected UMUC3 cells were seeded onto coverslips at 3.0 x 10^4 cells/well. In addition, empty vector-transfected control cells stimulated with 20 ng/ml of Wnt3a (Proteintech) served as positive controls for vector-transfected control cells stimulated with 20 ng/ml of Wnt3a. Details are provided in Data S1.

2.15 Microarray analysis

Microarray analysis using Affymetrix Clariom D Assay, Human (Affymetrix, Inc.) was conducted by Cell Innovator Inc. Details are provided in Data S1. To identify genes upregulated or downregulated by CA2 overexpression we calculated z-scores and ratios (nonlog-scaled fold change) from the normalized signal intensities of each probe for comparison of samples from CA2-transfected and empty vector-transfected cells (control). Then we established the criteria for regulated genes: (upregulated genes) z-score ≥ 2.0 and compared ratio (nonlog-scaled fold change) ≥ 1.5; (downregulated genes) z-score ≤ -2.0 and compared ratio ≤ 0.5. To investigate the functional significance of the upregulated or downregulated genes in the CA2-transfected cells, the list of differentially expressed genes was analyzed using Ingenuity Pathway Analysis (IPA) (Ingenuity Systems, Inc.).

2.16 Statistical analysis

Mean values in mouse experiments and gene expression analyses are reported as mean ± standard deviation (SD). In the in vitro assays, mean values are reported as mean ± SE. Statistical analysis was performed using GraphPad Prism version 5 software (GraphPad Software). Fisher’s exact test was used to evaluate the differences in UC incidence in the mouse experiments. The difference in means between groups was evaluated by Student’s t test when variance was homogeneous and Welch’s t test when variance was heterogeneous.

3 RESULTS

3.1 Experiment 1: 0.025% BBN-induced urinary bladder carcinogenesis in mice

Final mouse body weights and the incidences and T stages of UC in the urinary bladder are summarized in Table 1. There were no significant differences in final body weights among the groups. Total UC incidence was significantly decreased in the BBN→Ace group (8%) and the BBN→Cis+Ace group (4%) compared with the BBN alone group (32%). Total UC incidence in the BBN→Cis group (16%) tended to decrease compared with the BBN alone group, albeit without significant difference. Only three muscle invasive UCs (≥pT2) were observed in the 0.025% BBN-initiated groups that were not treated with Ace: one in the BBN alone group and two in the BBN→Cis group. Therefore, the inhibitory effects of Ace on the invasiveness of UC could not be evaluated. Accordingly, in experiment 2 the concentration of BBN was increased to 0.05%.

3.2 Experiment 2: 0.05% BBN-induced urinary bladder carcinogenesis in mice

Final body weights, bladder weights, and the incidences and T stages of UC in the urinary bladder are summarized in Table 1. There were no significant differences in final body weights among the groups. In contrast, bladder weights were significantly decreased in the BBN→Cis+Ace group compared with the BBN alone group. Bladder weights also tended to be decreased in the BBN→Cis and BBN→Ace groups compared with the BBN alone group, albeit without statistical difference.

Macroscopically, fewer and smaller UCs were observed in the BBN→Cis+Ace group compared with the BBN alone group (Figure 1). Cis alone (BBN→Cis) did not significantly affect total UC incidence or the incidence of muscle invasive UC (≥pT2). Ace alone (BBN→Ace) did not significantly affect total UC incidence, but did significantly decrease muscle invasive UC (6.7%) compared with the BBN alone group (30%). Concurrent administration of Cis and Ace (BBN→Cis+Ace) markedly decreased both total UC incidence and muscle invasive UC (10% and 3.3%, respectively) compared with the BBN alone group (53.3% and 30%, respectively). These results clearly demonstrated that Ace treatment inhibited tumor invasion and that combined treatment with Cis and Ace was more effective than Cis or Ace alone in inhibiting the development of UCs.
The immunoreactivity of plasma and nuclei of the urothelial cells in the control mice (Figure 2) did not affect the expression of CA.

Some umbrella cells in the simple hyperplasia were positive for CA. The incidence of CA-positive UCs between the 0.025% BBN and 0.05% BBN- induced UCs when the same stages of the UCs were compared. Expression patterns of CA2 in the Bladder urothelium of mice in the BBN→Cis group (2/3: 66.7%), the BBN alone group (12/12: 100%), the BBN→Ace group (7/9: 77.7%), and the BBN alone group (7/9: 77.7%), and the BBN alone group (11/14: 78.6%). This suggests that CA2 is a CA activity inhibitor and does not affect the expression of CA.

As expected, 0.05% BBN induced more tumors than 0.025% BBN, indicating that 0.05% BBN is a stronger initiating agent than 0.025% BBN. The results of the present study suggested that the inhibitory potential of Ace-mediated CA2 inhibition was capable of inhibiting UC initiation induced by 0.025% BBN, but was not capable of inhibiting UC initiation induced by 0.05% BBN. These results also suggested that one or more pathways involved in progression of UC were effectively inhibited by Ace-mediated CA2 inhibition.

### 3.3 | Expression of CA2 and β-catenin in 0.05% BBN-induced urinary bladder lesions

Representative HE and immunohistochemical findings for CA2 in the urinary bladder are shown in Figure 2. CA2 was negative in normal bladder epithelium in the control mice (Figure 2E). In the BBN alone mice, positive staining of CA2 was localized to the cytoplasm of urothelial cells in morphologically normal appearing urothelium (Figure 2F), simple hyperplasia (Figure 2G), and UCs (Figure 2H). Some umbrella cells in the simple hyperplasia were positive for CA2 in both the nucleus and cytoplasm.

The incidence of CA2-positive UC in the urinary bladder is summarized in Table S1. High expression of CA2 was noted in most of the BBN-induced UCs regardless of T stage. There were no significant differences in the incidences of CA2-positive UCs between the groups. This can be explained by the fact that Ace is a CA activity inhibitor and does not affect the expression of CA.

Weakly positive β-catenin staining was observed in the cytoplasm and nuclei of the urothelial cells in the control mice (Figure 2I). The immunoreactivity of β-catenin was increased in the nuclei of urothelial cells in the morphologically normal appearing urothelium (Figure 2J), simple hyperplasia (Figure 2K) and UCs (Figure 2L) of BBN alone mice. As summarized in Table S2, the majority of BBN-induced UCs were positive for β-catenin. As dysregulation of Wnt/β-catenin signaling is associated with numerous tumor types, the expression of β-catenin in BBN-induced UCs suggests that β-catenin may be involved in BBN-induced bladder carcinogenesis in mice. Whereas treatment with Cis+Ace significantly reduced tumor formation (Table 1), in the tumors that did develop there were no significant differences in the incidences of β-catenin-positive UCs between the BBN→Cis+Ace group (2/3: 66.7%), the BBN→Ace group (12/12: 100%), the BBN→Cis group (7/9: 77.7%), and the BBN alone group (11/14: 78.6%). This suggests that Cis and Ace-resistant UC and supports the premise that β-catenin may be involved in BBN-induced bladder carcinogenesis in mice.

In addition, there were no apparent histological differences between the 0.025% BBN and 0.05% BBN-induced UCs when the same stages of the UCs were compared. Expression patterns of CA2 and β-catenin were also similar between 0.025% BBN and 0.05% BBN-induced UCs (data not shown).

### 3.4 | Effects of Cis and Ace treatment on cell proliferative actives in the bladder urothelium of mice

To evaluate the effects of Ace and/or Cis on proliferation, the Ki-67 index was determined in normal and morphologically normal appearing urothelium. As shown in Figure 3, the Ki-67 index was significantly decreased in the morphologically normal appearing urothelium of mice in the BBN→Ace group and the BBN→Cis+Ace group.

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**Table 1** Body weights, bladder weights and incidence of urothelial carcinoma in the urinary bladders of mice in the BBN-induced urinary bladder carcinogenesis study

| Group                  | No. of mice | Final body weight (g) | Bladder weight (g) | Incidence of UC (%) |
|------------------------|-------------|-----------------------|--------------------|---------------------|
|                        |             |                       |                    | pTa  | pT1  | ≥pT2 | Total |
| Experiment 1           |             |                       |                    |      |      |      |       |
| 0.025% BBN alone       | 25          | 29.07±0.43            | –                  | 6 (24.0) | 1 (4.0) | 1 (4.0) | 8 (32.0) |
| 0.025% BBN→Cis         | 25          | 27.88±0.75            | –                  | 2 (8.0) | 0 (0) | 2 (8.0) | 4 (16.0) |
| 0.025% BBN→Ace         | 25          | 29.44±0.60            | –                  | 1 (4.0) | 0 (0) | 1 (4.0) | 2 (8.0) |
| 0.025% BBN→Cis+Ace     | 25          | 27.50±0.57            | –                  | 1 (4.0) | 0 (0) | 0 (0) | 1 (4.0) |
| Ace alone              | 10          | 29.82±1.16            | –                  | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| Control                | 10          | 29.04±0.91            | –                  | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| Experiment 2           |             |                       |                    |      |      |      |       |
| 0.05% BBN alone        | 30          | 30.25±0.52            | 0.107±0.052        | 4 (13.3) | 3 (10.0) | 9 (30.0) | 16 (53.3) |
| 0.05% BBN→Cis          | 30          | 28.98±0.67            | 0.062±0.008        | 1 (3.3) | 3 (10.0) | 6 (20.0) | 10 (33.3) |
| 0.05% BBN→Ace          | 30          | 30.17±0.66            | 0.068±0.022        | 3 (10.0) | 8 (26.7) | 2 (6.7) | 13 (43.3) |
| 0.05% BBN→Cis+Ace      | 30          | 29.54±0.77            | 0.054±0.006        | 1 (3.3) | 1 (3.3) | 1 (3.3) | 3 (10.0) |

Abbreviations: –, not examined; UC, urothelial carcinoma.
*p < 0.05; **p < 0.01 versus the BBN alone group.
group compared with the BBN alone group. There was also a nonsignificant decrease in the Ki-67 index in the BBN→Cis group. In addition, the Ki-67 index was significantly decreased in the BBN→Cis+Ace group compared with the BBN→Cis group. Decreased proliferation of cells in morphologically normal appearing urothelium indicate that Ace exerted inhibitory effects on proliferation in the early stages of BBN-induced bladder carcinogenesis. These findings agree with the results of the 0.05% BBN-induced bladder carcinogenesis experiment in which the most potent inhibitory effects were observed in the BBN→Cis+Ace group.

3.5 | Effects of CA2 on migration and invasion capabilities of T24 and UMUC3 cells

T24 and UMUC3 cells were transfected with pCDNA3.1+/C-(K)-DYK-CA2 (CA2) and pCDNA3.1+/C-(K)-DYK (empty vector, control). CA2 was overexpressed in the CA2-transfected cells compared with the empty vector-transfected cells (Figure 4A,B). Migration and invasion capabilities were significantly increased in CA2-transfected T24 and UMUC3 cells (Figure 4C,D and Figure S2). Notably, there was no significant difference in cell proliferative capabilities between the CA2-transfected T24 and UMUC3 cells.
and their respective controls (Figure 4E). These results indicate that the promoting effects of CA2 on migration and invasion of T24 and UMUC3 cells were not associated with the regulation of cell proliferation. Ace treatment significantly inhibited the migration and invasion capabilities of CA2-transfected UMUC3 and T24 cells compared with empty vector-transfected cells (Figure 4F and Figure S3).

3.6 | Effects of CA2 on expression and nuclear translocation of β-catenin and expression of EMT-related proteins in T24 and UMUC3 cells

Wnt/β-catenin-regulated EMT is a key mechanism that promotes migration and invasion of BCs. Therefore, we investigated the expression of β-catenin and EMT-related proteins in T24 and UMUC3 cells. As shown in Figure 4G, β-catenin expression was increased in CA2-transfected T24 and UMUC3 cells. Furthermore, immunofluorescence and confocal image analyses confirmed that β-catenin was increased and expressed predominantly in the nuclei of CA2-transfected cells, whereas β-catenin was expressed in the cytoplasm in empty vector-transfected controls (Figure 4H). This agrees with the finding of increased nuclear expression of β-catenin in 0.05% BBN-induced bladder lesions (Figure 2J,K,L).

In addition to β-catenin expression, Figure 4G shows the effects of CA2 overexpression on vimentin and N-cadherin expression: vimentin expression was increased in CA2-transfected T24 cells but not in UMUC3 cells; N-cadherin expression was increased in CA2-transfected UMUC3 cells but not in T24 cells. This is consistent with the microarray results described below: the genes displaying altered expression caused by CA2 overexpression are considerably different between transfected-T24 and UMUC3 cells. This is most likely to be due to differences in the clones from which T24 and UMUC3 cell lines were derived and showed that the effect of CA2 on EMT-related proteins can be different in different cell lines.

3.7 | Microarray gene expression analyses of CA2-transfected T24 and UMUC3 cells

The number of differently expressed genes in CA2-transfected T24 and UMUC3 cells is shown in Figure 5A. In total, 38 genes were increased in CA2-transfected T24 cells and 186 genes were increased in UMUC3 cells compared with their respective controls. Six of these
genes were commonly upregulated (Table 2). In addition, 180 genes were downregulated in CA2-transfected T24 cells and 118 genes were downregulated in CA2-transfected UMUC3 cells. Three genes were commonly downregulated (Table 2). Phosphatidylinositol 4-phosphate 5-kinease 1B (PIP5K1B) was one of the commonly upregulated genes. PIP5K1B is associated with stabilization of β-catenin and activation of Wnt/β-catenin signaling. Real-time RT-PCR showed significantly increased mRNA expression of the PIP5K1B gene in CA2-transfected T24 and UMUC3 cells (Figure 5B). These results suggest that overexpression of CA2 may activate the Wnt/β-catenin signaling pathway by upregulating the expression of PIP5K1B.

**4 | DISCUSSION**

In the present study, we found that Ace treatment significantly inhibited the development of muscle invasive UC and that the combined treatment with Cis and Ace significantly inhibited both overall UC incidence and the development of muscle invasive UCs in mice. We also showed that Ace significantly inhibited the migration and invasion of CA-2-transfected human UCs. These findings demonstrated that CA2 was functionally associated with UC in mice and suggested that combined treatment with Cis and Ace, a CA2 inhibitor, may provide a new therapeutic strategy to inhibit UC muscle invasion and improve the outcomes of UC patients.

In BBN-treated mice, expression of β-catenin was increased in the nuclei of urothelial cells in morphologically normal appearing urothelium, simple hyperplasia, and UCs, suggesting that β-catenin is involved in BBN-induced bladder carcinogenesis in mice. High expression of CA2 was also observed in the cytoplasm of urothelial cells in morphologically normal appearing urothelium, simple hyperplasia, and UCs in these mice. This finding suggests that overexpression of CA2 may activate the Wnt/β-catenin signaling pathway. Wnt/β-catenin signaling pathway-regulated EMT is a key mechanism that promotes migration and invasion of BCs. Wnt signaling inhibits the ubiquitination and proteasome degradation of β-catenin, enhancing β-catenin accumulation in the cytoplasm and its translocation to the nucleus. In the nucleus, β-catenin binds to TCF transcription factors and regulates the expression of EMT-related genes such as N-cadherin, E-cadherin, and vimentin. In the present study, overexpression of CA2 increased the expression and nuclear translocation of β-catenin in T24 and UMUC3 cells, providing evidence for an association between overexpression of CA2 and β-catenin signaling in UCs. Overexpression of CA2 increased the migration and invasion capabilities of T24 and UMUC3 cells that, along with increased expression of EMT-related genes, provide further evidence that CA2 overexpression is
FIGURE 4 Effects of CA2 overexpression on migration and invasion capabilities of T24 and UMUC3 cells. (A) Increased protein levels of CA2 in CA2-transfected T24 and UMUC3 cells. (B) Increased mRNA levels of CA2 in CA2-transfected T24 and UMUC3 cells. (C) Overexpression of CA2 increased the migration and invasion capabilities of T24 cells. (D) Overexpression of CA2 increased the migration and invasion capabilities of UMUC3 cells. (E) CA2 had no effect on the proliferation of T24 or UMUC3 cells. (F) Ace significantly inhibited migration and invasion capabilities in T24 (A,B) and UMUC3 cells (C,D). (G) Protein expression levels of β-catenin, vimentin, N-cadherin, and CA2 in CA2-transfected T24 and UMUC3 cells. Band intensity was measured with a Fusion SOLO7S and expressed as a ratio to empty vector-transfected cells in each lane. Data are representative of two separate experiments yielding similar results. (H) Overexpression of CA2 (Flag-tagged CA2, red) induced nuclear translocation of β-catenin (green) in UMUC3 cells. Wnt3A stimulation served as the positive control. Arrowheads: nuclear localization of β-catenin. Bars, 10 μm. *p < 0.05, **p < 0.01, ***p < 0.001, versus controls, respectively.
associated with the activation of the Wnt/β-catenin signaling pathway. These data provide indirect evidence that Ace inhibited the invasion of UCs in mice by inhibiting CA2 activity and are in accordance with the finding that Ace had a stronger inhibitory effect on invasion by UC, compared with its inhibition of BBN-mediated initiation of UC (Table 1). Although the detailed mechanism by which CA2 activates the Wnt/β-catenin signaling pathway remains to be elucidated, PIP5K1B activates the production of PtdIns4,5P2, and PtdIns4,5P2 stabilizes β-catenin in the cytoplasm. The finding that PIP5K1B was one of six genes commonly upregulated in CA2-transfected T24 and UMUC3 cells suggests that PIP5K1B may play an important role in the CA2-activation of Wnt/β-catenin signaling, possibly via stabilization of β-catenin. Little information is known about expression of PIP5K1B and its roles in bladder UC muscle invasion. Further study to determine the role of PIP5K1B in BC is warranted.

In our previous study we demonstrated that overexpression of CA2 expression was associated with the grade of malignancy, invasiveness, and progression in human UC, and in the present study we demonstrated a functional association of CA2 with UC. As overexpression of CA2 has been reported in oligodendrogliomas, astrocytomas, uterine tumors, chromophobe renal cell carcinomas, and pulmonary neuroendocrine tumors, CA2 might also be a useful therapeutic target for these cancers. It should be noted that Ace is a potent inhibitor of CA2, CA9, and CA12. Further studies to determine the expression of different CAs and evaluation of the inhibitory effects of selective CA inhibitors are necessary.

In the present study, the combined treatment of Cis and Ace was more potent than treatment with Ace or Cis alone. Previous studies have also reported the synergistic effects of Ace and Cis against laryngeal carcinomas and neck squamous cell carcinomas. The cytotoxicity of Cis is mediated by DNA damage and results

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**TABLE 2** List of genes commonly differentially expressed in T24 and UMUC3

| Gene symbol | Gene description                        | Ratio<sup>a</sup> |
|-------------|----------------------------------------|-------------------|
| CA2         | Carbonic anhydrase II                   | 803.18            |
| PIP5K1B     | Phosphatidylinositol-4-phosphate 5-kinase, type I, beta | 2.12              |
| FAM20C      | Family with sequence similarity 20, member C | 1.67              |
| ZDHHC14     | Zinc finger, DHHHC-type containing 14   | 1.59              |
| COX4I1      | Cytochrome c oxidase subunit IV isoform 1 | 1.55              |
| HIST1H4A    | Histone cluster 1, H4a                  | 1.54              |
| TGFA        | Transforming growth factor alpha         | 0.60              |
| RPS6KA1     | Ribosomal protein S6 kinase, 90kDa, polypeptide 1 | 0.48              |
| ART1        | ADP-ribosyltransferase 1                | 0.40              |

<sup>a</sup>Fold changes versus respective empty vector-transfected control cells.
in apoptosis of the target cell.\textsuperscript{39} Cis uptake is markedly increased by low extracellular pH.\textsuperscript{39,40} Under hypoxic conditions, CA2 catalyzes the hydration of carbon dioxide and water into bicarbonate and protons (H\textsuperscript{+}), thereby inducing an acidic microenvironment.\textsuperscript{41} CA2 is also reported to activate the monocarboxylate transporter MCT1, which supports intracellular lactate transport in cancer cells and acidifies the extracellular environment.\textsuperscript{42} Consequently, Ace-mediated inhibition of CA2 activity would appear to inhibit cellular uptake of Cis and decrease its antitumor effect. Therefore, further investigation of the mechanisms by which Ace and Cis act in concert is needed.

In conclusion, inhibition of CA2 by Ace inhibits the development and muscle invasion of BBN-induced UC in mice and inhibits the migration and invasion of human UC cells in vitro, confirming a functional association between CA2 and UC development and progression. One possible mechanism by which CA2 promotes UC progression is the upregulation of Wnt/\beta-catenin signaling and the consequential induction of EMT. Taken together, these findings suggest that Ace may suppress Wnt/\beta-catenin signaling and the consequential EMT in vivo and that this played a role in Ace-mediated suppression of muscle invasion by UC in the present study. Ace has already been used in clinical practice to treat glaucoma\textsuperscript{43} and heart failure.\textsuperscript{44} Drug Repositioning of Ace may have clinical application as a therapeutic agent for invasive UC.

AUTHOR CONTRIBUTIONS
Conceptualization and design of the experiments: Taisuke Matsue, Min Gi, and Hideki Wanibuchi. Performance of experiments: Taisuke Matsue, Min Gi, Masayuki Shiota, Hirokazu Tachibana, Shugo Suzuki, Masaki Fujikoa, Anna Kakehashi, and Tomoki Yamamoto. Data analysis: Taisuke Matsue, Min Gi, and Minoru Kato. Writing of the paper: Taisuke Matsue and Min Gi. Writing-review: Junji Uchida and Hideki Wanibuchi. Supervision: Hideki Wanibuchi. Taisuke Matsue and Min Gi contributed equally to this work.

ACKNOWLEDGMENTS
We gratefully acknowledge the technical assistance of Rie Onodera, Keiko Sakata, Yuko Hisabayashi, and Yukiko Iura (Department of Molecular Pathology, Osaka Metropolitan University Graduate School of Medicine School) and Kumi Takemura (Research Support Platform of Osaka Metropolitan University Graduate School of Medicine).

CONFLICT OF INTEREST
The authors have no conflict of interest. Hideki Wanibuchi, the corresponding author, is an Associate Editor of Cancer Science.

ETHICS STATEMENT
Approval of the research protocol by an Institutional Reviewer Board: N/A. Informed Consent: N/A. Registry and the Registration No. of the study/trial: N/A. Animal Studies: All animal studies were approved by the Institutional Animal Care and Use Committee of Osaka City University Graduate School of Medicine.

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**SUPPORTING INFORMATION**

Additional supporting information can be found online in the Supporting Information section at the end of this article.

**How to cite this article:** Matsue T, Gi M, Shiota M, et al. The carbonic anhydrase inhibitor acetazolamide inhibits urinary bladder cancers via suppression of β-catenin signaling. *Cancer Sci.* 2022;113:2642-2653. doi: [10.1111/cas.15467](https://doi.org/10.1111/cas.15467)