Carbonic anhydrase 2 is a novel invasion-associated factor in urinary bladder cancers

Hirokazu Tachibana,1,2 Min Gi,1 Minoru Kato,1,2 Shotaro Yamano,1,3 Masaki Fujioka,1 Anna Kakehashi,1 Yukihiro Hirayama,1,2 Yuki Koyama,1,2 Satoshi Tamada,2 Tatsuya Nakatani2 and Hideki Wanibuchi1

1Department of Molecular Pathology, Osaka City University Graduate School of Medicine, Osaka; 2Department of Urology, Osaka City University Graduate School of Medicine, Osaka, Japan

Key words
Carbonic anhydrase 2, Hras128 rats, invasion-associated factor, invasive urinary bladder cancer, N-butyl-N-(hydroxybutyl)nitrosamine

Correspondence
Hideki Wanibuchi, Department of Molecular Pathology, Osaka City University Graduate School of Medicine, 1-4-3 Asahi-machi, Abeno-ku, Osaka 545-8585, Japan. Tel: +81-6-6645-3737; Fax: +81-6-6646-3093; E-mail: wani@med.osaka-cu.ac.jp

Funding Information
Japan Society for the Promotion of Science (JSPS KAKENHI Grant Number 25462499), and Health Labour Sciences Research Grants (Research on Risk of Chemical Substances H26-chemistry-shitei-001) from Ministry of Health, Labour and Welfare, Japan.

Received September 23, 2016; Revised December 11, 2016; Accepted December 17, 2016

Cancer Sci 108 (2017) 331–337
doi: 10.1111/cas.13143

Urinary bladder cancer is the 9th most frequent cancer worldwide and the 13th most common cause of cancer death.1 Approximately 90% of bladder cancers are urothelial carcinomas (UC), and 25% of these tumors are diagnosed as muscle-invasive bladder cancer (MIBC).2-3 MIBC is fatal with a 5-year survival rate of approximately 50% due to lethal metastasis.4 The other 75% of UC are diagnosed as non-MIBC (NMIBC). NMIBC include pTis (generally referred to carcinoma in situ [CIS]), pTa and pT1. CIS and pTa tumors are non-invasive and localized in the epithelium of the urinary bladder, whereas pT1 tumors invade subepithelial connective tissue (lamina propria).5 NMIBC frequently recur following treatment, and 15% of them progress to fatal MIBC.6 It has been suggested that MIBC can arise de novo7-10 or progress from NMIBC.6-8

Muscle-invasive bladder cancer has been extensively studied and recent studies have revealed that MIBC can be grouped into basal and luminal subtypes based on the gene expression profile, with the basal MIBC being associated with poor outcomes because they tend to have more invasive and metastatic characteristics.11 In addition, mutation of p53 is frequently observed in CIS, and aberrant RB is also considered a key molecule in the progression of CIS to MIBC.7 However, the exact mechanisms by which pTa and pT1 tumors progress to MIBC remains unclear. Histopathologically, tumor grade is a good prognostic indicator of progression for UC.12-14 However, predicting progression of pTa and pT1 tumors remains a challenge as bladder tumors with the same pathologic grade have a heterogeneous clinical outcome. Therefore, identification of invasion-associated factors during the process of acquiring invasive capability by pTa and pT1 tumors may provide new therapeutic strategies, improving the outcomes of UC patients.

Chemically-induced rat urinary bladder cancer is nearly always non-invasive UC that closely resembles the pathologic characteristics of its human counterpart, non-invasive pTa tumors.15-16 In the present study, we aim to establish a novel medium-term chemically-induced invasion model mimicking the invasive UC that arise from non-invasive UC and to use this model to identify invasion-associated factors. The BBN-induced rat bladder cancer model is a widely used orthotropic model of human UC with short-term treatment of BBN being capable of inducing high incidences of non-invasive UC,18,19 and Hras128 rats exhibit high susceptibility to
N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN)-induced bladder carcinogenesis,(20,21) however, unlike other rat models, Hras128 rats treated with BBN also develop invasive UC.(20,21) In addition, it was reported that preneoplastic lesions induced by the rat urinary bladder carcinoma phenylethyl isothiocyanate (PEITC) were prone to dysplasia in rats.(22,23) In an attempt to efficiently induce invasive UC, we treated separate groups of Hras128 rats with BBN followed by PEITC (BBN→PEITC), PEITC followed by BBN (PEITC→BBN), BBN alone, or PEITC alone.

The results of the present study demonstrated that BBN→PEITC treatment is an effective regimen to induce invasive UC in the Hras128 rat. Proteomic comparison of invasive and non-invasive UC induced by BBN→PEITC treatment and analysis of human UC revealed that carbonic anhydrase 2 (CA2) is an invasion-associated factor and suggest that it could serve as a potential therapeutic target for UC.

Materials and Methods

Chemicals. N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN) (purity >98%) was obtained from Wako Pure Chemical Industries, Osaka, Japan. Phenylethyl isothiocyanate (PEITC) (purity = 98%) was obtained from Sigma-Aldrich (Tokyo, Japan). Corn oil was obtained from Nakarai Chemicals (Kyoto, Japan).

Animals. Fifty-two male human c-Ha-ras proto-oncogene transgenic (Hras128) rats at 5 weeks of age were obtained fromCLEA Japan (Tokyo, Japan). 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). Animals were housed in polycarbonate cages (3 rats/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 to the animal room environment for 7 days before being used for experiments.

Animal study protocol. 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). For the present study, 0.05% BBN was dissolved in tap water. To prepare a 0.1% PEITC diet, PEITC was dissolved in 1% corn oil (10 g/kg diet), and the solution was mixed with basal diet (Oriental MF [Oriental Yeast, Tokyo, Japan]). Diet and tap water were available ad libitum throughout the study. Male Hras128 rats were divided into five groups and treated as follows: BBN→PEITC group (13 rats), 8 weeks treatment with BBN followed by 8 weeks treatment with PEITC after 3 days of BBN cessation; PEITC→BBN group (13 rats), 8 weeks treatment with PEITC followed by 8 weeks treatment with BBN after 3 days of PEITC cessation; BBN alone group (9 rats), 16 weeks treatment with BBN; PEITC alone group (9 rats), 16 weeks treatment with PEITC; and control group (8 rats), no treatment. At the end of week 16, rats were killed by administration of an overdose (50 mg/kg of body weight, i.p.) of pentobarbital sodium (Somnopentyl, Kyoritsu Seiyaku, Tokyo, Japan). Urinary bladders were inflated by intraluminal administration of an overdose (50 mg/kg, i.p.) of pentobarbital sodium (Somnopentyl, Kyoritsu Seiyaku, Tokyo, Japan.) Urinary bladders were inflated by intraluminal administration of an overdose (50 mg/kg, i.p.) of pentobarbital sodium (Somnopentyl, Kyoritsu Seiyaku, Tokyo, Japan) and then fixed in the same PFA solution at 4°C for 4 h. PFA-fixed urinary bladders were cut into eight strips and routinely processed for embedding in paraffin.

Patients. Immunohistochemical analysis was performed on samples from 235 patients with urothelial carcinomas who were treated for bladder cancer by radical cystectomy or first transurethral resection of bladder tumor (TURBT) at Osaka City University Hospital between 2000 and 2009. There were 189 men and 46 women, and the median age was 67 years (range, 33–90 years). Pathologic staging and grading was performed according to 2004 WHO/1998 ISUP classification.(5) The Institutional Review Board at Osaka City University Graduate School of Medicine approved the use of the specimens and clinical data in accordance with the Declaration of Helsinki and guidelines of Osaka City University Graduate School of Medicine.

Protein extraction and QSTAR Elite LC/MS/MS analysis. Six non-invasive UC from 5 rats and 6 invasive UC from 6 rats in the PEITC→BBN group, and 4 normal bladder urothelium from 4 rats in the control group were processed for proteomic analysis. The six invasive UC included 4 non-muscle invasive and 2 muscle invasive UC. Ten serial UC sections and twenty serial normal urinary bladder sections (10-μm thickness) were cut from paraffin-embedded urinary bladder specimens. The first and the last sections in each bladder sample were stained with H&E to identify the area for needle microdissection. After deparaffinization, normal bladder urothelium and non-invasive and invasive UC were collected using seriele toothpicks under a light microscope and transferred immediately to Eppendorf Tubes containing buffer for the Liquid Tissue MS Protein Prep Kit (Expression Pathology, Gaithersburg, MD, USA). An image of representative normal urothelial tissue separated from the urinary bladder of a control rat is shown in Figure S1.

Protein extraction was performed using Liquid Tissue MS Protein Prep Kit according to the manufacturer’s instructions. Protein concentrations were determined using the BCA Protein Assay kit (Pierce, IL, USA). Forty-eight μg of protein from normal urothelium (12 μg each rat) and 48 μg of protein from 6 non-invasive and 6 invasive UC (8 μg each UC) were used for proteome analysis as described previously.(24,25) Briefly, protein reduction, alkylation, digestion and subsequent peptide labeling were performed using the AB Sciex iTRAQ Reagent Multi-Plex Kit (AB Sciex, Foster City, CA, USA), according to the manufacturer’s instructions. Peptides were fractionated by six concentrations of KCl solutions using the ICAT cation exchange cartridge (AB Sciex). Desalting and concentrating, peptides of each fraction were quantified using a DiNa-Al nano LC System (KYA Technologies, Tokyo, Japan) coupled to the QSTAR Elite MS/MS through a NanoSpray ion source (AB Sciex, Concord, ON, Canada). Protein Pilot 2.0 software (AB Sciex) with the Paragon Algorithm was used for the identification and relative quantification of proteins. Protein quantitative ratio statistics were calculated as the median of all peptide ratios. Proteins showing a fold-change of at least 1.2 at a P-value < 0.05 were considered differentially expressed. The LC-MS/MS and ProteinPilot results were further analyzed byIngenuity Pathway Analysis (Ingenuity Systems, Mountain View, CA, USA) to investigate protein functions and cellular location.

Immunohistochemical analysis. Serial sections (4-μm thickness) cut from paraffin-embedded urinary bladder specimens of the BBN alone group, BBN→PEITC group, and human UC specimens were examined for expression of CA2 by immunohistochemical staining using the avidin–biotin–peroxidase complex (ABC) method. Antigen retrieval was performed for rat sections by microwaving at 98°C for 20 min in 0.01 M citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked with 3% H2O2 in distilled water for 5 min. After blocking
non-specific binding with goat serum at 37°C for 30 min, rat sections were incubated with rabbit monoclonal anti-CA2 antibody (ab124687, Abcam, Cambridge, MA, USA) diluted 1:1000 and human sections were incubated with rabbit polyclonal anti-CA2 antibody (ab191343, Abcam, Cambridge, MA, USA) diluted 1:1000 overnight at 4°C. Immunoreactivity was detected using a VECSTAIN Elite ABC Kit (PK-6101, Vector Laboratories, Burlingame, CA, USA) and 3,3’-diaminobenzidine hydrochloride (Sigma Chemical, St Louis, MO, USA). Omission of the primary antibody served as the negative control and was included with each staining procedure.

Overexpression of CA2 in rat and human UC were defined as positive when cytoplasmic staining was evident in >10% of the cells. In addition, semiquantitative estimation of CA2 staining in rat UC with multiple values for extent and intensity for a score of 0 to 9 was performed as previously described. The extent of staining was scored on a semiquantitative scale of 0 to 3, using the following criteria: 0, no detectable staining; 1, <10% stained cells; 2, >10% but ≤50% stained cells; 3, homogeneous staining in >50% of cells. The intensity of staining was scored using the following criteria: 0, no detectable staining; 1, weakly stained cytoplasm; 2, moderately stained cytoplasm; 3, strongly stained cytoplasm. Final scores were derived from multiplication of extent by intensity.

**Results**

**Induction of invasive urothelial carcinoma in Hras128 rats.** Macroscopically, tumors were observed in BBN→PEITC and BBN alone groups but not in the PEITC→BBN (c) or PEITC alone (d) groups. The incidence and multiplicity of UC in the urinary bladders of Hras128 rats were derived from multiplication of extent by intensity. The extent of staining was scored on a semiquantitative scale of 0 to 3, using the following criteria: 0, no detectable staining; 1, <10% stained cells; 2, >10% but ≤50% stained cells; 3, homogeneous staining in >50% of cells. The intensity of staining was scored using the following criteria: 0, no detectable staining; 1, weakly stained cytoplasm; 2, moderately stained cytoplasm; 3, strongly stained cytoplasm. Final scores were derived from multiplication of extent by intensity.

**Statistical analysis.** All mean values are reported as mean ± SD. Statistical analyses were performed using the GraphPad Prism 6 program (GraphPad Software, CA, USA). Fisher’s exact test was used to evaluate the differences in incidence of UC and CA2-positive cancers in the animal study and incidence of CA2 expression pattern among clinical and pathological parameters in the human study. The nonparametric Mann–Whitney U-test was used for the evaluation of CA2 staining scores in rat UC. Differences in mean values of multiplicity of bladder cancers in the animal study were evaluated by Student’s t-test when variance was homogeneous and Welch’s t-test when variance was heterogeneous. Progression-free survival was defined as the time between the date of surgery and the last date of follow up or date of progression in pT status. The curves were done using the Kaplan–Meier method with the log-rank test to assess the hazard ratio. P-values < 0.05 were considered significant.

**Identification of differentially expressed proteins by proteomic comparison analysis of invasive and non-invasive urothelial carcinoma induced by BBN→PEITC treatment.** Proteomic analysis was conducted for 6 invasive UC and 6 non-invasive UC in the rats treated with BBN→PEITC and 4 normal urothelium in the non-treatment group by QSTA Elite LC-MS-MS. As shown in Figure 2a, there were 232 and 217 proteins differentially expressed in the invasive and non-invasive UC compared to the normal urothelium, respectively. A total of 183 of these proteins, 100 overexpressed and 83 underexpressed, were differentially expressed in both the invasive and non-invasive UC compared to the normal urothelium, respectively. A total of 34 proteins, 23 overexpressed and 25 underexpressed proteins, were differentially expressed in the invasive but not non-invasive UC (Tables S1 and S2). A total of 49 proteins, 24 overexpressed and 25 underexpressed proteins, were differentially expressed in the non-invasive but not invasive UC (Tables S3 and S4). There were 34 proteins, 23 overexpressed and 11 underexpressed proteins, differentially expressed in the non-invasive but not invasive UC (Tables S5 and S6).

**Selection of carbonic anhydrase 2 as a candidate invasive factor.** Of the 49 proteins differentially expressed in the invasive but not the non-invasive UC, 18 are coded by genes categorized as cancer related-genes by Ingenuity Pathway Analysis (Fig. 2b). Of these 18 proteins, we selected CA2 as a candidate invasive factor for further studies: CA2 have been expressed in rat UC with multiple values for extent and intensity for a score of 0 to 9 was performed as previously described. The extent of staining was scored on a semiquantitative scale of 0 to 3, using the following criteria: 0, no detectable staining; 1, <10% stained cells; 2, >10% but ≤50% stained cells; 3, homogeneous staining in >50% of cells. The intensity of staining was scored using the following criteria: 0, no detectable staining; 1, weakly stained cytoplasm; 2, moderately stained cytoplasm; 3, strongly stained cytoplasm. Final scores were derived from multiplication of extent by intensity.

**Statistical analysis.** All mean values are reported as mean ± SD. Statistical analyses were performed using the GraphPad Prism 6 program (GraphPad Software, CA, USA). Fisher’s exact test was used to evaluate the differences in incidence of UC and CA2-positive cancers in the animal study and incidence of CA2 expression pattern among clinical and pathological parameters in the human study. The nonparametric Mann–Whitney U-test was used for the evaluation of CA2 staining scores in rat UC. Differences in mean values of multiplicity of bladder cancers in the animal study were evaluated by Student’s t-test when variance was homogeneous and Welch’s t-test when variance was heterogeneous. Progression-free survival was defined as the time between the date of surgery and the last date of follow up or date of progression in pT status. The curves were done using the Kaplan–Meier method with the log-rank test to assess the hazard ratio. P-values < 0.05 were considered significant.

**Results**

**Induction of invasive urothelial carcinoma in Hras128 rats.** Macroscopically, tumors were observed in BBN→PEITC and BBN alone groups but not in the PEITC→BBN or PEITC alone groups (Fig. 1). The incidence and multiplicity of UC in the urinary bladder are summarized in Table 1. Non-invasive UC were observed in the all animals in the BBN→PEITC and BBN alone groups. The incidence of invasive UC showed a tendency to increase in the BBN→PEITC group compared to the BBN alone group. The multiplicity of invasive UC as well as non-invasive UC was significantly higher in the BBN→PEITC group compared to the BBN alone group. The incidence and multiplicity of muscle invasive UC was also increased in the BBN→PEITC group (69.2%; 0.5 ± 0.8/rat) compared to the BBN alone group (11.1%; 0.1 ± 0.3/rat), albeit without statistical difference. In contrast, no UC were observed in the PEITC→BBN, PEITC alone or control groups, although papillary or nodular hyperplasia was observed in the PEITC→BBN and PEITC alone groups. These results show that BBN→PEITC treatment is the most effective regimen to induce invasive UC in Hras128 rats, and we used this model of invasive bladder cancer to identify invasion-associated proteins.
implicated in cancer invasiveness;\textsuperscript{(27,28)} CA2 was the only overexpressed CA in the present study and its aberrant expression has not been reported in UC; CA2 was the 4th most highly overexpressed protein; and CA inhibitors are commercially available that can be used for future studies. We did not select the top 3 overexpressed proteins (S100A9, ALDH3A1 and NDUFV2) for further examination because: (i) S100A9 has already been reported to be an invasion-associated factor in UC;\textsuperscript{(29–31)} and (ii) we tried several ALDH3A1 and NDUFV2 antibodies, but they were not satisfactory for immunohistochemistry of either rat or human UC specimens.

Expression of CA2 in rat urothelial carcinoma. Normal epithelial cells in the control rats did not show immunoreactivity for CA2 (Fig. 3a). CA2 staining was localized to the cytoplasm of UC (Fig. 3b,c), and some papillary and nodular hyperplasias, but was not present in morphologically normal urothelial cells of the BBN→PEITC-treated rats. The incidence of CA2-positive UC was significantly higher for invasive UC (78.9\%) compared to non-invasive UC (28.9\%). The expression score of CA2 was also significantly higher for invasive UC (5.2±2.7) compared to non-invasive UC (2.4±1.7). In UC induced by BBN alone, the incidence of CA2-positive UC was also significantly higher for invasive UC (75\%) compared to non-invasive UC (31.9\%). While the multiplicity of invasive UC as well as non-invasive UC was significantly higher in the BBN→PEITC group compared to the BBN alone group, the incidence of CA2-positive invasive and non-invasive UC was comparable between these two groups. These results suggest that CA2 is an invasion-associated factor for rat UC. These results also show that BBN→PEITC treatment is the most effective regimen to induce invasive UC in Hras128 rats, but PEITC had no effect on the expression of CA2 and may promote BBN-induced invasive UC in a CA-2 independent manner.

Expression of carbonic anhydrase 2 in human urothelial carcinomas and its correlation with histopathological parameters and progression. Carbonic anhydrase 2 expression and clinical and pathological characteristics of the 235 bladder cancer patients used in the present study are summarized in Table 2. Normal urothelium was negative for CA2 (Fig. 4a). CA2 staining was localized to the cytoplasm of UC (Fig. 4b,c). The incidence of CA2-positive UC was 0, 15.2, 13.2 and 54.8\% in pTis, pTa, pT1 and ≥pT2 UC, respectively. The similar incidence of CA2-positive UC between pTa and pT1 UC suggested the possibility that CA2-positive pTa may have the potential to invade the lamina propria. The incidence of CA2-positive UC was significantly higher in MIBC (≥pT2) compared to pTis, pTa and ≥pT1 UC, respectively. The similar incidence of CA2-positive UC between pTa and pT1 UC suggested the possibility that CA2-positive pTa may have the potential to invade the lamina propria. The findings that the incidence of CA2-positive UC was significantly higher in MIBC (≥pT2) compared to pTis, pT1 and ≥pT2 UC, respectively. The similar incidence of CA2-positive UC between pTa and pT1 UC suggested the possibility that CA2-positive pTa may have the potential to invade the lamina propria. The findings that the incidence of CA2-positive UC was significantly higher in MIBC (≥pT2) compared to pTis, pT1 and ≥pT2 UC, respectively.
Table 2. Pathological characteristics and CA2 expression in human UC

| Characteristic | Number of patients | Incidence of CA2-positive UC |
|----------------|-------------------|-----------------------------|
| Patients (mean age ± SD) | 235 (67 ± 10) | 50/235 (21.2%) |
| Gender | | |
| Male (Mean age ± SD) | 189 (67 ± 10) | 40/189 (21.2%) |
| Female (Mean age ± SD) | 46 (67 ± 13) | 10/46 (21.7%) |
| Pathological T stage | | |
| pTis | 8 | 0/8 (0) |
| pTa | 132 | 20/132 (15.2%) |
| ≥pT2 | 53 | 7/53 (13.2%) |
| NMIBC (pTis<pTa<pT1) | 193 | 27/193 (14.0%) |
| MIBC(≥pT2) | 42 | 23/42 (54.8%) |
| Tumor grade | | |
| Low grade | 104 | 8/104 (7.7%) |
| High grade | 131 | 42/131 (32.1%) |

†P < 0.01 versus pTis; ‡P < 0.0001 versus pTa and pT1; §P < 0.0001 versus NMIBC, ¶P < 0.0001 versus low grade. CA2, carbonic anhydrase 2; UC, urothelial carcinoma.

Fig. 4. Expression of carbonic anhydrase 2 (CA2) in the human urothelial carcinoma (UC). Normal epithelial cells were negative for CA2 (a). CA2 staining was localized to the cytoplasm of UC (b) HE staining; (c) CA2 staining). Analysis of the cumulative incidence of stage progression of 95 non-muscle-invasive bladder cancer (NMIBC) patients after transurethral resection of bladder tumor (TURBT) showed that CA2-positive UC had a more rapid disease progression than CA2-negative UC: progression being defined as an increase in stage of pTa to ≥pT1 or pT1 to ≥pT2, (P < 0.0001, HR = 10) (d).

Discussion

In the present study, we established a new 16-week rat invasive bladder cancer model using Hras128 rats treated with BBN followed by PEITC. Chemically-induced rat urinary bladder cancer in the existing models is nearly always non-invasive UC that closely resembles the pathologic characteristics of its human counterpart, non-invasive pTa tumors.15–18,32,33 The advantages of our model are that it mimics invasive UC that arises from non-invasive UC and the short period of time required for inducing invasive UC. Our model is useful in understanding mechanisms by which non-invasive UC progresses to invasive UC; using this model, we identified 49 proteins differentially expressed in invasive rat UC by comparing invasive and non-invasive UC. In the present study, we demonstrated that CA2 was overexpressed in invasive UC in rats, and using this result, we found that the incidence of CA2-positive UC was also significantly higher in human MIBC compared to NMIBC, and expression of CA2 is positively associated with the progression of NMIBC. These findings suggest that CA2 is an invasion-associated factor of UC.

The extracellular pH of tumor tissues is often acidic, and an acidic microenvironment is closely associated with migration and invasion of tumor cells, leading to more aggressive behavior.28,34,35 CA enzymes catalyze the chemical equilibration among CO2, HCO3⁻ and H⁺, and play an important role in maintaining pH homeostasis.25 Hypoxic conditions induce the expression of CA, which lowers the extracellular pH and increases invasion, progression and metastasis of cancer cells.26 Overexpression of CA2 has been reported in astrocytomias, oligodendrogliomas and medulloblastomas.36 However, little is known about the exact roles of CA2 in these cancers. Our finding that CA2 expression is increased in invasive UC supports a hypothesis that CA2 plays a role in making the surrounding environment acidic and promoting UC invasiveness. This hypothesis supports the proposal stated above that CA2 is an invasion-associated factor of UC and suggests that CA2 may provide new therapeutic strategies to improve the outcomes of UC patients.

The rat model of invasive UC described in this report will also be useful for evaluating potential chemopreventive and therapeutic agents in the treatment of invasive UC. A study to investigate the effects of CA2 inhibitors using this model is currently underway. In a separate ongoing study using a mouse model of BBN-induced invasive UC, a non-specific CA inhibitor significantly inhibited the development of invasive UC, while it did not have a significant effect on the development of non-invasive UC, which supports the proposals put forth in this report: CA2 is an invasion-associated factor of UC that may provide new therapeutic strategies to improve the outcomes of UC patients, and the model of invasive UC described in this report will be useful in evaluating potential chemopreventive and therapeutic drugs.

Hras128 rats carry three copies of the human c-Ha-ras proto-oncogene and exhibit high susceptibility to BBN-induced bladder carcinogenesis.20,21 Taken together with the fact that activating mutations in Hras are an early event in the development of a subset of human UC,17–20 this suggests that mutation of the Hras transgene is a primary factor in the development of invasive UC in the animal model described in this study. However, it has been demonstrated that enhanced UC development is not primarily due to mutations occurring in the transgene in Hras128 rats.21 Another factor in the susceptibility of Hras128 to UC could be relatively high expression of the human Hras transgene. A recent study using mice with an active HRAS gene showed that RTK/RAS pathway activation alone in urothelial cells causes hyperplasia, and RAS pathway activation and P53 pathway inactivation together confer invasive properties to
Comparative proteome analysis of invasive and non-invasive UC provide insight into the development and progression of UC in this model. Proteins other than CA2 differentially expressed in the invasive rat UC (Tables S3 and S4) may also be involved in development of invasive UC. Exploring the role of these proteins in UC invasion and analysis of their interactions and relationships with CA2 in the animal model described here will facilitate understanding not only of the role of CA2 but also provide insight into the mechanism of UC invasion. It also should be noted that CA2 was the only CA that was increased in the invasive rat UC compared to the non-invasive UC and normal urothelium. Considering that expression of CA9 has been associated with the invasion and progression of human UC and that CA9 and CA12 are overexpressed in human colon cancer, glioblastoma and breast cancer, further study is necessary to determine the relationship of CA2 to CA9 and CA12 in human UC.

BBN followed by PEITC treatment was the most effective regimen to induce invasive UC in the Hras128 rat in the present 16-week study. Under the conditions of this study, BBN (at the dose of 0.05% in the drinking water) showed much higher invasive activity of bladder carcinogenesis compared to PEITC (at the dose of 0.1% in the diet), as evidenced by the result that a high incidence of UC was noted in the 16-week BBN treatment group but not in the 16-week PEITC treatment group. Therefore, a possible reason that no UC were induced in the PEITC→BBN group is the lower bladder cancer initiation activity of PEITC compared to BBN and the shorter duration of the BBN treatment after PEITC treatment (8 weeks) compared to BBN treatment alone (16 weeks).

In conclusion, the findings of the present study indicate that CA2 is an invasion-associated factor and suggests that it could serve as a potential therapeutic target for bladder cancers. Hras128 rats treated with BBN followed by PEITC is a useful model of invasive bladder cancer that can be used for identifying invasion-associated proteins, and it is also applicable for evaluation of potential chemopreventive and therapeutic agents for the treatment of invasive UC.

Acknowledgments
The authors gratefully acknowledge the technical assistance of Rie Onodera, Keiko Sakata, Azusa Inagaki, Yuko Hisabayashi, and Yukiko Iura (Department of Molecular Pathology, Osaka City University Graduate School of Medicine). We are grateful to Dr. Mitsuji Fukui for his statistical consultant (Laboratory of Statistics, Osaka City University Graduate School of Medicine). This work was supported by a Grant-in-Aid for Scientific Research from Japan Society for the Promotion of Science (JSPS KAKENHI Grant Number 25462499), and Health Labour Sciences Research Grants (Research on Risk of Chemical Substances H26-chemistry-shitei-001) from Ministry of Health, Labour and Welfare, Japan.

Disclosure Statement
The authors have no conflict of interest to declare.

References
1 Ploeg M, Aben KK, Kiemeney LA. The present and future burden of urinary bladder cancer in the world. World J Urol 2009; 27: 289–93.
2 Epstein JI, Amin MB, Reuter VR, Mostofi FK. The World Health Organization/International Society of Urological Pathology consensus classification of urothelial (transitional cell) neoplasms of the urinary bladder. Bladder Con-
  sensus Conference Committee. Am J Surg Pathol 1998; 22: 1435–48.
3 Soloway MS. Overview of treatment of superficial bladder cancer. Urology 1983; 26: 18–26.
4 Lotan Y, Kamat AM, Porter MP et al. Key concerns about the current state of bladder cancer: a position paper from the bladder cancer think tank, the bladder cancer advocacy network, and the society of urologic oncology. Cancer 2009; 115: 4096–103.
5 Eble JN, Sauter G, Epstein JI, Sesterhenn IA. World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of the Urinary System and Male Genital Organs. Lyon: IARC Press, 2004.
6 Dinney CP, McConkey DJ, Millikan RE et al. Focus on bladder cancer. Cancer Cell 2004; 6: 111–6.
7 Wu XR. Urothelial tumorigenesis: a tale of divergent pathways. Nat Rev Cancer 2005; 5: 713–25.
8 Knowles MA, Harst CD. Molecular biology of bladder cancer: new insights into pathogenesis and clinical diversity. Nat Rev Cancer 2015; 15: 25–41.
9 Vaidya A, Soloway MS, Hawke C, Tigges R, Civantos F. De novo muscular bladder cancer: is there a change in trend? J Urol 2001; 165: 47–50.
10 Hidas G, Pode D, Shapiro A et al. The natural history of secondary muscle-invasive bladder cancer. BMC Urol 2013; 13: 23.
11 Choi W, Porten S, Kim S et al. Identification of distinct basal and luminal subtypes of muscle-invasive bladder cancer with different sensitivities to frontline chemotherapy. Cancer Cell 2014; 25: 152–65.
12 Althausen AF, Prout GR Jr, Daly JJ. Non-invasive papillary carcinoma of the bladder associated with carcinoma in situ. J Urol 1976; 116: 575–80.
13 Holmang S, Helelin H, Anderstrom C, Johansson SL. The relationship among multiple recurrences, progression and prognosis of patients with stages Ta and T1 transitional cell cancer of the bladder followed for at least 20 years. J Urol 1995; 153: 1823–6; discussion 6–7.
14 Millan-Rodriguez F, Chechile-Toniolo G, Salvador-Bayarri J, Palou J, Vicente-Rodriguez J. Multivariate analysis of the prognostic factors of primary superficial bladder cancer. J Urol 2000; 163: 73–8.
28 Damaghi M, Wojtkowiak JW, Gillies RJ. pH sensing and regulation in cancer. *Frontiers Physiol* 2013; 4: 370.
29 Yao R, Lopez-Beltran A, Macleanman GT, Montironi R, Eble JN, Cheng L. Expression of S100 protein family members in the pathogenesis of bladder tumors. *Anticancer Res* 2007; 27: 3051–8.
30 Dokun OY, Flori AR, Seifert HH, Wolff I, Schulz WA. Relationship of SNCG, S100A4, S100A9 and LCN2 gene expression and DNA methylation in bladder cancer. *Int J Cancer* 2008; 123: 2798–807.
31 Kim WT, Kim J, Yan C et al. S100A9 and EGFR gene signatures predict disease progression in muscle invasive bladder cancer patients after chemotherapy. *Annals Oncol* 2014; 25: 974–9.
32 Shibata MA, Hasegawa R, Shirai T, Takesada Y, Fukushima S. Chemoprevention by indomethacin of tumor promotion in a rat urinary bladder carcinogenesis model. *Int J Cancer* 1993; 55: 1011–7.
33 Grubbs CJ, Labet RA, Koki AT et al. Celecoxib inhibits N-butyl-N-(4-hydroxybutyl)-nitrosamine-induced urinary bladder cancers in male B6D2F1 mice and female Fischer-344 rats. *Cancer Res* 2000; 60: 5599–602.
34 Gatenby RA, Gawlinski ET, Gmitro AF, Kaylor B, Gillies RJ. Acid-mediated tumor invasion: a multidisciplinary study. *Cancer Res* 2006; 66: 5216–23.
35 Kato Y, Ozawa S, Miyamoto C et al. Acidic extracellular microenvironment and cancer. *Cancer Cell Int* 2013; 13: 89.
36 Nordfors K, Haapasalo J, Korja M et al. The tumour-associated carbonic anhydrases CA II, CA IX and CA XII in a group of medulloblastomas and supratentorial primitive neuroectodermal tumours: an association of CA IX with poor prognosis. *BMC Cancer* 2010; 10: 148.
37 Fujita J, Yoshida O, Yuasa Y, Rhim JS, Hatanaka M, Aaronson SA. Ha-ras oncogenes are activated by somatic alterations in human urinary tract tumours. *Nature* 1984; 309: 464–6.
38 Visvanathan KV, Pocock RD, Summerhayes IC. Preferential and novel activation of H-ras in human bladder carcinomas. *Oncogene Res* 1988; 3: 77–86.
39 Senger DR, Perruzzi CA, Ali IU. T24 human bladder carcinoma cells with activated Ha-ras protooncogene: nontumorigenic cells susceptible to malignant transformation with carcinogen. *Proc Natl Acad Sci USA* 1988; 85: 5107–11.
40 Jebar AH, Hurst CD, Tomlinson DC, Johnston C, Taylor CF, Knowles MA. FGFR3 and Ras gene mutations are mutually exclusive genetic events in urothelial cell carcinoma. *Oncogene* 2005; 24: 5218–25.
41 He F, Melamed J, Tang MS, Huang C, Wu XR. Oncogenic HRAS activates epithelial-to-mesenchymal transition and confers stemness to p53-deficient urothelial cells to drive muscle invasion of basal subtype carcinomas. *Cancer Res* 2015; 75: 2017–28.
42 Tafreshi NK, Bui MM, Bishop K et al. Noninvasive detection of breast cancer lymph node metastasis using carbonic anhydrases IX and XII targeted imaging probes. *Clin Cancer Res* 2012; 18: 207–19.

**Supporting Information**

Additional Supporting Information may be found online in the supporting information tab for this article:

**Fig. S1.** An image of representative normal urothelial tissue (↑) separated from urinary bladder of a control Hras128 rat (HE staining).

**Table S1.** Proteins overexpressed in both non-invasive and invasive urothelial carcinoma (UC) compared to normal urothelium in Hras128 rats.

**Table S2.** Proteins underexpressed in both Non-invasive and invasive urothelial carcinoma (UC) compared to normal urothelium in Hras128 rats.

**Table S3.** Proteins overexpressed in the invasive urothelial carcinoma (UC) but not in the non-invasive UC compared to the normal urothelium in Hras128 rats.

**Table S4.** Proteins underexpressed in the invasive urothelial carcinoma (UC) but not in the non-invasive UC compared to the normal urothelium in Hras128 rats.

**Table S5.** Proteins overexpressed in the noninvasive urothelial carcinoma (UC) but not in the invasive UC compared to the normal urothelium in Hras128 rats.

**Table S6.** Proteins underexpressed in the noninvasive urothelial carcinoma (UC) but not in the invasive UC compared to the normal urothelium in Hras128 rats.