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ORIGINAL ARTICLES

Potential therapeutic significance of increased expression of aryl hydrocarbon receptor in human gastric cancer

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Abstract

AIM: To determine the functional significance of aryl hydrocarbon receptor (AhR) in gastric carcinogenesis, and to explore the possible role of AhR in gastric cancer (GC) treatment.

METHODS: RT-PCR, real-time PCR, and Western blotting were performed to detect AhR expression in 39 GC tissues and five GC cell lines. AhR protein was detected by immunohistochemistry (IHC) in 190 samples: 30 chronic superficial gastritis (CSG), 30 chronic atrophic gastritis (CAG), 30 intestinal metaplasia (IM), 30 atypical hyperplasia (AH), and 70 GC. The AhR agonist tetrachlorodibenzo-para-dioxin (TCDD) was used to treat AGS cells. MTT assay and flow cytometric analysis were performed to measure the viability, cell cycle and apoptosis of AGS cells.

RESULTS: AhR expression was significantly increased in GC tissues and GC cell lines. IHC results indicated that the levels of AhR expression gradually increased, with the lowest levels in CSG, followed by CAG, IM, AH and GC. AhR expression and nuclear translocation were significantly higher in GC than in precancerous tissues. TCDD inhibited proliferation of AGS cells *via* induction of growth arrest at the G1-S phase.

CONCLUSION: AhR plays an important role in gastric carcinogenesis. AhR may be a potential therapeutic target for GC treatment.

Key words: Apoptosis; Aryl hydrocarbon receptor; Cell cycle; Cell proliferation; Gastric cancer

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INTRODUCTION

Gastric cancer (GC) is the fourth most common malignancy and the second most frequent cause of cancer-related death in the world. It is often diagnosed at advanced stages when treatment options are limited, leading to a poor prognosis[1]. The development of human GC is a multi-step process where normal mucosa progresses to chronic gastritis, precancerous lesions (including gastric atrophy, intestinal metaplasia, dysplasia), and invasive cancer[2,3]. The carcinogenesis of GC involves numerous genetic and epigenetic alterations, as well as many environmental risk factors[4]. Environmental pollutants such as polycyclic aromatic hydrocarbons (PAHs) and halogenated hydrocarbons (HAHs) are well-known carcinogens that play important roles in GC development[5,6]. The toxic effects of PAHs and HAHs are mediated by a conserved signaling

pathway that binds and activates the aryl hydrocarbon receptor (AhR)[7].

AhR is a ligand-activated transcription factor of the basic helix-loop-helix/Per-Arnt-Sim family. PAHs and HAHs are exogenous AhR ligands, among which 2,3,7,8-tetrachlorodibenzo-para-dioxin (TCDD) is the most potent[8]. The ligand-AhR complex is translocated to the nucleus and heterodimerizes with the AhR nuclear translocator. The complex binds to the cognate enhancer sequence and subsequently activates downstream gene expression. AhR regulates genes that code for xenobiotic metabolizing enzymes, such as cytochrome P450 1A1 (CYP1A1), cytochrome P450 1B1 (CYP1B1), and growth-regulatory proteins[9]. Inappropriately modified expression, and/or abnormally sustained expression of critical genes by the xenobiotic-activated AhR leads to various toxicities which were observed in exposed organisms: teratogenicity, immunotoxicity, tumor promotion, as well as various metabolic dysfunctions[10,11].

Many studies in recent years have demonstrated a close relationship between AhR and mammary gland tumorigenesis[12,13]. AhR gene polymorphisms have been linked to an increased risk of lung and breast cancers[14,15]. Increased expression of AhR has been reported in lung, breast, and pancreatic cancers in humans[9,12,16]. Studies also suggest that constitutively active AhR may promote hepatocarcinogenesis in mice[17]. On the other hand, more and more studies have indicated that AhR-mediated responses are anti-proliferative in some cell types and that AhR might function as a potential target for cancer treatment[16,18]. However, the role of AhR in gastric tumorigenesis is still unclear. Andersson *et al*[19-21] reported that constitutively active AhR could induce stomach tumors and mediate down-regulation of osteopontin gene expression in a mouse model. In our previous study, we found increased expression of AhR in two human GC cell lines (RF1 and RF48) using microarray analysis[22]. A recent study suggested that concurrent expression of AhR and CYP1A1 is correlated with GC development[23].

The aim of our current study was to further determine the functional significance of AhR in gastric carcinogenesis, and to explore the potential role of AhR as a therapeutic target for GC treatment.

MATERIALS AND METHODS

Tissue specimens

Tissues of chronic superficial gastritis (CSG), chronic atrophic gastritis (CAG), intestinal metaplasia (IM) and atypical hyperplasia (AH) were obtained from 120 patients undergoing upper gastrointestinal endoscopy. Tissues of gastric tumors and their corresponding adjacent non-tumor tissues were collected from 70 GC patients who underwent GC surgery. Written, informed consent was obtained from all patients before sample collection. None of the GC patients had received preoperative chemotherapy or radiotherapy. Tissue samples were fixed in 10% neutralized formalin and embedded in paraffin for histological processing or snap-frozen in liquid nitrogen and stored at -80℃ for RT-PCR and Western blot analysis. All tissue specimens were histologically verified by a pathologist. Chronic gastritis specimens were classified according to the updated Sydney System[24]. GCs were classified according to the WHO classification[25] and Lauren's classification[26]. The clinical and histological characteristics of the study population are shown in Table 1. The study was approved by the Ethics Committee of the university hospital.

Table 1 Clinical and histological characteristics of the study population

Histology type	Patient number	Gender		Age (yr)
		Male	Female	mean ± SD
CSG	30	20	10	50.47 ± 11.63
CAG	30	12	18	53.27 ± 16.36
IM	30	16	14	52.38 ± 10.26
AH	30	15	15	55.67 ± 16.88
GC	70	37	33	56.59 ± 13.24
i-GC	32	17	15	57.27 ± 14.56
d-GC	38	20	18	55.91 ± 11.62

CSG: Chronic superficial gastritis; CAG: Chronic atrophic gastritis; IM: Intestinal metaplasia; AH: Atypical hyperplasia; GC: Gastric cancer; i-GC: Intestinal-type gastric cancer; d-GC: Diffused-type gastric cancer.

GC cell lines

Five GC cell lines- MKN28, MKN45, AGS, NCI N-87 (N87), and KATO Ⅲ-were obtained from the Riken Cell Bank (Tsukuba, Japan) and the American Type Culture Collection (ATCC, Rockville, MD, USA). All five cell lines were maintained in RPMI-1640 medium (Hyclone, USA) supplemented with 2 mmol/L glutamine, 100 mL/L fetal bovine serum (Hyclone, USA), 1×10^5 U/L of penicillin, and 0.1 g/L of gentamycin. The cellular environment was maintained at 50 mL/L CO_2 and 37℃. Cells were harvested from the exponential growth phase and total RNA and protein were prepared as described below.

RNA isolation, RT-PCR and real-time PCR

Gastric tissue specimens and cell pellets were homogenized with an ultrasound homogenizer. Total RNA in cells and tissues was extracted using the Qiagen RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. cDNA was synthesized with 1 μg total RNA using reverse transcriptase, ReverTra AceTM (Toyobo Co., Osaka, Japan) under the following conditions: 30℃ for 10 min, 42℃ for 20 min, 99℃ for 5 min, and 4℃ for 5 min. PCR of cDNA was carried out in a reaction mixture (30 μL) containing 2 μL of template cDNA, 2.5 mmol/L $MgCl_2$, 200 μmol/L dNTPs, 0.3 μmol/L primer 1 and 2, and 1 U of Taq DNA polymerase (New England Biolabs, China). Amplification was performed using the following conditions: 94℃ for 5 min, followed by 25-32 cycles (denaturation for 45 s at 94℃, annealing for 30 s, and extension for 30 s at 72℃), and then 72℃ for 7 min. Details of primers, annealing temperature, amplification cycles, and

Table 2 Primer sequences and PCR amplification conditions

Gene	Primers (5'→3')	Annealing temperature (℃)	Cycles	Product size (bp)
AhR	S: ACTCCACTTCAGCCACCATC A: ATGGGACTCGGCACAATAAA	55	25	204
CYP1A1	S: CCATGTCGGCCACGGAGTT A: ACAGTGCCAGGTGCGGGTT	59	32	174
β-actin	S: CTCGCTGTCCACCTTCCA A: GCTGTCACCTTCACCGTTC	52	30	256

S: Sense primer; A: Antisense primer.

PCR product size for each gene are listed in Table 2. The PCR products were electrophoresed on 15 g/L agarose gel, stained with ethidium bromide, and visualized with an UV transilluminator. The positive rate of mRNA expression was calculated. mRNA expression levels of AhR were further detected by quantitative real-time PCR with beta-actin as the internal reference, using the Stratagene MX3000P system (Stratagene, USA). cDNA was mixed with SYBR Green QPCR master mix (Stratagene) and primers. The thermal cycling comprised of an initial step at 95℃ for 10 min, then 40 intermediate cycles (95℃ for 30 s, 55℃ for 30 s, and 72℃ for 30 s), and one final cycle (95℃ for 1 min, 55℃ for 30 s, and 95℃ for 30 s). Real-time PCR was performed using AhR primers (5'-TACCCTGGACTTGCCTCTGC-3' and 5'-TGAAGCCAGTCAGCACCCTC-3'), and beta-actin primers (5'-TCATGAAGTGTGACGTGGACATC-3' and 5'-CAGGAGGAGCAATGATCTTGATCT-3'). Relative quantitation was calculated using the comparative threshold cycle (C_T) method. C_T indicates the fractional cycle number at which the amount of amplified target genes reaches a fixed threshold within the linear phase of gene amplification, and is inversely related to the abundance of mRNA transcripts in the initial sample. Mean C_T of duplicate measurements was used to calculate ΔC_T as the difference in C_T for target and internal reference (β-actin) genes. ΔC_T for each sample was compared to the corresponding ΔC_T of the experiment control and expressed as $\Delta\Delta C_T$. Relative quantitation was expressed as fold changes of the gene of interest compared to the experimental control according to the formula $2^{-\Delta\Delta CT}$: fold change = $2^{-\Delta\Delta CT}$

Western blot analysis

Gastric tissue specimens and cell pellets were homogenized in a lysis buffer containing 20 mmol/L HEPES, 1 mmol/L EGTA, 50 mmol/L β-glycerophosphate, 2 mmol/L sodium orthovanadate, 100 mL/L glycerol, 10 mL/L Triton X-100, 1 mmol/L DTT, and 1 × Protease Inhibitor Cocktail (Roche, Mannheim, Germany). The lysate was centrifuged at 13 000 *g* and 4℃ for 10 min. The supernatant was the total cell lysate. Protein concentration was measured using the BCA protein assay kit (Pierce Chemical Co., Rockford, IL, USA). Thirty micrograms of protein was loaded per lane, separated by 100 g/L SDS-PAGE, and transferred onto equilibrated polyvinylidene difluoride membrane by electroblotting. Membranes were blocked with TBS-T buffer containing 50 g/L non-fat dry milk. AhR, CYP1A1, and beta-actin were detected for 2 h using antibodies against AhR (SC-5579, Santa Cruz Biotechnology, USA, working dilution 1:150), CYP 1A1 (AB1258, Chemicon International, USA, working dilution 1:500), and beta-actin (4970, Cell Signaling Technology, USA, working dilution 1:1000). After secondary antibody incubation (working dilution 1:2000), enhanced chemiluminescence (Pierce Biotechnology, Inc., USA) was determined by exposure to x-ray film. Band intensities in Western blotting were quantified using Quantity One imaging analysis software. Band intensities of AhR and CYP 1A1 were normalized with corresponding band intensities of beta-actin. Data was reported as mean ± SD.

Immunohistochemistry

Paraffin sections (4 μm thickness) were dewaxed in xylene and rehydrated in graded alcohols. Antigen retrieval was performed by heating the sections for 10 min at 100℃ in 0.01 mol/L citrate buffer (pH 6.0). Endogenous peroxidase activity was quenched with 30 mL/L H_2O_2 for 15 min and non-specific staining was reduced using a blocking serum for 10 min. The sections were then incubated with rabbit anti-human AhR antibodies (SC-5579, Santa Cruz Biotechnology, working dilution 1:100) overnight at 4℃. The next day, a two-step detection method (EnVision™ Detection Kit, Gene Tech Company Limited, China) was used according to the manufacturer's instructions. Briefly, after incubation with primary antibodies the tissues were incubated with the ChemMate™ EnVision™/HRP for 30 min at room temperature. The reaction was visualized using the CheMate™ DAB plus Chromogen. Hematoxylin was used as a counterstain. Negative controls were carried out using a similar process, however, the first antibodies were omitted.

A scoring system with two categories was used to evaluate the immunohistochemical results[27]. Category A documented the number of immunoreactive cells: 0 (< 5%), 1 (5%-25%), 2 (26%-50%), 3 (51%-75%), and 4 (> 75%). Category B documented the intensity of the immunostaining: 0 (no immunostaining), 1 (weak), 2 (moderate), and 3 (strong). A final score was calculated by adding the individual scores for each category. The staining results were measured semi-quantitatively based on the final combined score: 0 (score less than 2), 1+ (score range from 2 to 3), 2+ (score range from 4 to 5), and 3+ (score range from 6 to 7). Immunostaining was assessed by an experienced histopathologist who was blinded to the clinical data of the patients.

Treatment of cells

TCDD and resveratrol were purchased from Sigma

Chemical Company (Bellefonte, PA, USA). Cells were plated on 60 mm diameter plates (for RNA preparation) and 100 mm diameter plates (for cytosolic preparation) at 80%-90% confluence in RPMI-1640. After incubating for 24 h, one group of cells was treated with TCDD at different concentrations (0, 0.01, 0.1, 1, 10, 100 nmol/L) for 24 h. A second group was also treated for an additional 24 h with TCDD (1 nmol/L) plus resveratrol (0, 1, 5, 10, 20 μmol/L). Another group was treated with TCDD (1 nmol/L) for different time intervals (0, 1, 6, 24, 48, 72 h), respectively. All drugs were dissolved in dimethyl sulfoxide (DMSO). Control cells received 1 mL/L DMSO only.

MTT Assay

A total of 1×10^4 trypsin-dispersed cells in 0.1 mL culture medium were seeded into each well of a 96-well plate and cultured for 24 h. Next, the cells were incubated with medium alone or with medium plus TCDD at different concentrations (0, 0.01, 0.1, 1, 10, 100 nmol/L) for another 12, 24 or 48 h. Then, 20 μL of MTT (5 g/L, Sigma) was added to each well and the incubation was continued for 4 h at 37℃. Finally, the culture medium was removed and 200 μL of DMSO was added to each well. The absorbance was determined with an ELISA reader at 490 nm. The cell viability percentage was calculated as: Viability percentage (%) = (Absorption value of TCDD treatment group)/(Absorption value of control group) × 100%

Flow cytometric analysis

For flow cytometric analysis, AGS cells were plated on 60-mm diameter culture plates and treated with TCDD at different concentration (0.01, 0.1, 1, 10, 100 nmol/L) for 48 h. The control contained 1 mL/L DMSO only. Prior to harvesting, the cells were washed twice with 0.01 mol/L PBS, trypsinized, and pelleted. The cells were then fixed with ice-cold 700 mL/L ethanol at 4℃ overnight. Finally, the cells were washed twice with PBS and dyed with propidium iodide (PI). The DNA content was analyzed with a flow cytometer (Beckman-Coulter, USA). The cell cycle and apoptosis of AGS cells were analyzed using MULTYCYCLE and winMDI2.9 software (Phoenix, AZ, USA). The final data was reported as the mean ± SD for each of the three independent experiments.

Statistical analysis

All quantitative data were expressed as mean ± SD and analyzed using Student *t*-tests. Immunohistochemical results were analysed using the Kruskal-Wallis test and the Mann-Whitney test. The differences in positive rates were evaluated by Fisher's exact test. All statistical analyses were carried out using the SPSS statistical software package (version 11.0, SPSS Inc.). $P < 0.05$ was considered statistically significant.

RESULTS

Expression of AhR in gastric cancer and pre-malignant tissues

RT-PCR and Western blotting were performed to analyze AhR mRNA and protein expression in 39 GC tissues and their corresponding adjacent non-cancerous tissues. Five GC cell lines (MKN28, MKN45, AGS, N87, and KATO Ⅲ) were also analyzed. Compared with non-cancerous tissues both AhR mRNA and protein expression were significantly increased in cancer tissues. The AhR mRNA positive rate was significantly higher in GC tissues compared with their corresponding adjacent non-cancerous tissues (92.31%, 36/39 *vs* 66.67%, 26/39, $\chi^2 = 7.863$; $P = 0.005$). Quantitative real-time PCR and Western blotting results indicated that both AhR mRNA (Figure 1A and B) and protein levels (Figure 1C and D) in cancer tissues were significantly higher than levels in corresponding adjacent non-cancerous tissues ($P < 0.01$). AhR expression was high in MKN28, MKN45, AGS and KATO Ⅲ cells, but very weak in N87 cells (Figure 2A-D).The five GC cell lines were derived from different sources: MKN45 and AGS were derived from poorly differentiated primary carcinoma of the stomach; MKN28 was derived from a moderately differentiated primary gastric carcinoma; N-87 was derived from a liver metastasis of a well-differentiated carcinoma; KATO-Ⅲ was derived from metastasis of gastric carcinoma. Tumor stage did not appear to correlate with the level of AhR expression.

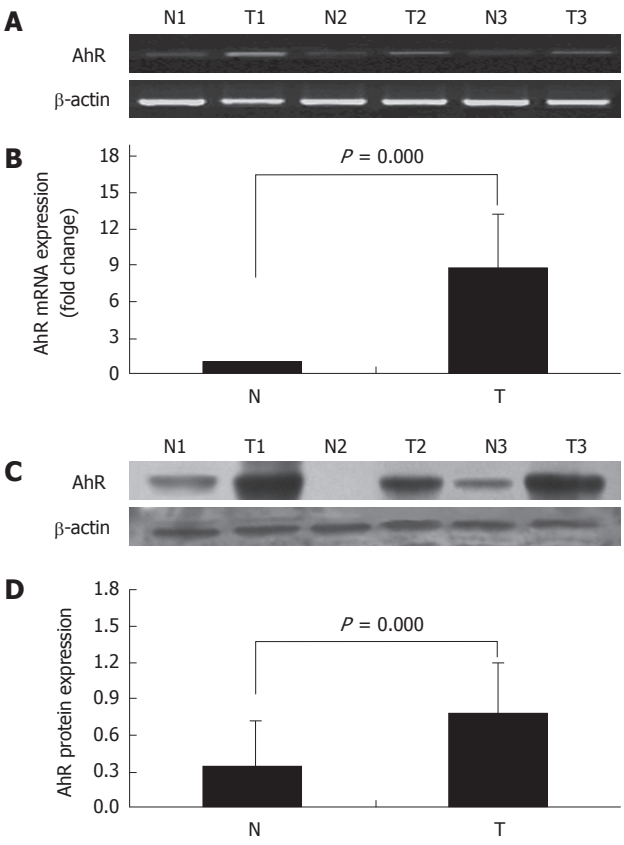

Figure 1 AhR mRNA and protein expression in GC tissues (T) and their corresponding adjacent non-cancerous tissues (N). A: AhR mRNA was detected by RT-PCR; B: AhR mRNA was detected by real-time PCR; C and D: AhR protein expression was detected by Western blotting and band intensities of AhR were normalized with corresponding band intensities of β-actin. A and C represent three cases; B and D summarize the overall mRNA and protein expression levels of AhR in all 39 cases.

Expression of AhR was further detected by

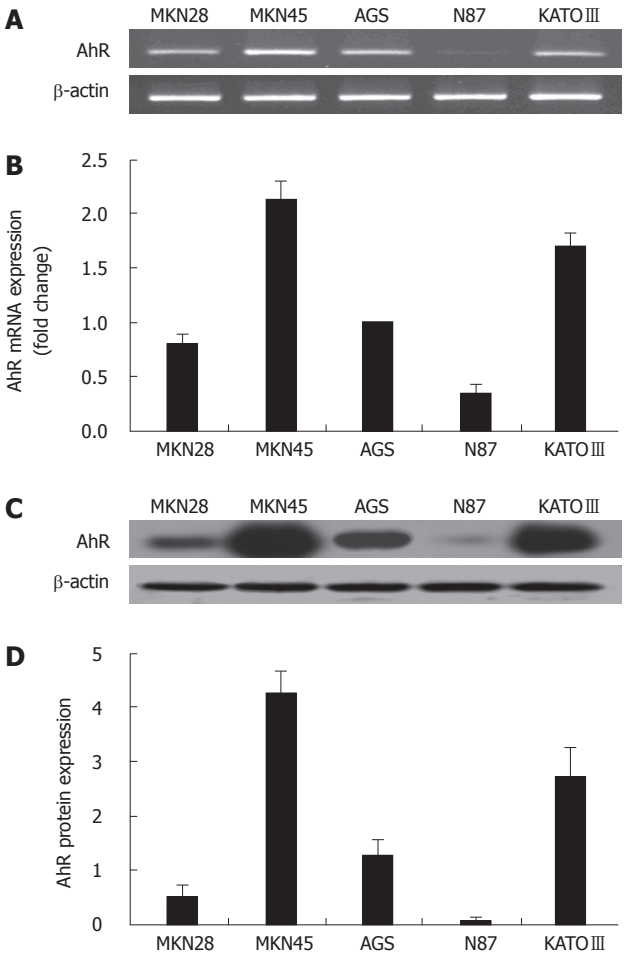

Figure 2 AhR mRNA and protein expression in five GC cell lines. A: AhR mRNA was detected by RT-PCR; B: AhR mRNA was detected by real-time PCR, AhR mRNA of AGS cells was used as the experimental control to calculate the fold changes; C and D: AhR protein expression was detected by Western blotting and band intensities of AhR were normalized with corresponding band intensities of beta-actin.

immunohistochemistry in 190 GC and pre-malignant gastric tissues: 30 CSG, 30 CAG, 30 IM, 30 AH, and 70 GC. Among the 70 GC patients, 38 suffered from Lauren diffuse type and 32 had intestinal type GC[25]. There were no significant differences in gender or age in the different groups in this study population (P = 0.095) (Table 1). Strong nuclear expression and weak cytoplasmic distribution of AhR were observed in epithelial cells of both GC and pre-malignant tissues. Interestingly, AhR expression was also found in some stroma cells of both GC and pre-malignant tissues (Figure 3). The levels of AhR expression gradually increased, with the lowest levels in CSG, followed by CAG, IM, AH and GC (Table 3). Considering the fact that AhR needs to move into the nucleus to trigger expression of its target gene, evaluation of nuclear translocation of AhR may be of more importance than assessing the overall AhR expression in both the nucleus and cytoplasm. Therefore, we further calculated nuclear expression of AhR in GC and pre-malignant tissues. As with overall AhR expression, nuclear translocation of AhR also showed an increasing trend, with the lowest expression in CSG, followed by CAG, IM, AH and GC (Table 4). Both AhR expression and nuclear translocation were significantly higher in GC than in precancerous tissues. There were no significant differences in AhR expression and nuclear translocation between diffuse type (d-GC) and intestinal type gastric cancers (i-GC) (Tables 3 and 4).

Table 3 Expression of AhR in gastric cancer and pre-malignant tissues

Histology type	Patient number	AhR expression				AhR positive rate (%)
		-	+	++	+++	
CSG	30	16	1	13	0	46.67
CAG	30	8	7	15	0	73.33
IM	30	7	8	15	0	76.67
AH	30	5	5	18	2	83.33
GC	70	2	7	35	26	97.14[1]
i-GC	32	1	3	16	12	96.88
d-GC	38	1	4	19	14	97.37

[1]Compared with CSG, CAG, IM and AH, $P < 0.05$.

Table 4 Nuclear translocation of AhR in gastric cancer and pre-malignant tissues

Histology type	Patient number	Nuclear expression of AhR				AhR nuclear positive rate (%)
		-	+	++	+++	
CSG	30	20	1	9	0	33.33
CAG	30	14	5	11	0	53.33
IM	30	13	6	11	0	56.67
AH	30	5	5	18	2	83.33[2]
GC	70	4	6	34	26	94.29[1]
i-GC	32	2	3	15	12	93.75
d-GC	38	2	3	19	14	94.74

[1]Compared with CSG, CAG, IM and AH, $P < 0.05$. [2]Compared with CSG, CAG and IM, $P < 0.05$.

Effects of AhR signal pathway activation in AGS GC cell line

To investigate the potential role of the AhR signal pathway in gastric carcinogenesis, we first treated the GC cell line AGS with a specific AhR agonist, TCDD. CYP1A1, a classic target gene of AhR, was utilized as the indicator of AhR signal pathway activation. Although a baseline level of CYP1A1 expression was observed in AGS cells, RT-PCR and Western blot analysis showed that both CYP1A1 mRNA and protein expression in AGS cells were increased in a dose- and time-dependent manner following TCDD treatment (Figure 4A-D). After TCDD treatment, while CYP1A1 protein expression increased, AhR protein in the total cell lysates gradually decreased (Figure 4C and D). To further confirm the activation of the AhR signal pathway in gastric carcinogenesis, we treated AGS cells with a specific AhR antagonist, resveratrol[28,29]. Controls included AGS cells treated with DMSO only. Experimental samples included AGS cells treated with resveratrol (10 μmol/L) only or TCDD (1 nmol/L) plus different concentrations of resveratrol (0, 1, 5, 10, 20 μmol/L), respectively for 24 h (Figure 5). In concordance with previous results, treatment of AGS cells with 1 nmol/L TCDD caused a

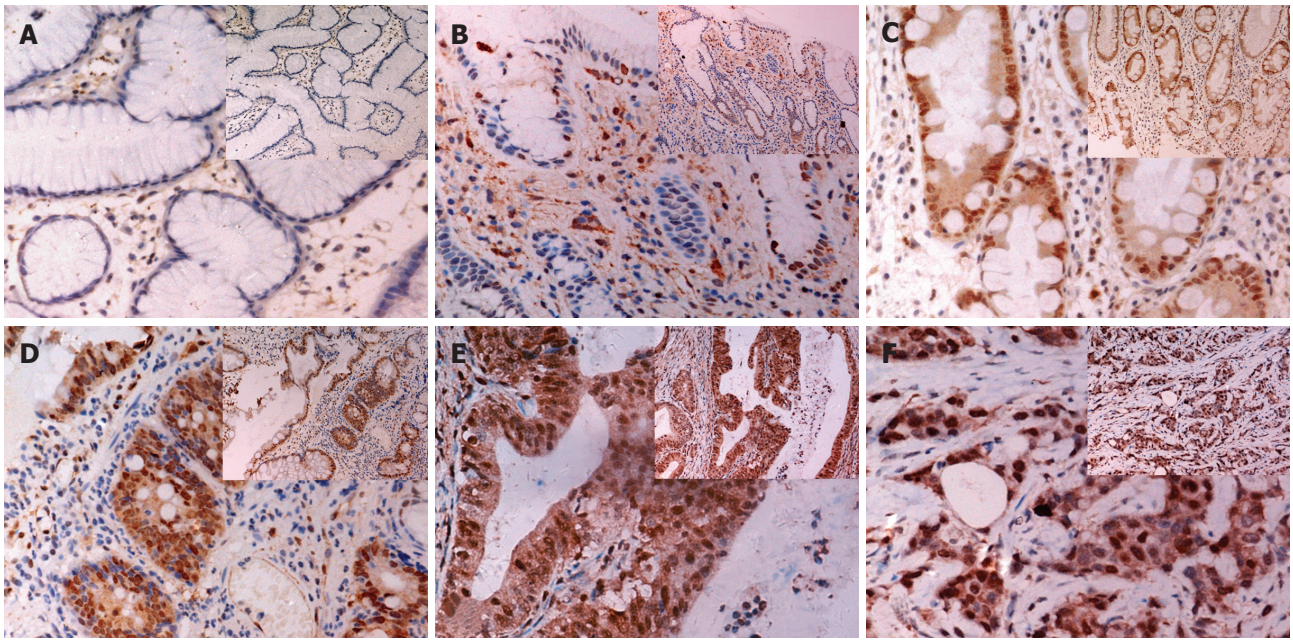

Figure 3 Immunohistochemical staining of AhR in gastric tissues. A: CSG; B: CAG; C: IM; D: AH; E: i-GC; F: d-GC (Original magnification × 400 and × 200). Strong nuclear expression and weak cytoplasmic distribution of AhR were observed in epithelial cells and some stroma cells of both GC and pre-malignant tissues.

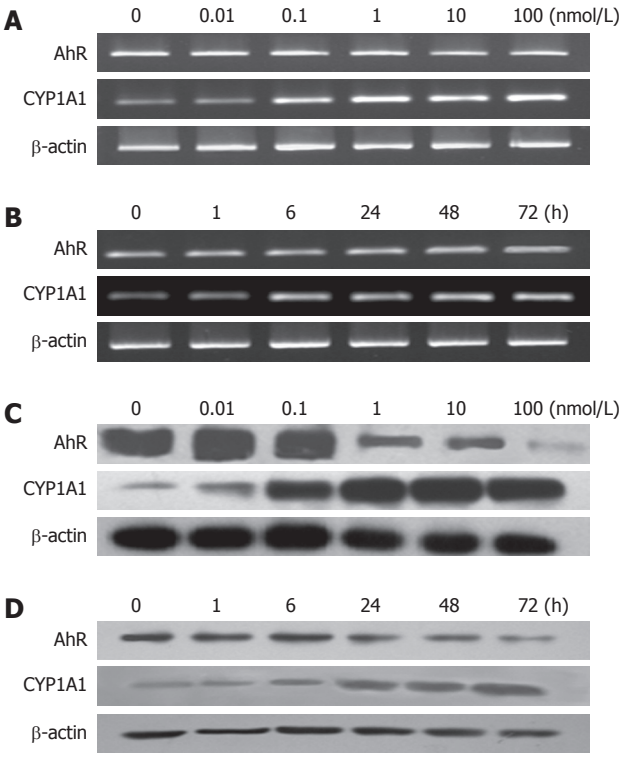

Figure 4 AhR and CYP1A1 expression in AGS cells after TCDD treatment. A and B: RT-PCR; C and D: Western blotting. Treatment of AGS cells with specific AhR agonist TCDD resulted in a dose- (A and C) and time-dependent (B and D) induction of CYP1A1 expression. The results shown are representative of three independent experiments.

remarkable increase in CYP1A1 expression. However, this TCDD-induced CYP1A1 expression was partially reversed by resveratrol in a dose-dependent manner (Figure 5A and B).

Effects of AhR activation by TCDD on the proliferation, cell cycle and apoptosis of AGS cells were further analyzed by MTT assay and flow cytometry. MTT assay demonstrated that the viability of AGS cells was significantly decreased in a dose- and time-dependent manner after TCDD treatment (Figure 6). Flow cytometric analysis demonstrated that TCDD caused a dose-dependent alteration in the cell cycle distribution of AGS cells 48 h after treatment. TCDD increased the proportion of cells in the G1 phase and correspondingly decreased the proportion in the S phase of the cell cycle. The proportion of cells in the G2 phase showed no significant change after TCDD treatment (Table 5, Figure 7). However, apoptosis of AGS cells was unable to be detected in this assay (Table 6, Figure 8). Thus, these results suggest that TCDD inhibits proliferation of AGS cells via induction of growth arrest at the G1-S phase.

DISCUSSION

AhR is an evolutionarily conserved ligand-activated transcription factor bound and activated by ubiquitous environmental pollutants. Historically, AhR has been studied for its transcriptional regulation of genes encoding xenobiotic metabolizing enzymes such as cytochrome P450 enzymes, which metabolize many of these chemicals into mutagenic and toxic intermediates. Therefore, it has been suggested that AhR may play a role in oncogenic processes, especially those initiated by environmental carcinogens[11-13]. Environmental carcinogens such as PAHs and HAHs are well-known exogenous AhR ligands that play important roles in GC[5,6]. In addition to synthetic and environmental chemicals, numerous naturally occurring dietary and endogenous AhR ligands have also been identified

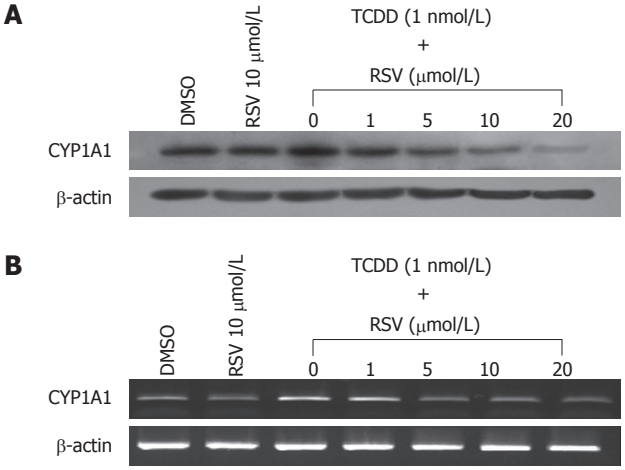

Figure 5 Inhibition of TCDD-induced CYP1A1 mRNA and protein expression by resveratrol. A: CYP1A1 protein was detected by Western blotting; B: CYP1A1 mRNA was detected by RT-PCR. The results shown are representative of three independent experiments. Treatment of AGS cells with 1 nmol/L TCDD caused a remarkable increase in CYP1A1 expression. This TCDD-induced CYP1A1 expression was partially reversed by resveratrol in a dose-dependent manner.

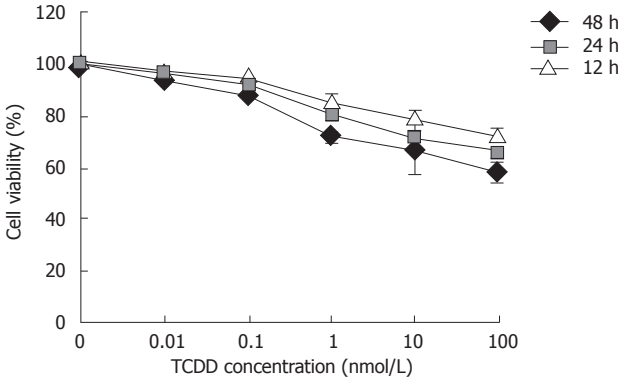

Figure 6 Viability of AGS cells after TCDD treatment was assessed by MTT assay. Viability of AGS cells was significantly decreased in a dose-dependent manner after TCDD treatment.

recently[8,10]. Since gastric epithelium may be constantly exposed to both exogenous and endogenous AhR ligands, it would be of significance to shed light on the essential role of AhR in gastric tumorigenesis. Andersson *et al*[19-21] first suggested that constitutively activated AhR could induce stomach tumors in a transgenic mouse model. In our previous study, we found increased expression of AhR in two human GC cell lines (RF1 and RF48) using microarray analysis[22]. A recent study by Ma *et al*[23] reported that concurrent expression of AhR and CYP1A1 is correlated with GC development. However, the role of AhR in human gastric tumorigenesis is still unclear.

In the current study, we first detected AhR mRNA and protein expression in 39 GC tissues and five GC cell lines using RT-PCR and Western blot analysis. Compared with their corresponding adjacent non-cancerous tissues, both AhR mRNA and protein expression were significantly increased in cancer tissues. Moreover, significantly different AhR levels in GC

Table 5 The effect of TCDD on AGS cell cycle

TCDD concentration (nmol/L)	Percentage of cell cycle (%)		
	G_0/G_1	S	G_2/M
Control	54.47 ± 0.45	39.10 ± 1.39	6.43 ± 1.48
0.01	60.47 ± 3.11[a]	33.20 ± 2.51	6.33 ± 1.12
0.1	66.07 ± 0.80[b]	28.67 ± 3.08[b]	5.33 ± 2.34
1	67.53 ± 2.57[b]	25.73 ± 4.56[b]	6.73 ± 2.06
10	67.20 ± 4.33[b]	25.03 ± 5.31[b]	7.77 ± 1.99
100	68.57 ± 5.57[b]	25.10 ± 7.41[b]	6.33 ± 1.96

Values given are the mean ± SD of three independent experiments. [a]$P < 0.05$, [b]$P < 0.01$, compared with respective control value.

Table 6 The effect of TCDD on AGS cell apoptosis

TCDD concentration (nmol/L)	Sub-G_1	*P*
Control	8.33 ± 1.59	
0.01	9.10 ± 2.46	0.583
0.1	8.20 ± 1.65	0.924
1	7.97 ± 0.31	0.792
10	6.30 ± 1.71	0.161
100	9.57 ± 1.52	0.382

Values of Sub-G_1 given are the mean ± SD of three independent experiments.

cell lines from different derivations suggest that AhR expression may not be correlated with tumor stage. Since the development of human GC is a multi-step process, we further detected the expression and distribution of AhR using immunohistochemistry in a series of GC and pre-malignant gastric tissues. Ma *et al*[23] performed similar examinations in their study. However, their study included only 39 GC tissues, 17 pre-malignant tissues, and six non-cancerous mucosa samples which were detected using immunohistochemistry. In addition, atypical hyperplasia, the most important pre-malignant histology type, was not included in their study. The small sample size in that study may not accurately reflect the real expression pattern of AhR in GC and pre-malignant tissues. In our study, we included a larger sample size and included 30 atypical hyperplasia tissues. Similar to the findings of Ma *et al*[23], our data also demonstrated a close correlation of AhR with tumor formation via enhanced expression levels and frequent nuclear translocation from pre-malignant lesions to GC. Significantly increased nuclear translocation of AhR was found even early in AH (Table 4). There were no significant differences in AhR expression and nuclear translocation between i-GC and d-GC. Our findings suggest that activation of AhR signaling may be an early event in gastric carcinogenesis. Interestingly, besides strong expression of AhR in epithelial cells, AhR expression was also found in some stroma cells of both GC and pre-malignant tissues. Trombino *et al*[30] reported similar findings in their study of mammary tumorigenesis. Using a rat model of PAH-induced mammary tumorigenesis, they demonstrated that AhR expression levels were significantly elevated in PAH-induced mammary tumors as well as in stroma elements surrounding these tumors. Since stroma

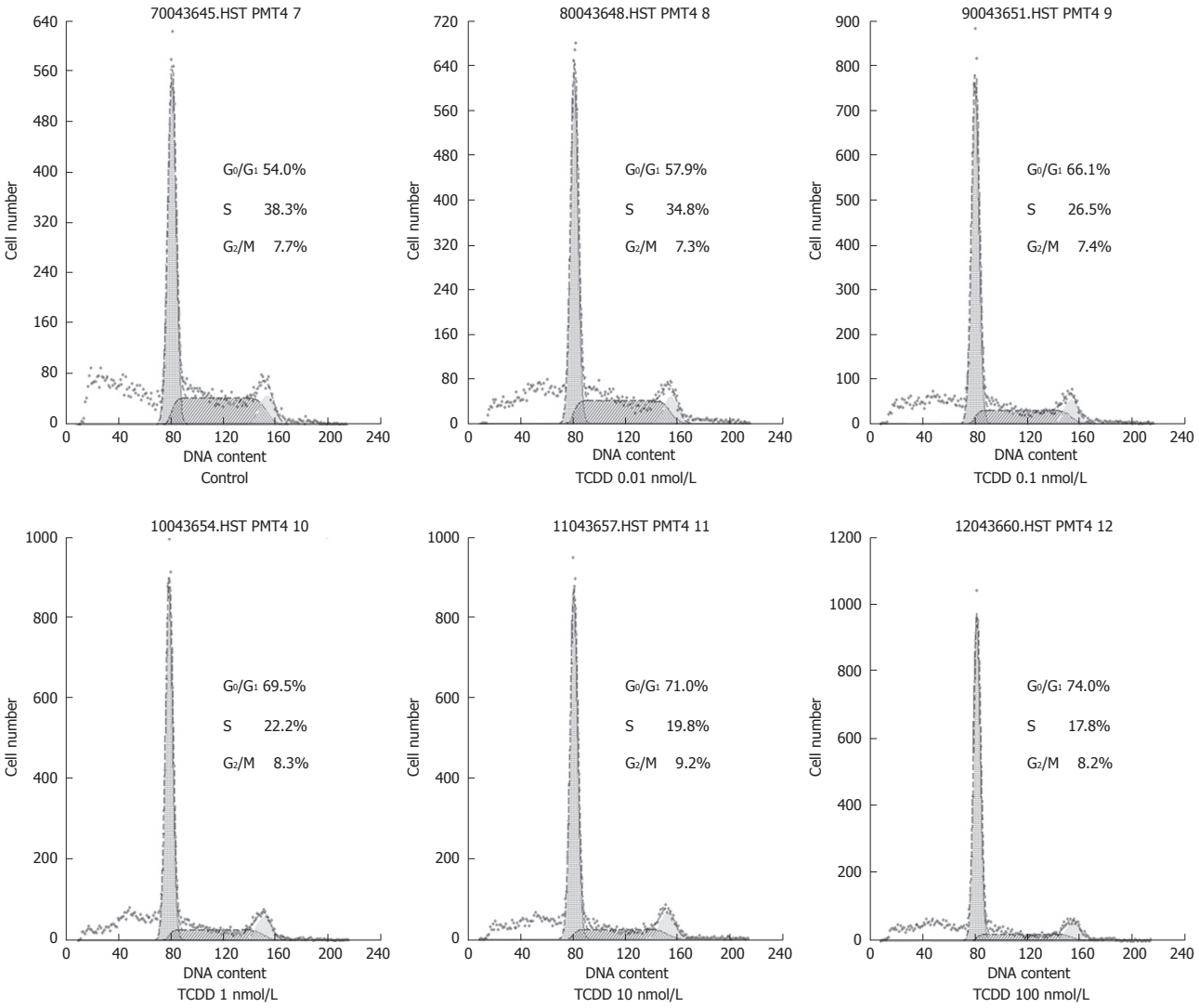

Figure 7 The effect of TCDD on AGS cell cycle distribution. AGS cells were treated with different concentrations of TCDD and subjected to flow cytometric analysis. The percentage of each phase is indicated in each panel. The results shown are representative of three independent experiments.

elements play important roles in maintaining the microenvironment and regulating growth of epithelial cells, expression of AhR in stroma cells may have a bearing on malignant transformation of gastric epithelial cells.

To further investigate the potential role of the AhR signal pathway in gastric carcinogenesis, we treated GC cell line AGS with the most potent AhR agonist, TCDD, and chose CYP1A1, a classic target gene of AhR, as the indicator of AhR signal pathway activation. Although both CYP1A1 and CYP1B1 are classic target genes of AhR, cell-specific expression of these two genes have been reported previously[30-32]. Over-expression of CYP1A1, but not CYP1B1 in GC has been reported by Ma *et al*[23] and Zhang *et al*[33]. Baseline levels of CYP1A1 expression were also observed in AGS cells in the present study. However, expression of CYP1A1 was significantly increased in a dose- and time-dependent manner after TCDD treatment, indicating the activation of AhR. Interestingly, while CYP1A1 protein expression increased, AhR protein in the total cell lysates gradually decreased (Figure 4C and D). Similar phenomena have been reported by several other groups[34-36]. Recent studies have demonstrated that the down-regulation of AhR following ligand binding is ubiquitin mediated and occurs *via* the 26S proteasome pathway following nuclear export of AhR. The degradation of AhR is the endpoint and would be one of the key factors controlling gene regulations by the AhR signal pathway[37]. To confirm the activation of the AhR signal pathway by TCDD, we treated AGS cells with a specific AhR antagonist, resveratrol. Previous studies suggested that resveratrol can regulate the transcription of AhR targeted genes by preventing AhR from binding to the enhancer sequences of the gene promoter[28,29]. Our results showed that TCDD-induced CYP1A1 expression was partially reversed by resveratrol in a dose-dependent manner. The incomplete reversal of CYP1A1 expression by resveratrol may be due to the fact that AhR is not the only regulator of CYP1A1 transcription[38,39]. Taken together, these results suggest that the AhR signal pathway could be activated in GC cells and that abnormal activation of the AhR signal pathway may be involved in gastric carcinogenesis.

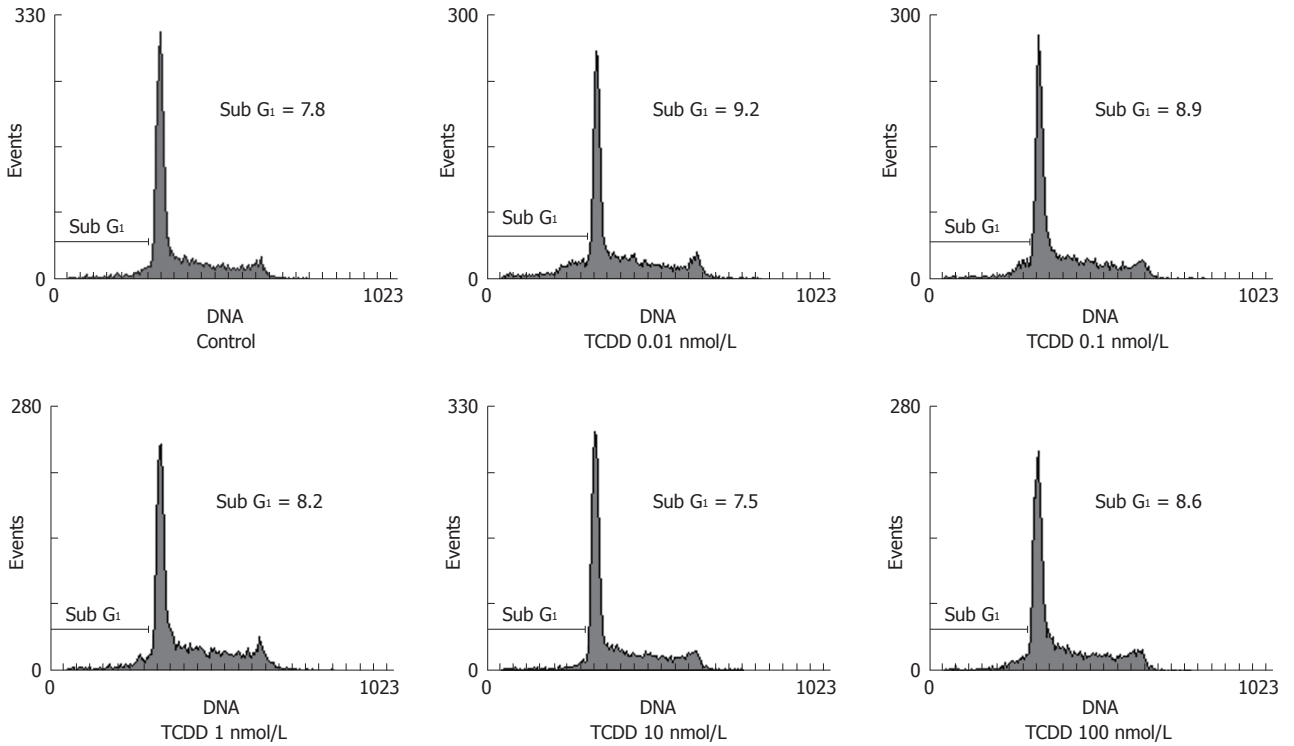

Figure 8 The effect of TCDD on apoptosis of AGS cells. AGS cells were treated with different concentrations of TCDD and subjected to flow cytometric analysis. Cellular apoptosis was evaluated by fragmented DNA (sub-G_1) analysis using winMDI2.9. The results shown are representative of three independent experiments.

Since AhR is significantly up-regulated in GC and may be involved in the early stage of gastric carcinogenesis, regulation of the AhR pathway may have a potential role in the treatment of GC. Interestingly, our MTT assay demonstrated that the viability of AGS cells was significantly decreased in a dose- and time-dependent manner after TCDD treatment. Further flow cytometric analysis indicated that TCDD inhibited growth of AGS cells *via* induction of growth arrest at the G1-S phase. As far as we know, this is the first report suggesting an inhibitory role of AhR agonists on human GC cell growth. Similar results have been reported in the treatment of pancreatic cancer and mammary tumors by AhR agonists[16,18,40]. However, previous studies by Andersson *et al*[19,20] showed that constitutively active AhR may result in significant proliferation in the parietal/chief cell region of glandular gastric mucosa in transgenic mice. These contradictory outcomes indicate that AhR appears to contribute to processes in both cell cycle arrest as well as cell proliferation. Recent studies on the cellular signal pathway may partly explain this complex phenomenon. As an evolutionarily conserved transcription factor, outside its well-characterized role in the induction of xenobiotic metabolizing enzymes, AhR also functions as a modulator of cellular signaling pathways. By interacting with different signal pathway effectors, AhR activation may result in completely different effects on cell growth[12,13]. Our present findings on the inhibitory effect of TCDD on GC cell growth suggest that AhR may be a potential therapeutic target for gastric cancer.

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COMMENTS

Background

The carcinogenesis of gastric cancer (GC) involves numerous genetic and epigenetic alterations, as well as many environmental risk factors. Environmental pollutants such as polycyclic aromatic hydrocarbons (PAHs) and halogenated hydrocarbons (HAHs) are well-known carcinogens that play important roles in GC development. The toxic effects of PAHs and HAHs are mediated by a conserved signaling pathway that binds and activates the aryl hydrocarbon receptor (AhR).

Research frontiers

AhR is a ligand-activated transcription factor and can mediate the carcinogenic and other toxic effects of a variety of environmental pollutants. Many studies in recent years have demonstrated a close relationship between AhR and tumorigenesis. However, the role of AhR in gastric tumorigenesis is still unclear. In this study, the authors demonstrate a close correlation of AhR with GC formation and the potential role of AhR as a therapeutic target for GC treatment.

Innovations and breakthroughs

AhR functions as a modulator of cellular signaling pathways. By interacting with different signal pathway effectors, AhR activation may result in completely different effects on cell growth. This is the first report suggesting an inhibitory role of AhR agonists on human GC cell growth. Furthermore, the present findings suggest that AhR may be a potential therapeutic target for GC.

Applications

By understanding how cell growth of human GC is influenced by AhR activation, this study may represent a future strategy for therapeutic intervention in the treatment of patients with GC.

Terminology

AhR is a ligand-activated transcription factor of the basic helix-loop-helix/Per-

Arnt-Sim family. As an evolutionarily conserved transcription factor, outside its well-characterized role in the induction of xenobiotic metabolizing enzymes, AhR also functions as a modulator of cellular signaling pathways.

Peer review

The authors investigated the functional significance of AhR in gastric carcinogenesis. This paper is interesting and written well.

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