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The Dual Function of Aryl Hydrocarbon Receptor in Bladder Carcinogenesis

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Abstract. *Background/Aim: Although Aryl hydrocarbon receptor (AhR) may be critical to several types of cancers, the function of AhR for carcinogenesis of bladder cancer (BC) is still inconclusive. We, therefore, sought to examine the involvement of AhR in bladder carcinogenesis. Materials and Methods: We examined the AhR expression of human BC and N-butyl-N-(4-hydroxybutyl)- induced bladder carcinogenesis in AhR-deficient mice. Results: There was a significantly higher expression of AhR in non-muscle-invasive BC compared to normal tissue and muscle-invasive BC (MIBC). The incidence of MIBC in AhR-deficient mice (87.5%) was significantly higher than wild-type mice (9.5%, p<0.01). In cell invasion assay, the induction of AhR signaling resulted in attenuation of BC cell invasiveness and proliferation. Conclusion: These results suggest that AhR may be essential for the initiation of carcinogenesis and attenuated the invasion of BC cells; this signaling may have a dual function in bladder carcinogenesis.*

Aryl hydrocarbon receptor (AhR) is known as a dioxin receptor and a ligand-activated transcription factor. There have been many reports on AhR playing a central role in cell cycle and DNA damage (1, 2). AhR is activated by binding with a ligand such as various environmental contaminants including 3-methylcholanthrene (3-MC) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (3). AhR is translocated to the nucleus where it dimerizes with AhR nuclear translocator (ARNT). Within the nucleus, the AhR/ARNT heterodimer acts as a fully qualified transcription factor capable of binding to xenobiotic response element (XRE), stimulates transcription of xenobiotic target genes, including cytochrome *P450* CYP1A1 (4) and CYP1B1. Animal and human data support the relationship between abnormal AhR function and cancer. Iwanari *et al*. (5). showed that AhR mRNAs were detected in all cell lines with similar expression levels. However, depending on the type of cancer, the potential roles of AhR for carcinogenesis is controversial. The AhR mechanism for carcinogenesis has not been yet established. In several cancers including glioblastoma (6), pancreatic cancer (7), lung cancer (8), and liver cancer (9), several lines of evidence suggest that the AhR plays a critical role in cancer progression. On the other hand, many other AhR-related studies indicated that AhR might act as a tumor suppressor for breast cancer (10), intestinal cancer (11), liver cancer (12), lung cancer (13), and pituitary adenoma (14). Brantley *et al*. indicated that AhR signaling impedes mammosphere formation (15).

AhR is important in regulating environmental exposure to xenobiotics, including benzopyrenes, a major polycyclic aromatic hydrocarbon present in cigarette smoke. Cigarette smoking is one of the major risk factors for human bladder cancer (BC) (16). Therefore, it is easy to assume that AhR is closely related to human bladder carcinogenesis. However, the mechanism has yet to be established.

Although many molecular markers associated with BC prognosis have been investigated (17), there is no potent biomarker for early detection of bladder carcinogenesis (18). In this study, we found AhR highly expressed in non-muscle-invasive BC (NMIBC) compared to muscle-invasive BC (MIBC). In addition, in an N-butyl-N-(4-hydroxybutyl) (BBN)-induced BC mouse model, AhR-knockout mice ($AhR^{-/-}$) on a C57BL/6J background developed MIBC with much greater frequency than AhR wild-type mice ($AhR^{+/+}$). This indicates that AhR is important for the initiation of carcinogenesis, but its activation may selectively inhibit processes required for tumor progression. The AhR signaling pathway may be a worthwhile target for therapeutic intervention in BC.

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Key Words: Bladder cancer, aryl hydrocarbon receptor, carcinogenesis.

Materials and Methods

All experiments were conducted in accordance with institutional guidelines and approved by the Ethics Committee (Approval No.: 09-026). Studies involving experiments with animals must state that their care was in accordance with institution guidelines. The informed consent was obtained for experimentation with human subjects. All animal experiments carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

RT-PCR (reverse transcriptase-polymerase chain reaction). Total RNA of RT4 and T24 cell lines were extracted with RNA-Bee isolation reagent (Tel-TEST, Inc., Friendswood, TX, USA) according to the manufacturer's instructions. RT4 is derived from NMIBC and T24 is derived from MIBC. The sequences of primers for human AhR were forward 5' GTAAGTCTCCCTTCATACC 3' and reverse 5' AGGCACGAATTGGTTAAGAG 3'. RT-PCR reaction was carried out in a 20-μl reaction mixture [One Step RNA PCR Kit (AMV), TaKaRa, Shiga, Japan] at 50˚C for 30 min and 95˚C for 2 min, followed by 35-cycle amplification by denaturing at 95˚C for 30 s, annealing at 58˚C for 30 s, and extending at 72˚C for 1 min 30 s. The RT-PCR products were electrophoresed in 1.5% agarose (TaKaRa) and visualized by staining with ethidium bromide.

Human BC specimen. A total of 31 BC samples were used in this study. Bladder tissues were pathologically classified as normal, NMIBC (pTa+pT1) and MIBC (≥pT2). These samples were used for experiments for western blot and immunohistochemistry staining (NMIBC=19, MIBC=12).

Western blot analysis. Proteins of cell lines and tissue specimens were homogenized and lysed in RIPA buffer on ice for 10 min. The cell lysates were centrifuged at 13,000 rpm for 10 min at 4˚C, and the supernatant was collected. Protein concentrations were measured by a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Inc., Lake Charles, LA, USA). Proteins were resolved by SDS-PAGE (5-20%, SuperSep Ace, Wako, Richmond, VA, USA) and transferred to a PVDF membrane (Millipore, Burlington, MA, USA). The membrane was blocked in 1% bovine serum albumin (BSA) in Tris-buffered saline (TBS), for 1 h at room temperature and then blotted overnight with AhR (SantaCruz Biotech, Santa Cruz, CA, USA; 1:1,000 dilution) rabbit polyclonal antibody, phospho-ataxia-telangiectasia mutated (p-ATM: Abcam, Burlingame, CA, USA; 1:1,000 dilution) rabbit polyclonal antibody, poly (adp-ribose) polymerase (PARP, Cell Signaling Technology, Inc., Danvers, MA, USA; 1:1,000 dilution) rabbit polyclonal antibody, at 4˚C. β-actin antibody (Sigma-Aldrich Co., St Louis, MO, USA; 1:10,000 dilution) was used as an internal loading control. Blots were washed three times (15 min each) with Tris-buffered saline containing 0.1% Tween-20 (TBST). The blots were then incubated with HRP-conjugated secondary antibody (Bio-Rad, Philadelphia, PA, USA) in 3,000 dilution for 1 h at room temperature and washed three times (15 min each) with TBST. Bands were visualized with ECL Western Blotting Detection Reagents (GE Healthcare, Marlborough, MA, USA) and LAS-3000 mini detection system (Fujifilm, Lemoyne, PA, USA).

To quantify AhR expression, the electrophoresis bands were compared on densitometric scans using the ImageJ software (NIH, Bethesda, MD, USA).

Immunohistochemistry. Immunostaining was carried out according to standard procedures. In brief, 4-μm paraffin sections were deparaffinized and hydrated, 3% H_2O_2 was used to remove endogenous peroxidase, and antigen retrieval was performed by boiling in 0.01 M citrated buffer and blocked with normal serum. Sections were incubated overnight with primary antibody to human AhR (SantaCruz, 1:100 dilution) or mouse AhR (SantaCruz, 1:100 dilution) at 4˚C, then incubated with second antibody (EnVision+ System Labelled Polymer-HRP, DakoCytomation, Glostrup, Denmark) for 30 min at room temperature. The enzyme reaction was developed with liquid chromogen-DAB (EnVision+ Kit/HRP, Dako, Santa Clara, CA, USA). Mayer's hematoxylin (Muto Pure Chemicals CO., Tokyo, Japan) was used as nuclear counterstain. HE staining was carried out according to standard protocol.

To quantify AhR expression, we used semiquantitative analysis. All epithelial cells (including cancer cells) observable at this magnification level were grouped into four classes depending on their staining intensity: 0 (negative)=not stained; 1 (weakly positive)=stained less strongly than the internal control; 2 (moderately positive)=stained as strongly as the internal control; and 3 (strongly positive)=stained more strongly than the internal control (19). The total cell number and the cell numbers in each class were counted in order to calculate composite scores as follows: composite score=(percentage of cells at intensity 0×0) + (percentage of cells at intensity 1×1) + (percentage of cells at intensity 2×2) + (percentage of cells at intensity 3×3). Thus, composite scores could range from 0 to 300. For example, 300 would indicate that all cancer cells were strongly positive. The internal control consisted of fibroblast cells and vascular endothelial cells.

Animals. All manipulations of mice were performed in accordance with the Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan, June 1st, 2006). C57BL/6 mice and heterozygous type $AhR^{tm1\ bra/+}$ were purchased from Jackson Laboratory (20). AhR-deficient type $AhR^{tm1\ bra/tm1\ bra}$ ($AhR^{-/-}$) mice were generated by mating females (C57BL/6 background) with non-transgenic C57BL/6 males. The $AhR^{-/-}$ mouse strain carries a targeted deletion of exon 2 of the AhR gene which was replaced by a neomycin selection cassette and has been backcrossed for 12 generations onto C57BL/6 (21). DNA extracted from tail samples were used for genotyping and the primers were as follows: AhR [forward 5' GGATTTGACTTAATTCCTTCAGCGG 3' and reverse 5' TCTTGGGCTCGATCTTGTGTCAGGAACAGG 3'] and neomycin cassette [forward 5' CTGAATGAACTGCAGGACGA 3' and reverse 5' ATACTTTCTCGGCAGGAGCA 3']. $AhR^{-/-}$ and $AhR^{+/+}$ littermates were used for the experiments.

All of them were housed in clear plastic cages, maintained on standard rodent chow diet and tap water at 23±1˚C with a 12/12-h light-dark cycle. The mice always had free access to food and tap water. When the mice reached 5 weeks old, they were separated to one mouse per cage; 0.05% BBN water in bottles was administered to the mice instead of tap water to induce BC. After administration of 0.05% BBN water for 15 weeks, the mice were sacrificed, and the bladders taken out and weighed. Each bladder was in 4% formaldehyde-fixation, gradient ethanol dehydration, xylene, embedded in paraffin, for immunohistochemistry.

Cell culture. Human BC cell lines, RT4 and T24, were obtained from ATCC, cultured in 10-cm diameter dishes with McCoy's 5A Medium (Invitrogen, Camarillo, CA, USA) containing 10% (v/v) fetal bovine

serum (FBS, Invitrogen), and 1% penicillin-streptomycin (Invitrogen) at 37˚C under 5% CO_2, passaged with TrypLE Select (Invitrogen) every three days to maintain cell monolayer. For transfection with siRNAs, RT4 or T24 cells were seeded in six-well plates and placed in OPTI-MEM I medium, transfected with 60 nmol/l AhR small interfering RNA (Dharmacon, Lafayette, CO, USA) or 60 nmol/l control siRNA (SantaCruz) for 24 h, siRNA transfection using Lipofectamine RNAiMAX reagent (Invitrogen). Expression plasmids for constitutively active AhR were previously described (22).

Cell proliferation assay. RT4 and T24 cells were transfected with control siRNA, AhR-siRNA, control plasmid (pGS5, Promega, Durham, NC, USA), constant-activated AhR plasmid (pAhR-C, kindly provided by Professor Kato, Tokyo University), for 24 h. Proliferation of T24 and RT4 cells were measured by MTS assay (CellTiter 96 AQueous One, Promega) at 0, 24, 48 and 72 h, respectively. Responses to all treatments were assayed in quintuplicate, and results were expressed as the means of three separate experiments.

Matrigel invasion assay. Invasion assay was carried out with BD BioCoat Matrigel Invasion Chamber (24-well BD Falcon TC Companion Plate with Falcon Cell Culture Inserts, BD Biosciences, San Jose, CA, USA), containing an 8-μm pore size PET membrane with a thin layer of MATRIGEL Basement Membrane Matrix. The lower compartment of the chamber was filled with 5% FBS-containing medium. The upper compartment (insert) was filled with 1×10^5/ml RT4 cells (0.5% FBS medium) transfected with control siRNA or AhR-siRNA, or filled with 5×10^4/ml T24 cells (0.5% FBS medium) transfected with pSG5 or pAhR-C. After 22 h of incubation at 37˚C, the inserts were washed with PBS, the filters removed with cotton tipped swab, then the membranes were fixed by adding 100% methanol (Wako) for 2 min and stained with 0.4% Trypan Blue (Invitrogen) for 2 min, washed with distilled water (MilliQ) two times, and cut and placed on a glass slide. Cells that migrated through the Matrigel and the filter pores to the lower surface were counted in five random low-power fields per insert using a light microscope. Each experimental point was analyzed in triplicate.

Transfection. For siRNA transfection, 3 μl of AhR-siRNA (10 μM) or control siRNA (10 μM) and 9 μl of RNAiMax transfection reagent (Invitrogen) were diluted in 100 μl of OptiMEM® I Reduced Serum medium, respectively. After these were mixed together, and underwent a 5-min incubation, the mixture was added to RT4 seeded onto a 6-well plate at 1×10^5/ml the day before the transfection. For AhR constitutive expression plasmid transfection, 2 μg DNA was mixed with 6 μl of FuGENE® HD transfection reagent (Promega), and after 10 min of incubation, the mixture was added to T24 cells seeded at 5×10^4/ml the day before the transfection. The cells were incubated for 48 h.

Luciferase reporter gene assay. For co-transfection of siRNA and DNA to RT4 cells, 0.5 pmol of AhR or control siRNA, 50 ng of pGL4.43 (luc2p/XRE) plasmid DNA (Promega), and 0.3 μl lipofectamin® 2000 (Invitrogen) for each well were used to transfect cells seeded in 96-well plate. For co-transfection of AhR expression plasmid or control vector and pGL4.43 (luc2p/XRE) plasmid DNA to T24 cells, 0.1 μg DNA and 0.3 μl FuGENE HD transfection reagent per well were used.

ApoLive-Glo® Multiplex assay for viability and caspase activation. Culture cells in 96-well plate at a density of 5×10^4 cells/ml were incubated overnight at 37˚C. Cells were incubated with H_2O_2 at a final concentration of 0, 100 and 250 μM for 6 h. As per manufacture instructions, following incubation, 20 μl of viability reagent were added to each well and incubated for 30 mins at 37˚C. Fluorescence was measured at the wavelength of 400EX/505EM for viability using fluorescence spectrophotometer F-7000 (Hitachi, Tokyo, Japan). 100 μl of Caspase-Glo 3/7 reagent was added to each well and incubated for 1 hour at room temperature. Luminescence was measured for apoptosis using a luminescent detector AB-2350 (Atto, New York City, NY, USA).

Statistical analysis. Data were expressed as mean±SD. For analysis, we used 2-tailed Student's *t*-test (SPSS), and $p<0.05$ was considered significant.

Results

We first analyzed the expression of AhR in RT4 and T24 cells by RT-PCR (Figure 1A) and western blotting (Figure 1B). Hela cells were used as positive control (23). RT4 cells which are NMIBC cell line showed relatively high AhR mRNA (Figure 1A) and AhR protein (Figure 1B) expression compared with T24 cells which are MIBC cell line.

We examined the expression of AhR in human bladder specimens by western blot (Figure 2). There was a significantly higher expression of AhR in NMIBC compared with normal epithelium ($p=0.0012$, Figure 2A and B). However, in MIBC, there were no significant differences in the expression of AhR between cancerous tissue and normal epithelium ($p=0.2454$, Figure 2A and 2B). There was a significant difference ($p=0.0097$) in AhR band density in cancerous tissue of NMIBC compared with MIBC (Figure 2B).

We also examined the expression of AhR in human bladder tissue specimens by immunohistochemistry with rabbit anti-human AhR antiserum. There was a higher expression of AhR (reactivity shown as brown color) in NMIBC than in normal epithelium in patients with NMIBC (Figure 3A and B). We counted a total of 5,834 cells (normal epithelium: 974, NMIBC: 2,970, MIBC: 1890) and checked the expression in cytoplasm. However, we found the expression of AhR in MIBC and normal epithelium in patients with MIBC to be similar ($p=0.8571$). As with western blotting, the expression of AhR in NMIBC was significantly higher than MIBC in immunohistochemistry ($p=0.0082$).

BC was induced in 5-week-old wild-type and AhR-deficient type mice by feeding 0.05 % BBN water continually for 15 weeks. After scarification, bladders were collected and weighed. Bladder weight for $AhR^{+/+}$ group (n=23, 0.0453±0.0336 g) was significantly lighter than $AhR^{-/-}$ group (n=8, 0.0644±0.0138 g) ($p=0.0258$, Figure 4A). Hematoxylin-eosin staining was used to analyze bladder carcinogenesis (data not show). In comparison of invasive

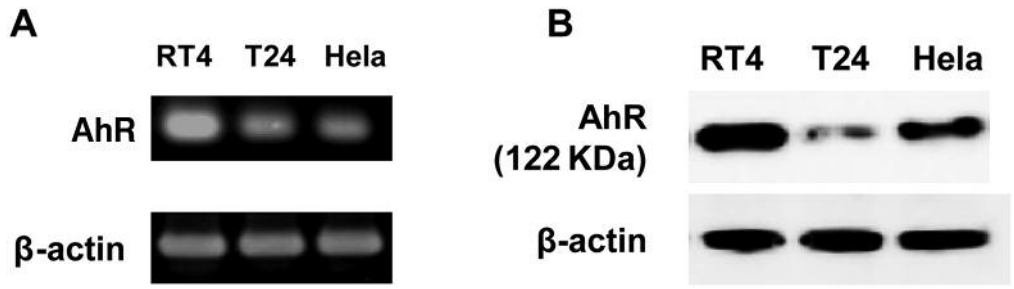

Figure 1. *A. RT-PCR analysis of AhR mRNA levels in BC cell lines. B. Western blot analysis of AhR protein expression in BC cell lines. Hela cells were used as positive control.*

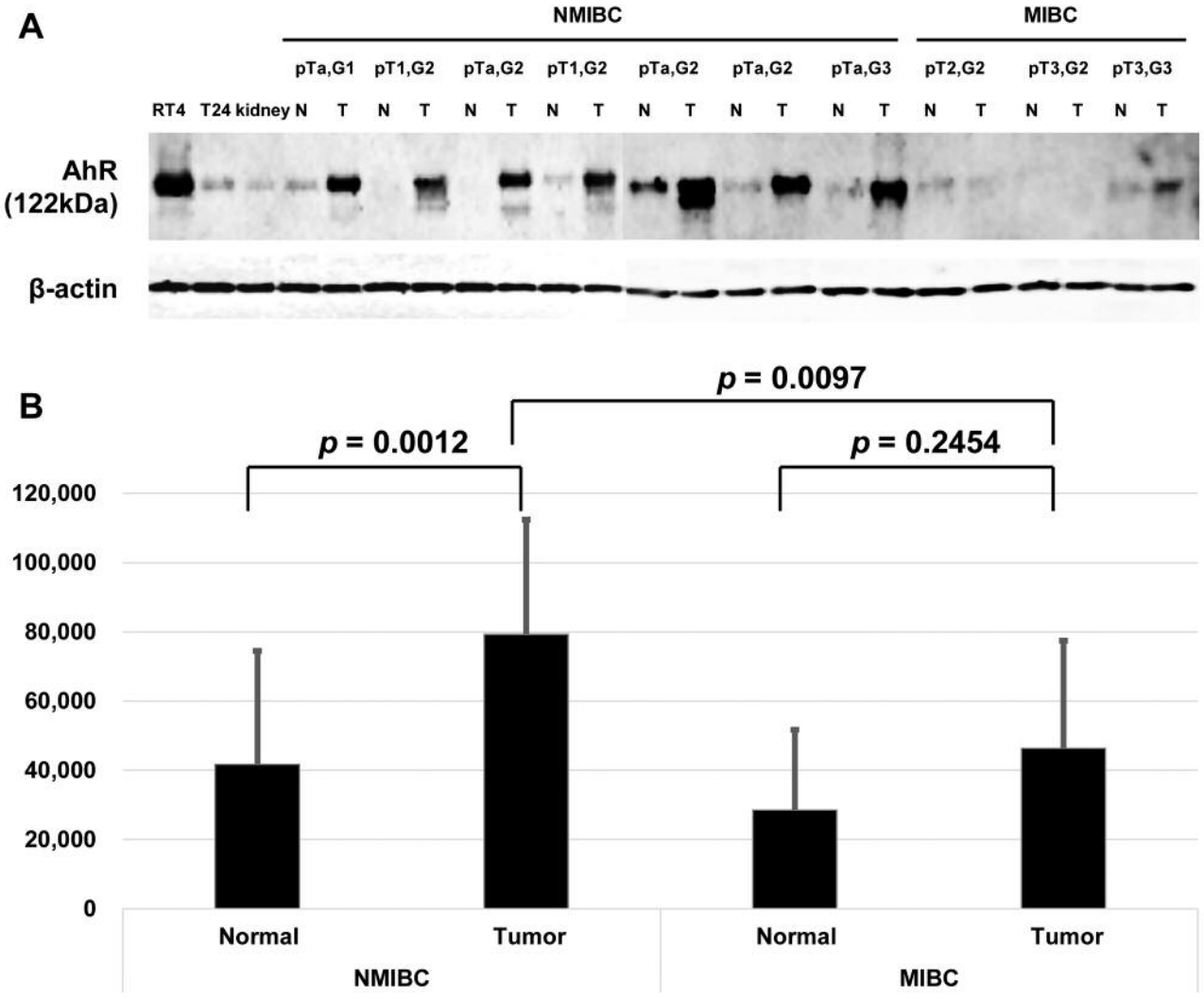

Figure 2. *A. AhR protein expression in BC tissue of clinical patients by western blot analysis. B. All quantitation of AhR band density were normalized to β-actin. The expression of AhR in NMIBC was significantly higher than normal epithelium (p=0.0012). However, there were no significant differences in the expression of AhR between MIBC and normal epithelium (p=0.2454). There was a significantly higher expression of AhR (p=0.0097) for NMIBC (pathologic stage of less than pT1) compared with MIBC (pathologic stage of more than pT2). N: Normal epithelium, T: tumor.*

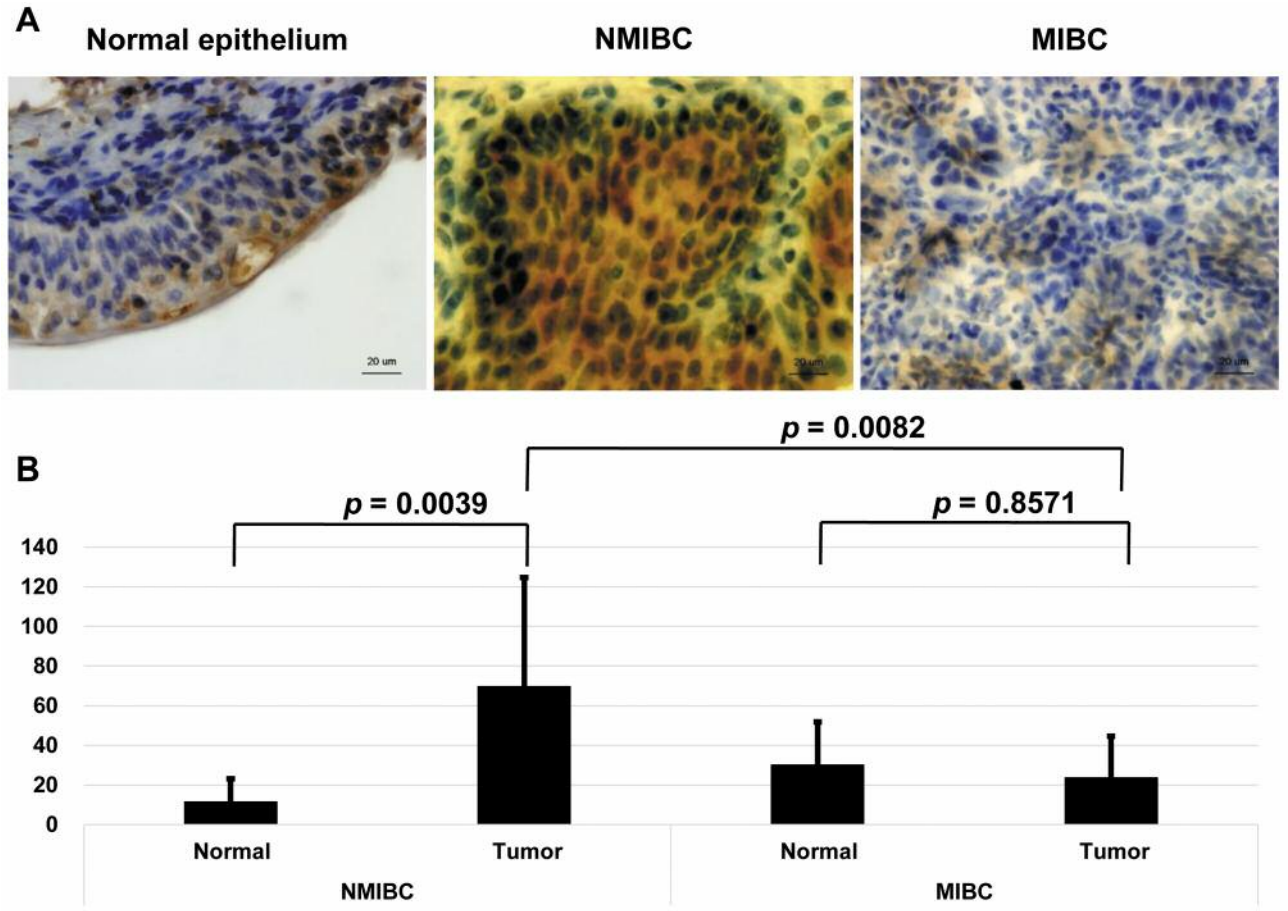

Figure 3. *A. Immunohistochemistry shows AhR proteins were expressed in human BC tissue. B. As is the case with western blot analysis, there were statistical significance differences in AhR expression for NMIBC (n=12) compared with normal epithelium (n=9, p=0.0039) or MIBC (n=8, p=0.0082), respectively. However, there were no significant differences in AhR expression between MIBC and normal epithelium (p=0.8571).*

BC alone, $AhR^{-/-}$ was significantly higher (87.5%) than $AhR^{+/+}$ (9.5 %) ($p<0.0001$, Figure 4B). CYP1A1 expression of bladder before BBN administration in $AhR^{-/-}$ was lower than $AhR^{+/+}$ (Figure 4C).

We investigated the role of AhR in T24 and RT4 cell proliferation with a 96-well-based MTS assay by modulating AhR signaling. Specifically, we either up-regulated or down-regulated AhR expression by transfecting AhR expression vectors or siRNA duplex targeting AhR, respectively. The experiments were carried out with 3 different batches of transfected cells. Although the expression of AhR was increased by transfection with pAhR-C (constitutively activated AhR) in T24 cells, the proliferation of the cells was unchanged, compared with pSG5 (control vector)-transfected cells (Figure 5A). Down-regulation of AhR expression in RT4 cells using AhR-siRNA did not alter the growth rate of RT4 cells (Figure 5B).

We used an AhR-responsive luciferase reporter (XRE reporter vector pGL4.43 [luc2p/XRE]) to confirm the direct involvement of AhR activation. T24 or RT4 cells transfected with AhR-constitutive expression plasmid or transfected with AhR-siRNA were incubated with 10 μM 3-MC for six hours (Figure 6). 3-MC is known to trigger the induction of CYP1A1 proteins *via* intracellular AhR activation (24). As shown in Figure 6A, whereas in the control vector AhR activation was only increased 1.5 times by 3-MC, AhR expression induced by 3-MC in T24 cells transfected with pAhR-C were significantly increased compared with control (5.91-fold). RT4 cell transfected with AhR-siRNA or control siRNA showed non-significant 2.50-fold and 1.58-fold increments compared with control, respectively (Figure 6B). These results, therefore, confirmed that pAhR-C and AhR-siRNA contributed to the expression of AhR.

We performed Matrigel invasion chamber assays to determine whether AhR is involved in promoting BC cell invasiveness three times. T24 and RT4 cells transfected with control siRNA or AhR-siRNA, and pSG5 or pAhR-C were placed in insert wells containing 0.5% FBS, with the lower

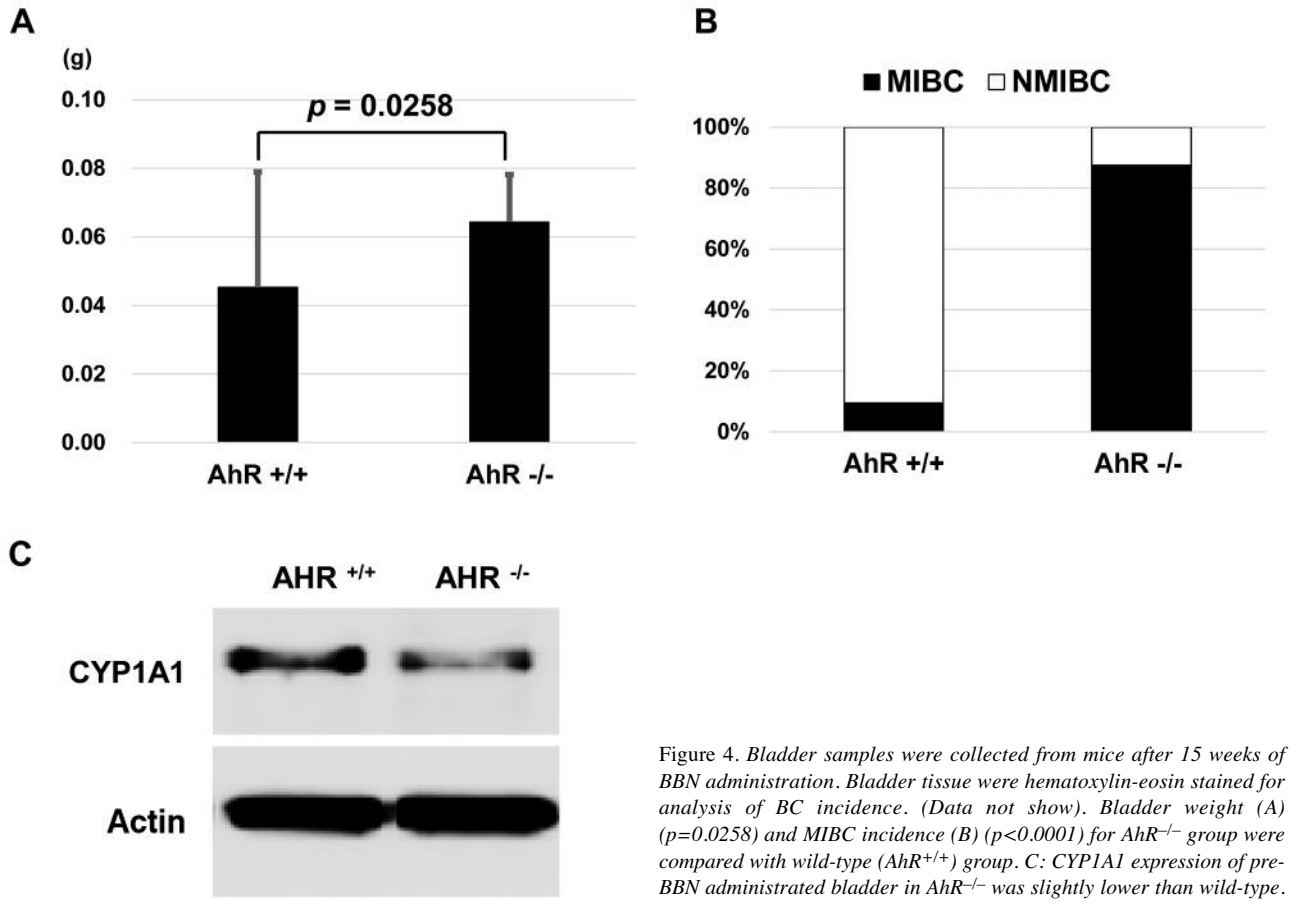

Figure 4. *Bladder samples were collected from mice after 15 weeks of BBN administration. Bladder tissue were hematoxylin-eosin stained for analysis of BC incidence. (Data not show). Bladder weight (A) (p=0.0258) and MIBC incidence (B) (p<0.0001) for AhR$^{-/-}$ group were compared with wild-type (AhR$^{+/+}$) group. C: CYP1A1 expression of pre-BBN administrated bladder in AhR$^{-/-}$ was slightly lower than wild-type.*

chamber filled with 5% FBS medium. After 22 h, cells that migrated through the Matrigel basement membrane matrix and the filter pores to the lower surface of the membrane were counted by light microscopy. The augmentation of AhR signaling, transfected with constitutive activation AhR, attenuated the invasiveness in T24 cells (Figure 7A), and the invasive ability was elevated in RT4 cells treated with AhR-siRNA compared with control siRNA (Figure 7B). These results indicated that AhR signaling is essential in attenuating the invasive potential of BC cells. However, the effect of activating or inactivating AhR signaling varies depending on the BC type, NMIBC or MIBC.

The activation of the DNA damage response pathway and PARP cleavage was increased by transfection with AhR-expression plasmid or decreased by transfection with AhR-siRNA. When T24 was transfected with control plasmid, the expression of AhR decreased depend on H_2O_2 dose. In addition, in spite of the transfection of AhR-constitutive expression plasmid, the expression of AhR also decreased depend on H_2O_2 dose (Figure 8A). Densitometric analysis showed higher levels of pATM (3.3-fold) and PARP cleavage (4.3-fold) in T24 cells transfected with AhR constitutive expression plasmid compared with control plasmid pSG5 (Figure 8A). In RT4 cells transfected with AhR-siRNA, the level of pATM decreased 37% and PARP cleavage decreased 24% compared with control siRNA (Figure 8B). Therefore, we investigated the relationship between AhR and DNA damage response pathway regulated apoptosis. Caspase-3/7 activity was consistent with apoptosis and ApoLive-Glo Multiplex Assay is a quantifiable measurement for apoptosis. Cells treated with H_2O_2 resulted in a dose-dependent decrease in viability (Figure 9 and Figure 10). Caspase-3/7 activity was normalized by viability. H_2O_2 treatment resulted in an increase in the ratio of caspase-3/7 activity (RLU) to viable cells (RFU). T24 cells transfected with pAhR-C treated with 100 μM and 250 μM H_2O_2 showed 1.6-fold and 2.8-fold increments in caspase-3/7 activity, while the cells transfected with control plasmid increased 1.3-fold and 1.7-fold, compared with control, respectively (Figure 9C). RT4 cell transfected with AhR-siRNA or control siRNA treated with various concentration of H_2O_2 showed similar increments of caspase-3/7 activity (Figure 10C).

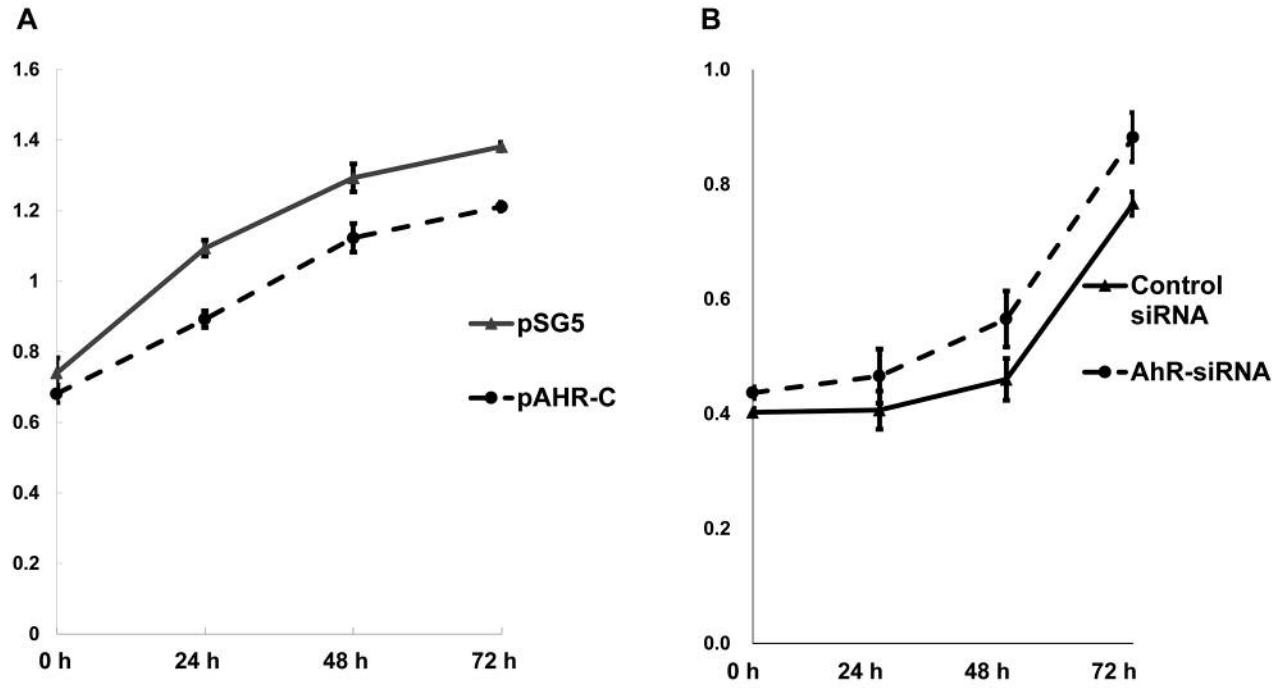

Figure 5. *Effects of AhR signaling on the growth of T24 (A) and RT4 (B) cells. After transfected with pSG5 and pAhR-C, or transfected with control siRNA and AhR-siRNA, cell viability was examined by MTS assay. Each point represents mean±SD for six wells during the 3-day culturing. There was no statistical significance between pSG5 and pAhR-C. pSG5: Control vector; pAhR-C: expression vector of constitutively active AhR.*

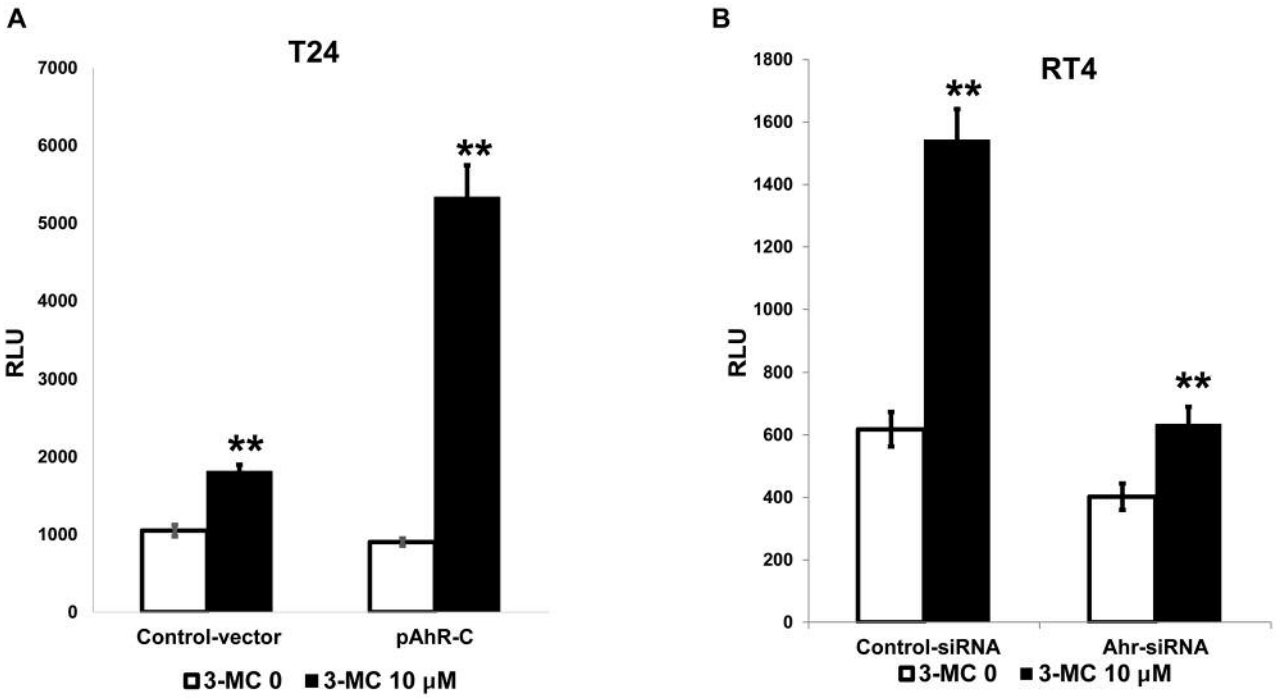

Figure 6. *AhR-responsive luciferase reporter (XRE reporter vector pGL4.43 [luc2p/XRE]) was transfected to T24 and RT4 cells, AhR activation was induced by 3-MC. A. T24 cells transfected with pAhR-C were significantly increased compared with control (**p<0.01). B. RT4 cell transfected with AhR-siRNA or control siRNA showed 1.58-fold and 2.5-fold increments, respectively.*

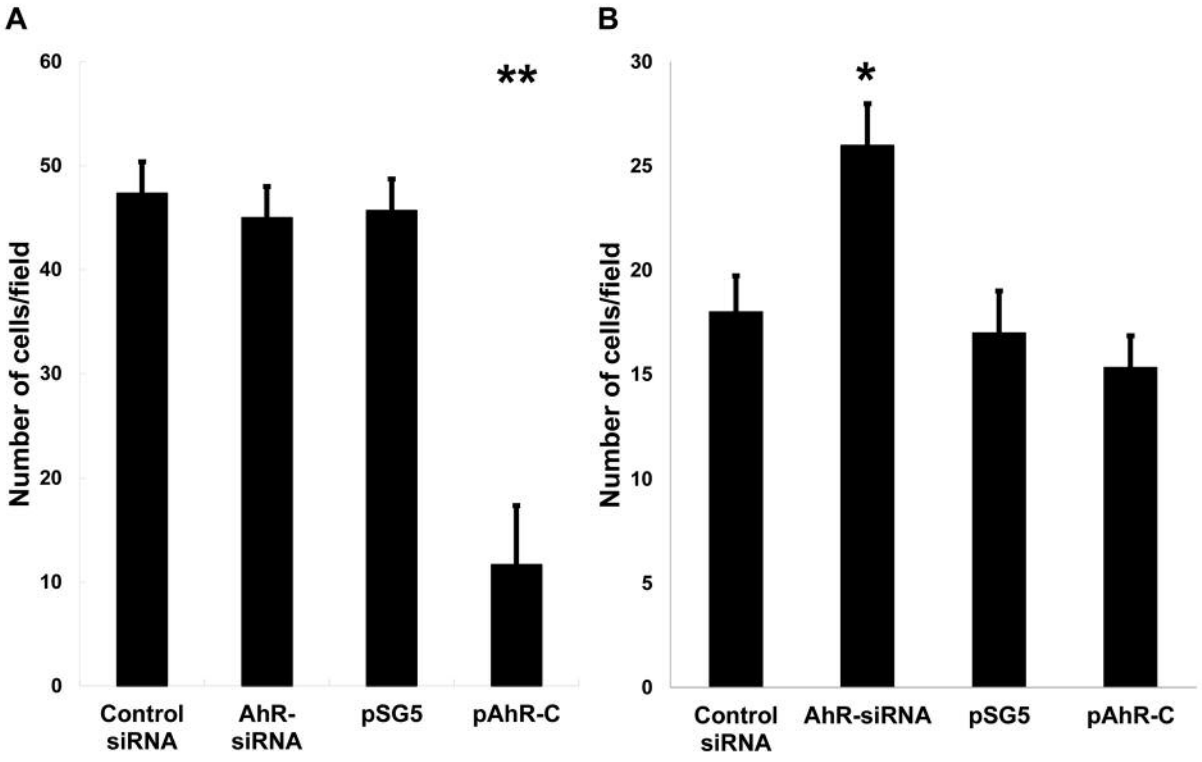

Figure 7. *Modulation of AhR signaling attenuates or stimulates the invasiveness of T24 and RT4 cells. A. The invasiveness of T24 cells was suppressed by transfection of pAhR-C compared to cells transfected with control vector. AhR-siRNA did not affect the invasiveness. B. Suppression of AhR expression with its siRNA stimulates the invasiveness of RT4 cells compared with cells transfected with control siRNA. Experiments were performed in triplicate. Statistical significance was obtained for pAhR-C transfected T24 cell (**p<0.01) or AhR-siRNA treated RT4 cell (*p<0.05) as compared with control, respectively.*

Discussion

To date, the mechanism of AhR carcinogenesis has been controversial. Our study showed that in the RT4 cell line (NMIBC cell line), the expression of AhR is strong, whereas in the T24 cell line (MIBC cell line) it is much weaker (Figure 1). In addition, although there was no difference in the total incidence of BBN - induced BC, the incidence of MIBC in $AhR^{-/-}$ mice was significantly higher than in wild-type mice (Figure 4B). CYP1A1 expression is mediated through AhR and have been identified as potential risk factors for human BC (25). However, Baker *et al*. reported that the expression of CYP1A1 differs considerably among patients even for normal urothelial cells. And they showed that CYP-function is related to differentiation status in BC (26). In mice experiments, our data showed that $AhR^{-/-}$ mice had a slightly decreased expression of CYP1A1 compared to $AhR^{+/+}$ mice (Figure 4c). In the incidence of MIBC produced by BBN, however, $AhR^{-/-}$ was significantly higher than $AhR^{+/+}$ (Figure 4B). As a consequence, we can consider that the AhR mechanism for muscle-invasive bladder carcinogenesis is not involved in the expression of CYP1A1 but may be caused by another CYP-function. As well, for example, there is strong evidence that sustained AhR activation is the molecular initiating event for rodent liver cancer promotion and the ensuing liver cancers. However, it is not known whether CYP induction and the following metabolic changes are required for this outcome (27). Although the cross-talk of AhR with different signal transduction pathways is apparent, the precise mechanisms by which AhR ligands elicit toxic responses that may contribute to carcinogenesis still remain unclear (28).

Although the interference of AhR signaling does not inhibit proliferation of BC cells (Figure 5), the constitutive activation of AhR attenuated the invasiveness in T24 cells derived from MIBC, and AhR-siRNA amplify the invasiveness in RT4 cells derived from NMIBC (Figure 7). Many chemical toxins can lead to cytotoxicity. So, it seems reasonable to suggest that treatment with AhR ligands may lead to negative control of cell proliferation. However, it is worth noting that increasing evidence suggests that AhR may also promote cell proliferation (29). In addition, as far as we are aware of, there are no previous reports on the involvement of AhR in the invasiveness of BC. In contrary

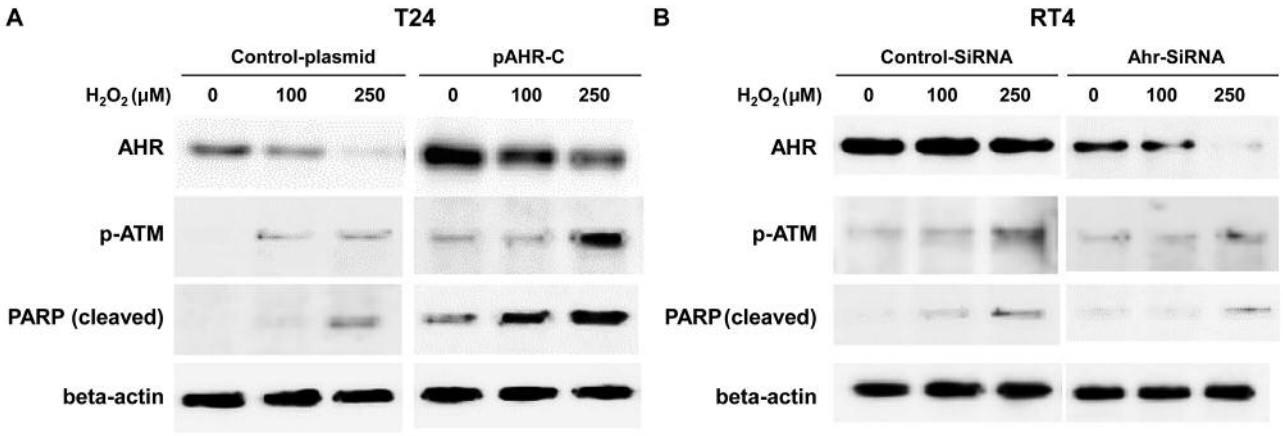

Figure 8. *DNA damage response was induced with H2O2 treatment. Activation of p-ATM (3.3-fold) and induction of PARP cleavage (4.3-fold) were increased in T24 cells transfected with pAhR-C (A) and the level of p-ATM (37%) was decreased in RT4 cells transfected with AhR-siRNA (B) compared with control, respectively.*

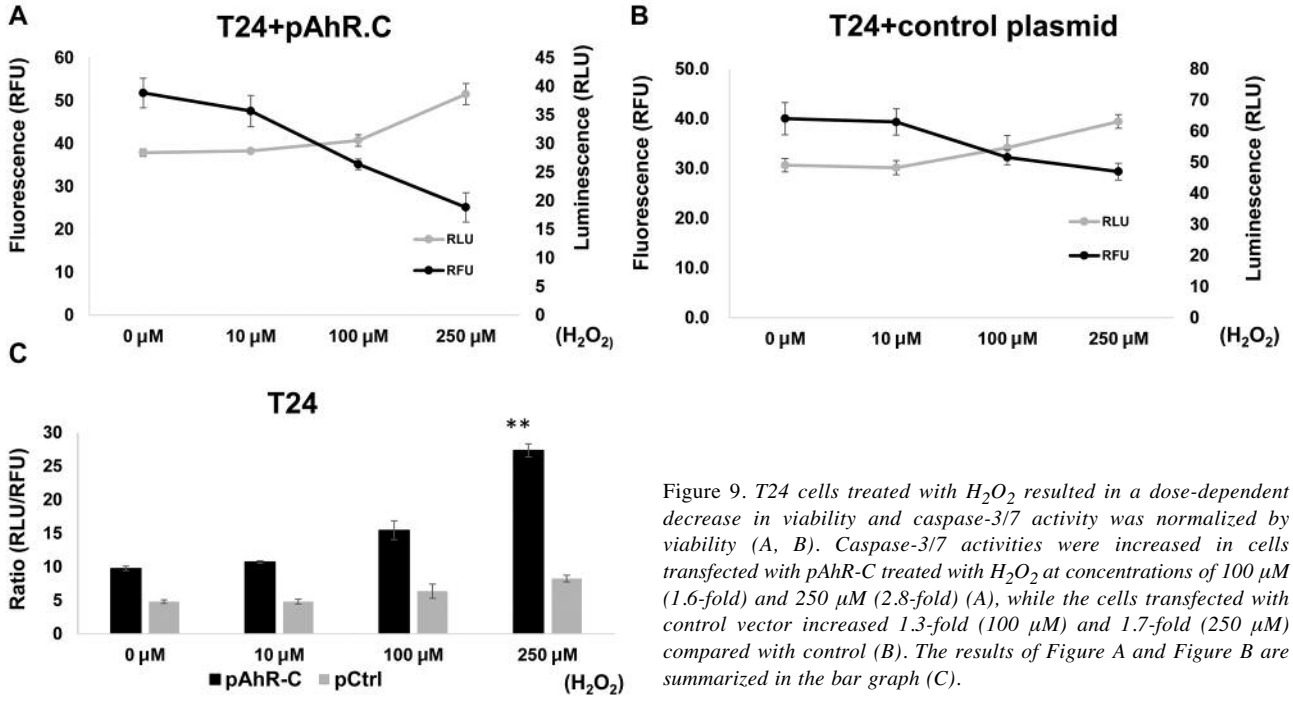

Figure 9. *T24 cells treated with H_2O_2 resulted in a dose-dependent decrease in viability and caspase-3/7 activity was normalized by viability (A, B). Caspase-3/7 activities were increased in cells transfected with pAhR-C treated with H_2O_2 at concentrations of 100 μM (1.6-fold) and 250 μM (2.8-fold) (A), while the cells transfected with control vector increased 1.3-fold (100 μM) and 1.7-fold (250 μM) compared with control (B). The results of Figure A and Figure B are summarized in the bar graph (C).*

to our results, regarding breast cancer (30) and glioblastoma (31), it has been reported that activated AhR promotes the invasiveness of tumor cells. However, Tsai *et al*. confirmed the differences in cell invasive potential between some NSCLC cells (32). The functional mechanism of AhR with regard to tumor cell proliferation and invasion is still under investigation.

Shen *et al*. reported that TCDD produces AhR-dependent oxidative stress in mitochondria (33). PARP has one of the primary repair mechanisms to resolve DNA lesions (34). Previous reports showed that ATM (35) and PARPs activation (36) was increased depending on the concentration of H_2O_2. Moreover, Diani-Moore *et al*. reported that the activation of the AhR by TCDD increased

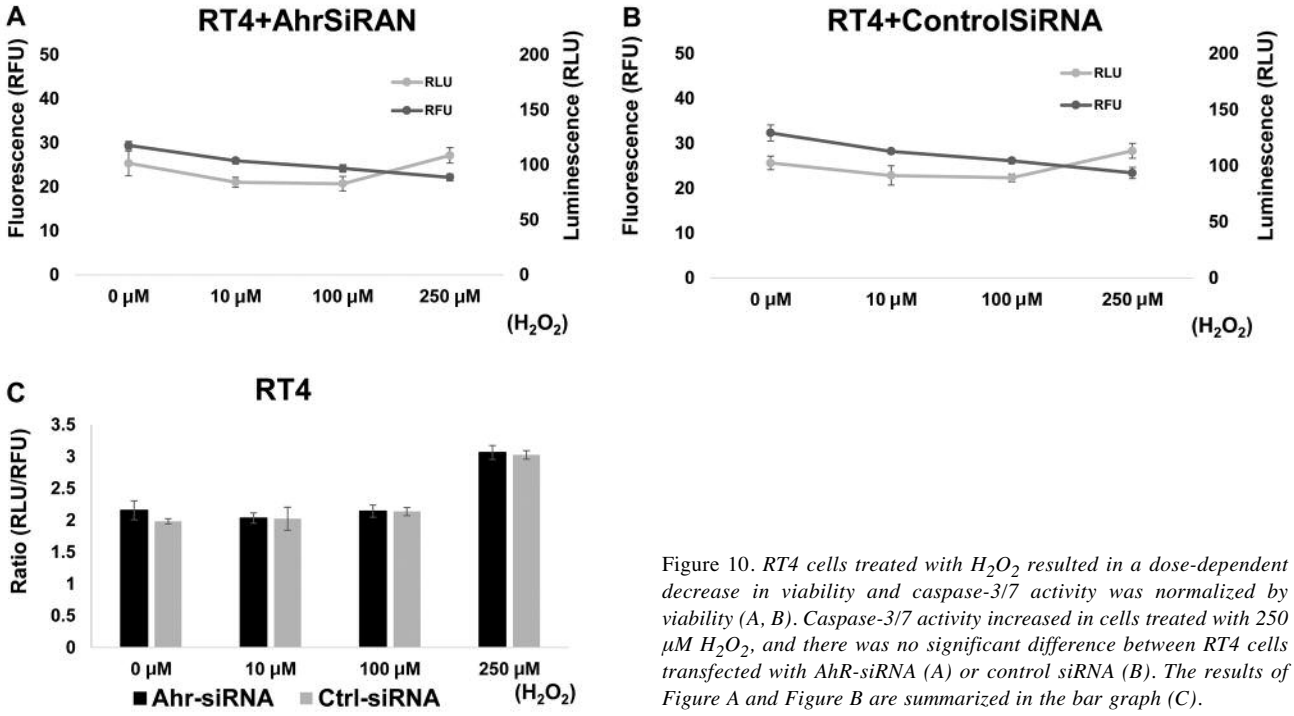

Figure 10. *RT4 cells treated with H_2O_2 resulted in a dose-dependent decrease in viability and caspase-3/7 activity was normalized by viability (A, B). Caspase-3/7 activity increased in cells treated with 250 µM H_2O_2, and there was no significant difference between RT4 cells transfected with AhR-siRNA (A) or control siRNA (B). The results of Figure A and Figure B are summarized in the bar graph (C).*

PARP activity (37). We could confirm the results similar to past reports in our control study, our data showed that higher levels of pATM (3.3-fold) and PARP cleavage (4.3-fold) in T24 cells transfected with AhR constitutive expression plasmid compared with control plasmid (Figure 8A), and in RT4 cells transfected with AhR-siRNA, the level of pATM and PARP cleavage decreased compared to control siRNA (Figure 8B). The cleavage of PARP inhibits PARP's DNA-repairing abilities. So, cleaved PARP may be considered a marker of apoptosis (38). Therefore, we suspected that the activation of ATM and apoptosis by oxidative stress may depend on AhR in BC cells. Although there are no previous reports on caspase-3/7 activity in BC, the induction of apoptosis by H_2O_2 clearly differs depending on the type of cell line. Previously, we were convinced the anti-apoptotic effect of the AhR, in part because exposure to environmental pollutants can lead to the development of various tumors. But now, on the contrary, we understand AhR can influence apoptosis by controlling the expression of apoptosis genes (39).

AhR has a dual ability in human tissues. With regard to urothelial cancer, Iida *et al*. reported that BBN can induce BC *via* suppression of the AHR signaling pathway (40). On the other hand, Ishida *et al*. reported that nuclear expression of AhR was significantly associated with histological grade, pathological T stage, and lymph node involvement (41). Similar results have been reported in prostate cancer. Richmond *et al*. found that the nuclear localization of the AhR in prostate cancer cells indicates its constitutive activation (42). On the other hands, $AhR^{-/-}$ transgenic adenocarcinoma of the mouse prostate (TRAMP) mice develop prostate tumors with much greater frequency than $AhR^{+/+}$ TRAMP mice (43). And Gluschnaider *et al*. reported that beta-transducin repeats-containing proteins inhibition reduces prostate cancer cell growth *via* upregulation of the AhR (44). Both results suggested that AhR has tumor suppressor properties. Both tumor-suppressor and pro-oncogenic functions have been reported for AhR also in lung cancer (32, 45).

There are certain limitations in this study. First, all outcomes presented in this study derived from the data using two cell lines T24 and RT4. In the future, it is necessary to consider using other cell lines. In addition, this study focused on the proliferation, invasiveness, DNA damage response and apoptosis. However, other molecular events, such as angiogenesis and epithelial mesenchymal transition should also be checked. Albini *et al*. reported that Matrigel invasion assay can provide evidence about migratory/invasive cellular functions that can then be verified *in vivo* and is still the most employed *in vitro* system for testing cells with different invasive abilities (46). Although the invasion includes cell migration, invasion requires to migrate beyond the extracellular matrix and settle in a new site. Consequently, strictly speaking,

migration and invasion is different. Unfortunately, for the purpose of examination of the impact of AhR on the invasiveness of BC cells, we experimented using only Matrigel invasion assay. In the future, we will have to experiment with other more accurate method such as 3D to investigate the status of invasion. In this study, we could not show the evidence to suggest which downstream signal pathways including growth factors and cytokines such as previously reported (47,48) are involved in cell proliferation, invasiveness, and so on.

In conclusion, in this study, we showed that AhR may potentially have dual function in bladder carcinogenesis. Various studies have reported that AhR can both negatively and positively regulate cancer cell, in either a ligand-dependent or endogenous AhR-dependent manner. The fundamental differences in carcinogenetic mechanism between NMIBC and MIBC may be a major cause of the difference in the role of AhR between NMIBC and MIBC in this study. For breast cancer, Romagnolo *et al*. indicated that there is a possibility that the AhR may be useful for treatment (49). In the same way, we hope that the elucidation of AhR mechanism for bladder carcinogenesis contributes to the progress of treatment for BC.

Conflicts of Interest

The Authors declare no conflicts of interest regarding this study. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Authors' Contributions

Conceptualization, Shigeo Horie; Data curation, Satoru Muto and Jingsong Yu; Formal analysis, Yan Lu; Methodology, Shigeo Horie; Project administration, Satoru Muto; Supervision, Shigeo Horie; Writing – original draft, Satoru Muto; Writing – review & editing, Hisamitsu Ide and Shigeo Horie.

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