Activation of the aryl hydrocarbon receptor pathway enhances cancer cell invasion by upregulating the MMP expression and is associated with poor prognosis in upper urinary tract urothelial cancer

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Aryl hydrocarbon receptor (AhR) and the activation of the AhR pathway are involved in xenobiotic-induced toxicity and carcinogenesis. Although xenobiotics, such as cigarette smoke, contribute to the development of urothelial carcinoma (UC), the relationship between AhR and UC is unclear. In the present study, we investigated AhR expression in 209 patients with upper urinary tract UC. The nuclear expression of AhR was significantly associated with histological grade, pathological T stage, lymphovascular invasion and lymph node involvement. A multivariate Cox analysis revealed that nuclear AhR expression was a significant and independent predictor for disease-specific survival (hazard ratio = 2.469, P = 0.013). To determine whether the AhR pathway can be activated in the T24 UC cell line, we examined the expression of cytochrome P450 (CYP) 1A1 and CYP1B1, which are target genes of the AhR pathway, following exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a ligand of AhR. TCDD treatment upregulated the expression levels of AhR, CYP1A1 and CYP1B1. TCDD enhanced T24 cell invasion associated with the upregulation of matrix metalloproteinase (MMP)-1 and MMP-9. Furthermore, targeting AhR messenger RNA (mRNA) expression in T24 cells with small interfering RNA (siRNA) downregulated the mRNA expression of AhR, CYP1A1 and CYP1B1. MMP-1 and MMP-2 and MMP-9; furthermore, the cells transfected with siRNA for AhR showed decreased invasion activity in comparison with the cells transfected with a non-targeting siRNA. Our results therefore suggest that AhR plays a role in the invasiveness of UC cells and can serve as a marker for the prognosis of upper urinary tract UC.

Introduction

Urothelial carcinoma (UC) occurs throughout the urinary tract. Although the urinary tract includes the renal pelvis, ureter, bladder and urethra, most cases of UC involve the bladder. UC of the renal pelvis is a relatively rare occurrence and constitutes ~5% of all urothelial tumors and 10% of all renal tumors (1). A ureteral tumor is even less common and its incidence is one-fourth that of renal pelvic tumors (2). In regard to bladder cancer, many molecular markers associated with cancer cell invasion and apoptosis (15–20).

Materials and methods

Patient population

A total of 228 patients were surgically treated for upper urinary tract UC at Keio University Hospital in Tokyo, Japan, from 1983 to 2007. The present study included 209 patients (121 patients with renal pelvic cancer, 78 patients with ureteral cancer and 10 patients with both types of cancer). The patients with distant metastasis at the time of diagnosis, a concomitant invasive bladder tumor or incomplete clinical data were excluded from this study.

The median age of the patients was 67 years (range 36–89 years) and the median follow-up period was 38.3 months (mean 60.9, range 0.5–246.3 months). A nephroureterectomy with the removal of the bladder cuff was the most common procedure (204 patients, 97.6%). A partial ureterectomy was performed in five patients. During the follow-up, 55 (26.3%) patients had distant metastases. We subdivided the pT3 (pathologically detected tumor invasion beyond the muscularis into peripelvic fat, renal parenchyma or periureteric fat) into two categories—a microscopic invasion (pT3a) and invasion (LVI) are associated with disease-specific survival (E. Kikuchi et al., personal communication); however, there are only a few molecular predictors for cancer recurrence or survival in patients with upper urinary tract UC (5–7). Therefore, the investigation of molecular markers that predict the prognosis of UC is considered to be a high priority.

Epidemiological studies have demonstrated that cigarette smoking contributes to the development of UC (8). Furthermore, the urinary tract is exposed to a number of xenobiotics other than the carcinogens of cigarette smoke due to the function of the kidneys as excretory organs. Aryl hydrocarbon receptor (AhR) is a ligand-activated transcriptional factor of the basic helix-loop-helix/Per-Arnt-Sim family (9). It is involved in xenobiotic-induced toxicity and carcinogenesis and is present in the cytosol in the absence of its ligand (10). Upon binding to a ligand such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), which is the most potent exogenous AhR-binding ligand, AhR translocates into the nucleus and dimerizes with AhR nuclear translocator (11). This complex binds to the specific DNA region and thereby activates a battery of gene expression, such as the expression of cytochrome P450s (CYPs), which are involved in the activation of procarcinogens (12). Furthermore, activation of the AhR pathway enhances the invasiveness of melanoma and gastric cancer cells by upregulating matrix metalloproteinases (MMPs) (13,14). Several studies have reported that activation of the AhR pathway is associated with cancer cell invasion and apoptosis (15–20).

Based on the above-mentioned evidence, AhR appears to be one of the key molecules involved in carcinogenesis and progression of smoking-related carcinomas. In fact, the constitutive activation of AhR has been reported in several tumors such as lung cancer (21) and pancreatic cancer (22). To our knowledge, there have so far been no reports on the expression and role of AhR in UC, even though cigarette smoke is one of the risk factors for UC (8).

In this study, we performed an immunohistochemical analysis of AhR in 209 cases of upper urinary tract UC and evaluated the association between the nuclear expression of AhR and clinicopathological parameters such as invasion depth and distant metastasis. To determine whether the AhR pathway can be activated in a urothelial cancer cell line, the T24 cell line was employed. The expression levels of CYP1A1 and CYP1B1, two target genes of the AhR pathway, were examined following TCDD exposure, and the effect of TCDD exposure on the invasiveness of T24 cells was investigated by MatrigelTM invasion assay. Furthermore, because AhR expression in T24 cells was downregulated by small interfering RNA (siRNA), the effect of siRNA on the AhR pathway and cancer cell invasion was also investigated.
a macroscopic fat invasion (pT3b), namely, because pT3 seems to comprise a heterogeneous group of patients (23–25). The patients were followed post-operatively with urinary cytology every 3 months for 2 years and thereafter for every 6 months. Computed tomography in addition to cystoscopy and/or magnetic resonance imaging and/or excretory urography were performed every 6 months for 5 years and thereafter were performed annually.

**Immunohistochemistry**

All the tissue samples were fixed in 10% formalin and embedded in paraffin. One representative paraffin block from each case was selected by observing the sections stained with hematoxylin and eosin. The paraffin sections (4 μm thick) were used for the immunohistochemistry. After deparaffinization in xylene and dehydration in a graded ethanol series, an antigen retrieval procedure was performed by heating the slides in 10 mM citrate buffer (pH 6.0) at 121°C for 10 min. The endogenous peroxidase activity was blocked by incubation with 0.3% hydrogen peroxide/methanol for 15 min. After incubation with 10% normal goat serum, the sections were incubated at 4°C overnight with the rabbit polyclonal anti-AhR antibody (1:100 dilution, sc-5579; Santa Cruz Biotechnology, Santa Cruz, CA). After washing with phosphate-buffered saline, they were incubated with secondary antibodies against rabbit IgG conjugated to a peroxidase-labeled dextran polymer (no dilution, anti-rabbit Envision, Dako Japan, Tokyo, Japan) for 30 min. The color was developed containing 0.005% hydrogen peroxide. The sections were counterstained with hematoxylin.

**Evaluation of immunostaining**

To evaluate the staining of AhR, the cancer cells with positive staining on the nuclei were counted in at least three representative fields (×400 magnification), and the mean percentage of the positive cancer cells was calculated. Nuclear AhR staining in more than half of the population of cancer cells was defined as a high AhR expression. Because the cytoplasmic AhR staining in the cancer cells was diffuse, it was evaluated and stratified from 0 to 3 (0, no staining; 1, slight staining; 2, moderate staining and 3, strong staining). The immunoreactivity for AhR was analyzed independently by two uropathologists. There were no discordances in regard to the evaluation of AhR staining in a few cases, and in those cases, the slides were reviewed again and a consensus opinion was achieved after a full discussion. The evaluation of immunostaining was made without any knowledge of the clinicopathological features in order to avoid any bias.

**Cell culture and treatment**

The human transitional cancer cell line, T24, was purchased from the American Type Culture Collection (Rockville, MD) and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 μg/ml streptomycin and 100 IU/ml penicillin (Sigma–Aldrich, St Louis, MO) in a humidified incubator with 5% CO2 at 37°C. T24 cells were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan) and was dissolved in dimethyl sulfoxide (DMSO). Cells were treated with different concentrations of TCDD (0.1, 1 and 10 nM) for 24 h. The control cells only received 0.1% DMSO.

**Small-interfering RNA**

Two designed siRNAs for AhR (Sigma–Aldrich Japan, Tokyo, Japan) and the non-targeting control siRNA (AllStars Negative Control siRNA; Qiagen K.K., Tokyo, Japan) were used for the study. The sense sequences of siRNA for AhR were as follows: AhR1, 5'-GCCCAGGUCUCCAUUCC-CAAUG-3' and AhR2, 5'-GACCGAGUGCCAAAGAUUCU-3'. The cells (5.0 × 10^4 cells per well) were cultured in RPMI-1640 without any antibiotics and 5% CO2 at 37°C in 5% CO2 and then transfected with AhR1, AhR2 or a non-targeting control using Lipofectamine™ 2000 (Invitrogen Co., Carlsbad, CA) as described previously (26). After 24 h, RPMI-1640 containing 10% FBS was added to each well and the cells were cultured for an additional 48 h.

**Cell proliferation and apoptosis assay**

Cell proliferation was estimated with the use of the Premix water-soluble tetrazolium salt-1 cell proliferation assay system (Takara Bio, Shiga, Japan), which analyzes succinate–tetrazolium reductase activity. The cells with or without siRNA for AhR were plated at 1 × 10^4 cells per well in 96-well plates. The next day, the cells without siRNA were incubated with various concentrations of TCDD or 0.1% DMSO for 24 h. During the analysis, water-soluble tetrazolium salt reagent was added to the culture medium and the cells were incubated at 37°C for 1 h with 5% of CO2 for the succinate–tetrazolium reductase reaction. To detect apoptosis, TCDD-treated cells or cells transfected with siRNA for AhR were incubated with terminal deoxynucleotidyl transferase and digoxigenin–deoxyuridine triphosphate at 37°C for 1 h and then washed and exposed to fluorescein isothiocyanate-labeled antidigoxigenin for 30 min at room temperature. They were subsequently washed with phosphate-buffered saline and subjected to flow cytometry.

**Real-time quantitative polymerase chain reaction**

Total RNA was extracted from the cells using ISOGEN (Nippon Gene, Toyama, Japan), and the quality and quantity of the RNA were evaluated by the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). The RNA (1 μg) was reverse transcribed with RevaTra Ace Reverse Transcriptase (TOYOBO Co., Tokyo, Japan) in a 20 μl reaction volume containing Oligo (dT) 12–18 primers. The reaction mixture (1 μl) was then used as template in a TaqMan real-time quantitative polymerase chain reaction assay using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. The cycling conditions were 50°C for 10 min, 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The primers and TaqMan probe sets (TaqMan Gene Expression Assays, Invitrogen) for AhR (Hs00907314_m1), CYP1A1 (Hs00053120_m1), CYP1B1 (Hs00164383_m1), MMP-1 (Hs00899658_m1), MMP-2 (Hs00234422_m1), MMP-9 (Hs00234579_m1) and human β-actin endogenous control (4310881E) were purchased from Applied Biosystems (sequences not disclosed). The AhR:β-actin messenger RNA (mRNA), CYP:β-actin and MMP:β-actin mRNA ratios were calculated for each sample to evaluate the relative mRNA expression.

**Matrigel™ invasion assay**

The degree of tumor cell invasion was assessed by counting the number of cells that migrated through the transwell inserts with an 8 μm pore size polycarbonate terephthalate membrane coated with a uniform layer of BD Matrigel™ Basement Membrane Matrix (BD Biosciences, San Jose, CA), according to the protocol recommended by the manufacturer. Briefly, the tumor cells (2.5 × 10^4 cells in 500 μl RPMI-1640) were seeded on top of the transwell inserts, whereas the lower chamber was filled with 0.75 ml RPMI-1640 with 5% FBS as the chemoattractant. The cells were cultured for 24 h at 37°C in a well-humidified incubator before the non-migrating cells in the inserts were scrapped off. The membranes were subsequently fixed and stained using a Diff-Quik™ staining kit (Sysmex Co., Hyogo, Japan). The cells that had invaded through the membranes were quantified by calculating the cell number in three randomly chosen visual fields at ×200 magnification.

**Statistical analysis**

The chi-square test was used to evaluate the association between the clinicopathological and the nuclear AhR expression. The associations between the clinicopathological features and the nuclear or cytoplasmic AhR expression were assessed with the Mann–Whitney U-test and the chi-square test. The Kruskal–Wallis test was used to evaluate the association between the nuclear expression of AhR and the pathological stage. The actuarial probabilities obtained using a Kaplan–Meier analysis were reported as the means ± 2 SEs (95% confidence intervals) and were compared by the log-rank test. The prognostic factors that we assessed included age (greater versus less than the patient’s mean age of 65 years), gender, tumor location (pelvis versus ureter), tumor grade (low versus high), pathological T stage (pTa–pT3a versus pT3b/pT4), LVI and AhR. We used the Cox proportional hazards regression analysis to assess the prognostic indicators for survival. The Mann–Whitney U-test was used to analyze the in vitro findings (AhR, CYPs and MMP’s mRNA levels). P values <0.05 were considered to be statistically significant. These analyses were performed using the STATA version 9.2 statistical software package (Stata Corporation, College Station, TX).

**Results**

**AhR expression in upper urinary tract UC and non-neoplastic urothelium**

Cytoplasmic AhR staining was observed in the non-neoplastic urothelium, albeit with a weak intensity (Figure 1A). The nuclear AhR staining was also very weak or negligible (Figure 1A). In the superficial UC, there was a moderate degree of cytoplasmic staining and weak nuclear AhR staining was observed in only a small number of the cancer cells (Figure 1B). In the invasive UC, strong AhR staining was observed in many of the nuclei of the cancer cells, but the cytoplasmic AhR staining was rather weak (Figure 1C). The lymphocytes adjacent to the carcinoma cells were also positive for AhR (Figure 1C). In the group of high nuclear AhR expression patients (n = 96), 14 (14.6%) cases displayed strong cytoplasmic staining and 82 (85.4%) had moderate to weak
cytoplasmic staining. The chi-square test statistically confirmed that the nuclear AhR expression inversely correlated with the cytoplasmic AhR staining \((P < 0.012)\).

**Associations between AhR expression and clinicopathological parameters**

The associations between the degree of nuclear or cytoplasmic AhR expression and the clinicopathological features are summarized in Table I. The nuclear AhR expression correlated with pathological tumor stage (pT) \((P < 0.001)\), histological grade \((P < 0.001)\), LVI \((P < 0.001)\) and lymph node involvement \((P = 0.015)\) (Table I). Although the cytoplasmic AhR expression was associated with the tumor location \((P = 0.003)\); Table I), no association was observed between the cytoplasmic AhR expression and the other clinicopathological parameters.

**Multivariate analysis for progression-free survival**

During the follow-up, 55 (26.3%) patients experienced distant metastases and 148 patients survived without disease progression. The 5 and 10 year progression-free survival rates were 70.4 and 66.1%, respectively. The 5 and 10 year progression-free survival rates were 87.4 and 82.7% with a low nuclear AhR and 48.1 and 44.1% with a high nuclear AhR, respectively \((P < 0.001); Figure 2A\). Cytoplasmic AhR was not a significant predictor of progression-free survival. According to the pathological T classification, the 5 and 10 year progression-free survival rates were 82.4 and 78.2% for pT3a or less and 42.4 and 36.3% for pT3b/pT4 disease, respectively \((P < 0.001); Figure 2B\). The 5 and 10 year progression-free survival rates were 87.2 and 82.4% without LVI and 42.5 and 38.2% with LVI, respectively \((P < 0.001); Figure 2C\). Age, tumor grade, pathological T stage, lymphovascular involvement and high AhR were significant predictors of progression-free survival in a univariate analysis (Table II). According to a multivariate analysis, the pathological T stage \((P = 0.023)\), LVI \((P = 0.019)\) and nuclear

| Table I. Association between the expression of AhR and the clinicopathological features |
|----------------------------------|-----------------|-----------------|-----------------|
|                                  | Nuclear AhR (%) | P value         | Cytoplasmic AhR | P value         |
| All patients (N = 209)           | 45.5 ± 32.9     | 0.38            | 162             | 0.20            |
| Age                              |                 |                 |                 |                 |
| <65 years (n = 88)               | 43.5 ± 32.0     | 0.38            | 72 (34.4)       | 0.20            |
| >65 years (n = 121)              | 47.2 ± 33.6     | 0.20            | 90 (43.1)       | 31 (14.8)       |
| Gender                           |                 |                 |                 |                 |
| Male (n = 158)                   | 44.7 ± 33.2     | 0.003           | 123 (58.9)      | 35 (16.7)       |
| Female (n = 51)                  | 48.8 ± 32.3     | 0.003           | 39 (18.7)       | 12 (5.7)        |
| Tumor location                   |                 |                 |                 |                 |
| Pelvis (n = 131)                 | 45.8 ± 34.2     | <0.001          | 101 (50.8)      | 20 (10.1)       |
| Ureter (n = 88)                  | 44.8 ± 31.5     | <0.001          | 51 (25.4)       | 27 (13.4)       |
| Histological grade               |                 |                 |                 |                 |
| Low (n = 45)                     | 23.3 ± 22.8     | <0.001          | 35 (17.4)       | 10 (4.8)        |
| High (n = 164)                   | 51.9 ± 32.6     | <0.001          | 127 (60.8)      | 37 (17.7)       |
| Pathological T                   |                 |                 |                 |                 |
| pTa (n = 39)                     | 21.2 ± 20.6     | <0.001          | 96 (45.9)       | 32 (15.3)       |
| pT1 (n = 32)                     | 31.9 ± 29.8     | <0.001          | 65 (31.6)       | 15 (7.2)        |
| pT2 (n = 39)                     | 48.2 ± 30.3     | <0.001          | 65 (31.6)       | 15 (7.2)        |
| pT3a (n = 36)                    | 47.6 ± 32.8     | <0.001          | 65 (31.6)       | 15 (7.2)        |
| pT3b (n = 55)                    | 61.8 ± 30.3     | <0.001          | 65 (31.6)       | 15 (7.2)        |
| pT4 (n = 8)                      | 88.8 ± 9.1      | <0.001          | 96 (45.9)       | 32 (15.3)       |
| pTa-pT3a (n = 146)               | 37.2 ± 30.6     | 0.20            | 65 (31.6)       | 15 (7.2)        |
| LVI                              |                 |                 |                 |                 |
| Negative (n = 127)               | 35.3 ± 29.8     | 0.63            | 97 (46.9)       | 30 (14.5)       |
| Positive (n = 82)                | 61.8 ± 31.1     | 0.63            | 65 (31.6)       | 17 (8.1)        |
| Lymph node involvement           | 0.015           | 0.977           | 148 (70.1)      | 43 (20.6)       |
| Negative (n = 191)               | 44.0 ± 33.1     | 0.977           | 148 (70.1)      | 43 (20.6)       |
| Positive (n = 18)                | 63.3 ± 26.0     | 0.977           | 14 (6.7)        | 4 (1.9)         |

SD, standard deviation.
AhR ($P = 0.001$) were found to be independent prognostic factors for a progression-free survival (Table II).

**Risk stratification analysis for progression-free survival**
Using the three statistically significant variables in the multivariate Cox regression analysis (pathological T stage, LVI and AhR), an equation to calculate the relative risk of death could be calculated with the following formula: $2.192 \times$ pathological T stage $+ 2.407 \times$LVI $+ 2.953 \times$AhR. Based on the relative risk of the progression of disease, the patients with upper tract UC were divided into low-risk (the relative risk of disease progression = 1), intermediate-risk (2.192–7.108) and high-risk (15.580) groups. According to the prognostic-factors-based risk stratification for upper tract UC, 82 patients were in the low-risk group ($\leq$pT3a, no LVI and low AhR), 38 patients were in the high-risk group ($\geq$pT3b, LVI and high AhR) and 89 patients were in the intermediate-risk group (all others). The 5 and 10 year progression-free survival rates were 95.4 and 89.1% in the low-risk group, 59.3 and 59.3% in the intermediate-risk group and 37.3 and 24.9% in the high-risk group, respectively. The differences among the groups for the progression-free survival were significant ($P < 0.001$ for the low- versus

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**Fig. 2.** The Kaplan–Meier analysis of the progression-free survival of the patients after surgery for UC according to the AhR expression (A), pathological T stage (B) and LVI (C), and the three risk groups that are stratified according to the risk factors (pathological stage, LVI and AhR) (D). The Kaplan–Meier analysis of the disease-specific survival of the patients after surgery for UC according to the AhR expression (E), pathological T stage (F), LVI (G) and the three risk groups that are stratified according to risk factors (pathological stage, LVI and AhR) (H).
intermediate-risk group, $P < 0.001$ for the low- versus high-risk group and $P = 0.018$ for the intermediate- versus high-risk group; Figure 2D).

**Multivariate analysis for disease-specific survival**

A total of 46 patients died from the disease and seven patients died from other causes. The overall disease-specific survival was 75.9% at 5 years and 68.6% at 10 years. The 5 and 10 year disease-specific survival rates were 89.0 and 84.8% with low AhR and 57.7 and 43.2% with high nuclear AhR, respectively ($P < 0.001$; Figure 2E). Cytoplasmic AhR was not a significant predictor of disease-specific survival. According to the pathological T classification, the 5 and 10 year survival rates were 86.9 and 81.7%, respectively, for pT3a or less and 49.7 and 33.1%, respectively, for pT3b/pT4 disease ($P < 0.001$; Figure 2F). The 5 and 10 year disease-specific survival rates were 90.1 and 87.4% without LVI and 52.3 and 35.1% with LVI ($P < 0.001$; Figure 2G). A univariate analysis revealed that age, tumor grade, pathological T stage, lymphovascular involvement and high AhR were significant predictors of disease-specific survival but cytoplasmic AhR was not. The pathological T stage, LVI and AhR provided independent prognostic information when the other variables were controlled in the multivariate analysis (Table II).

**Risk stratification analysis for disease-specific survival**

Based on the relative risk of death, the patients with upper tract UC were divided into low-risk (the relative risk of death = 1), intermediate-risk (2.180–7.585) and high (16.535)-risk groups. The 5 and 10 year disease-specific survival rates were 97.4 and 93.4% in the low-risk group, 65.5 and 59.8% in the intermediate-risk group and 47.9 and 19.9% in the high-risk group, respectively. The differences among the groups in regard to disease-specific survival were significant ($P < 0.001$ for low- versus intermediate-risk group, $P < 0.001$ for low- versus high-risk group and $P = 0.040$ for intermediate-versus high-risk group) (Figure 2H).

**Activation of the AhR pathway in T24 cells and its effect on cancer cell invasion in vitro**

Real-time polymerase chain reaction revealed that TCDD exposure upregulated AhR mRNA expression in T24 cells in a dose-dependent manner (Figure 3A). Because the cells treated with 10 nM TCDD showed the highest AhR mRNA expression, further experiments were performed with 10 nM TCDD. To determine whether TCDD exposure activates the AhR pathway in T24 cells, the expression levels of CYP1A1 and CYP1B1 were investigated. Their expression levels were upregulated by 73.0 and 38.1%, respectively ($P < 0.05$; Figure 3B and C).

TCDD exposure also upregulated the expression levels of MMP-1 and MMP-9 in T24 cells by 92.3, 21.8 and 85.4%, respectively ($P < 0.05$; Figure 3D and F). Furthermore, the MatrigelTM invasion assay revealed that TCDD exposure enhanced invasion activity ($106.7 \pm 8.4$) in comparison with that in the cells treated with 0.1% DMSO ($63.3 \pm 6.0$) ($P < 0.05$; Figure 3G and H). We also examined the effect of TCDD on cell proliferation and apoptosis in T24 cells. There was no significant change in proliferation and apoptosis between the cells treated with TCDD and the control cells (data not shown).

**Effect of siRNA for AhR on the AhR pathway and cancer cell invasion in vitro**

The transfection of two different siRNAs for AhR (AhR1 and AhR2) reduced the level of AhR mRNA expression by 75.2 and 89.0%, respectively, in T24 cells ($P < 0.05$) in comparison with the cells treated with the control siRNA (Figure 4A). AhR1 downregulated the mRNA expression of CYP1A1 and CYP1B1 in T24 cells by 71.9 and 19.9%, respectively ($P < 0.05$; Figure 4B and C). Similarly, AhR2 downregulated the mRNA expression of CYP1A1 and CYP1B1 in T24 cells by 78.1 and 75.9%, respectively ($P < 0.05$; Figure 4B and C).

Furthermore, AhR1 downregulated the mRNA expression of MMP-1, MMP-2 and MMP-9 in T24 cells by 76.6, 41.6 and 85.4%, respectively ($P < 0.05$; Figure 4D–F). AhR2 also downregulated the mRNA expression of MMP-1, MMP-2 and MMP-9 in T24 cells by 92.3, 21.8 and 94.8%, respectively ($P < 0.05$; Figure 4D–F). The MatrigelTM invasion assay revealed that T24 cells transfected with AhR1 or AhR2 exhibited decreased invasion activity ($30.7 \pm 3.1$ or $26.8 \pm 3.8$) in comparison with the cells that were transfected with control siRNA ($56.8 \pm 6.3$, $P < 0.05$; Figure 4G and H). We also examined the effect of AhR downregulation on cell proliferation by water-soluble tetrazolium salt-1 assay and on apoptosis by flow cytometry. There was no significant change in either proliferation or apoptosis between the cells transfected with siRNA for AhR and the control cells (data not shown).

**Discussion**

Inoue et al. (27) investigated a total of 55 nephroureterectomy specimens and reported that the MMP-9/E-cadherin ratio was an independent predictor for metastasis and survival. However, there have so far been no reports that investigated the molecular markers, including >100 cases analyzed by multivariate analysis. Because the major risk factor for UC is cigarette smoke, we investigated 209 cases of upper urinary tract UC and analyzed the effect of AhR on the disease prognosis. Our results demonstrated that nuclear AhR staining is predominantly observed in invasive UC and that the patients with high nuclear AhR expression had a significantly poorer prognosis in comparison with those with low nuclear AhR expression.

Because AhR acts as a transcriptional regulator with nuclear translocation (11), we evaluated both the nuclear and the cytoplasmic AhR...
staining in this study. Weak cytoplasmic staining without apparent nuclear staining was observed in non-neoplastic urothelium. The superficial UC showed a moderate degree of cytoplasmic AhR staining with spotted nuclear AhR staining. In contrast, the invasive UC showed strong nuclear AhR staining but the cytoplasmic staining was weak. In addition, the statistical analyses revealed that nuclear AhR expression was inversely associated with cytoplasmic AhR expression. Furthermore, a multivariate analysis demonstrated that nuclear AhR expression independently predicted the cancer recurrence and the disease-specific survival of patients with upper urinary tract UC. These results suggest that AhR acts as one of the transcriptional regulators involved in the progression of UC.

The lymphocytes adjacent to the carcinoma cells were also positive for AhR. Previous reports have demonstrated environmental AhR ligands to suppress a variety of B cell-mediated responses (28,29). Therefore, it is possible that nuclear AhR expression in lymphocytes is associated with the suppression of the immune response to cancer cells. However, the role of AhR in the lymphocytes adjacent to the cancer cells is unclear, and further studies are necessary to elucidate its role in upper urinary tract UC.

![Fig. 3. TCDD upregulated mRNA expression of AhR, CYPs and MMPs and invasiveness of T24 cells compared with the cells treated with 0.1% DMSO. After different concentrations of TCDD treatment for 24 h, AhR expression was upregulated in a dose-dependent manner (A). Ten nanometers TCDD treatment upregulated the mRNA expression of CYP1A1 (B), CYP1B1 (C), MMP-1 (D) and MMP-9 (F), but not MMP-2 (E) in comparison with cells treated with 0.1% DMSO. The cells that invaded through the BD Matrigel™ Basement Membrane Matrix were stained with Diff-Quik (G). The number of cells that invaded through the BD Matrigel™ Basement Membrane Matrix was significantly higher among the cells with TCDD treatment in comparison with the cells treated with 0.1% DMSO. (H). Bars, mean ± SE (n = 3); *P < 0.05; n.s., non-significant.](image-url)
The benefits of adjuvant therapy in patients undergoing surgery for the treatment of advanced upper urinary tract UC have not yet been established. Adjuvant radiotherapy seems to be of limited value, and systemic chemotherapy appears to provide some benefits (30,31). However, in these studies, the selection of an appropriate group of patients who had received and who should have benefited from these treatments was not analyzed. Czito et al. (32) recently demonstrated that adjuvant radiotherapy and chemotherapy improved the patient

Fig. 4. Two siRNA for AhR downregulated the mRNA expression of AhR, CYPs and MMPs in T24 cells and inhibited invasion in vitro. T24 cells transfected with siRNA for AhR showed significantly lower expression levels of AhR (A), CYP1A1 (B), CYP1B1 (C), MMP-1 (D), MMP-2 (E) and MMP-9 (F) in comparison with the cells with non-targeting siRNA. Bars indicate the mean ± SE. (G) The cells that invaded through the BD Matrigel™ Basement Membrane Matrix were stained with Diff-Quik. (H) The number of cells that invaded through the BD Matrigel™ Basement Membrane Matrix was significantly lower among the cells transfected with siRNA for AhR in comparison with those with non-targeting siRNA. Bars, mean ± SE (n = 3); * P < 0.05.
outcomes, especially in those patients with pT3/pT4 and/or node-positive upper tract UC. The current study indicates that investigating nuclear AhR expression may be useful for determining the treatment of upper urinary tract UC.

The efficacy of adjuvant therapy should therefore be re-evaluated after the selection of a more homogenous population. We established a prognostic-factor-based risk stratification for upper urinary tract UC using the independent predictors, pathological T stage, LVI and AhR. The patients were stratified into three groups according to statistical modeling based on the relative risk associated with the prognostic indicators derived from the multivariate analysis. The statistical significance was determined for the comparisons of all the group–pair combinations. This stratification can enable a more accurate prediction of patient outcomes. A more homogenous selection of patients for clinical trials is needed to clarify the benefits of integrated therapies involving surgery and post-operative systemic chemotherapy.

AhR plays a role in xenobiotic-related carcinogenesis by inducing AhR battery genes such as CYP enzymes, which are considered to be involved in the detoxication of drugs and xenobiotics and the activation of procarcinogens (12). However, Uno et al. (33,34) demonstrated that CYP1A1-knockout mice were paradoxically more sensitive to exposure to benz[a]pyrene (B[a]P), a ligand of AhR, than wild-type mice, and CYP1A1- and CYP1B1-double-knockout mice did not display B[a]P-mediated toxicity. They concluded that CYP1A1 may be important in B[a]P detoxification and that CYP1B1 may be responsible for metabolic activation of B[a]P. Meanwhile, Nebert et al. (35) reported that CYP enzymes produce reactive oxygen intermediates when they metabolize exogenous and endogenous substrates. Reactive oxygenate intermediates can cause genotoxicity and mutation by covalently binding to nucleic acids and proteins (35). In the current study, nuclear AhR staining was predominantly observed in invasive UC, but not in the normal epithelia, suggesting that the AhR pathway is activated in UC. Furthermore, we have demonstrated that the expression of T24 cells to TCDD increased CYP1A1 and CYP1B1 mRNA expression and that siRNA for AhR downregulated the mRNA expression of CYP1A1 and CYP1B1. These data suggest that the AhR activation pathway, especially reactive oxygenate intermediates produced by upregulated CYPs, may play a role in the carcinogenesis and progression of UC.

Park et al. (15) reported that exposure to TCDD, an AhR agonist, prevents mammary epithelial cells from undergoing apoptosis and promotes cell proliferation. Furthermore, TCDD induces hyperplasia of keratinocyte and ureteral epithelium (16,17). In contrast, other studies reported that TCDD induced AhR-mediated inhibition of cell proliferation and cell cycle arrest in rodent epithelial cells and human breast cancer cells (18–20). Although TCDD is well known as a powerful carcinoegenic pollutant, the effect of TCDD on epithelial cell proliferation remains controversial. In the present study, TCDD induced AhR activation and cancer cell invasion in vitro, but there was no significant change in proliferation and apoptosis between the cells treated with TCDD and the control cells. Therefore, TCDD may play a role in cancer cell invasion in T24 cells, but it plays no apparent role in cell proliferation and apoptosis.

Recent studies have shown AhR signaling to be related to cell invasion and migration (36). The AhR-signaling pathway mediates a number of genes that are unrelated to the metabolism of xenobiotics, such as the plasminogen activator inhibitor-2 and urokinase type plasminogen activator (37). The plasmin system can activate several MMPs by cleaving the prodomain (38). Furthermore, the activation of the AhR-signaling pathway upregulates the expression of MMPs (39,40). Villano et al. (13) reported that AhR signaling activated by TCDD contributed to melanoma progression, specifically through the stimulation of the expression and activity of MMP-1, MMP-2 and MMP-9 as well as through increased cancer cell invasion in the in vitro invasion assay. In the present study, we used membranes coated with reconstituted basement membrane (Matrigel™) to investigate the invasiveness of T24 cells. TCDD treatment activated the AhR-signaling pathway and increased the mRNA expression of MMP-1 and MMP-9. TCDD also promoted the invasion activity of T24 cells. The siRNA for AhR downregulated the mRNA expression of MMP-1, MMP-2 and MMP-9 and decreased the invasiveness of T24 cells. Because MMPs cleave the major components of the basement membrane, the reduction of the cancer cell invasion may be due to MMP downregulation.

In summary, this is the first report to investigate AhR expression in upper urinary tract UC and in the UC cell line. We demonstrated that strong nuclear AhR expression was observed in the invasive phenotype and an elevated nuclear AhR expression was associated with a poor prognosis. The risk stratification based on the independent prognostic factors obtained in this study might assist clinicians in predicting the disease outcome. The patients in a high-risk and/or intermediate-risk group may benefit from integrated therapies, including post-operative systematic chemotherapy. In vitro study revealed that activation of AhR by TCDD treatment increased the invasion activity of UC cells by the upregulation of MMP. Furthermore, the siRNA for AhR resulted in decreased invasion of cancer cells via MMP downregulation. These data suggest that AhR can be an attractive therapeutic target for the control of upper urinary tract UC.

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References


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