Functional analysis of six human aryl hydrocarbon receptor variants in a Japanese population

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ABBREVIATIONS: AhR, aryl hydrocarbon receptor; Arnt, aryl hydrocarbon receptor nuclear translocator; hAhR, human AhR; hArnt, human Arnt; SNP, single nucleotide polymorphism; PLSD, protected least significant difference; WT, wild type; BNF, β-naphthoflavone; 3MC, 3-methylcholanthrene; OME, omeprazole; DAPI, diamidino-2-phenylindole; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; DMSO, dimethylsulfoxide
Abstract

Aryl hydrocarbon receptor (AhR) is an important transcriptional regulator involved in the induction of CYP1A1, CYP1A2, CYP1B1, UGT1A1, and UGT1A6. In this study, functional properties of four novel naturally occurring human AhR variants (K401R, N487D, I514T, and K17T/R554K) were examined along with the single variants, K17T and R554K. The luciferase reporter assay using the CYP1A1 promoter reporter in HeLa cells treated with β-naphthoflavone or 3-methylcholanthrene, which are known as typical agonists for AhR, showed that reporter activities of the K401R and N487D variants were reduced to 40-58% of those of wild type but not of the other variants. Similarly, the K401R and N487D variants also reduced the omeprazole-induced reporter activities to approximately 56% and 74% of those of the wild type (WT), respectively. The reduced activities of the two variants were probably caused by the reduced protein expression levels, since the protein levels of the K401R and N487D variants were approximately 52% and 47% of the WT, respectively, without any changes in their mRNA levels. The reduced protein levels were recovered by treatment with a proteasome inhibitor MG-132, suggesting that the reduced protein levels were caused by the accelerated proteasomal degradation by a proteasome. Taken together, the current data demonstrate that the K401R and N487D variants reduce their apparent transcriptional activities, both ligand-induced and omeprazole-induced activation, probably through reduced protein expression. Thus, these two variants may influence drug metabolism through reduced induction of CYP1A1 and other target enzymes.
Human aryl hydrocarbon receptor (AhR), encoded by the \textit{AHR} gene, is a member of the basic helix-loop-helix/Per-Arnt-Sim (bHLH-PAS) family of transcriptional factors (Mimura and Fujii-Kuriyama, 1998). Its mRNA is dominantly expressed in the placenta, lung, heart, pancreas, and liver (Dolwick et al., 1993a). Under resting conditions, AhR exists as a cytosolic complex with Hsp90, the co-chaperone p23, and the immunophilin-like protein XAP2. Upon binding a ligand, following the replacement of its associated molecule with AhR nuclear translocator (Arnt), AhR translocates into the nucleus, and binds to the xenobiotic responsive elements (XRE) found in the regulatory elements of a variety of genes. For example, AhR ligands activate the transcription of drug metabolizing enzymes genes \textit{CYP1A1}, \textit{CYP1A2}, \textit{CYP1B1}, \textit{UGT1A1}, and \textit{UGT1A6} through XREs in the enhancer regions of these genes (Quattrochi et al., 1994; Emi Y et al., 1996; Munzel et al., 1998; Zhang et al., 1998; Whitlock, 1999; Yueh et al., 2003; Sugatani et al., 2004).

Human AhR protein consists of many functional domains, including bHLH-PAS (amino acid residues 13-81, 111-181, and 275-342), Hsp90 interacting (27-79 and 182-374), Arnt-interacting (40-79 and 182-374), nuclear localization (13-39), nuclear export (55-75), and transactivation (490-805) domains (Dolwick et al., 1993b; Fukunaga et al., 1995; Ikuta et al., 1998; Denison et al., 2003).

AhR has been reported to be activated by various exogenous aromatic hydrocarbons: e.g., 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), 3-methylcholanthrene (3MC), and \(\beta\)-naphthoflavone (BNF) (Song et al., 2002; Denison et al., 2003). Recently, several endogenous ligands were also identified, such as tryptophan derivatives (e.g., indirubin) and arachidonic acid metabolites (e.g., lipoxin A4) (Song et al., 2002; Denison et al., 2003).

The \textit{AHR} gene is located on chromosome 7p15 and consists of 12 exons, including a non-coding exon (exon 12) (Micka et al., 1997). Over 30-fold interindividual differences in the CYP1A1 inducibility by aromatic hydrocarbons have been reported in mitogen activated lymphocytes (Catteau et al., 1995; Kawajiri et al., 1995). Furthermore, it was suggested that the 7p15 region was involved in such interindividual differences (Micka et al., 1997). From these findings, it is possible that altered AhR transcriptional activity caused by genetic polymorphisms of the \textit{AHR} gene might affect the inducibility of target genes.
Several genetic polymorphisms have been reported in the \textit{AHR} gene (Harper et al., 2002). The most common single nucleotide polymorphism (SNP) is 1661G>A (R554K), which was first found by polymerase chain reaction (PCR)-single strand conformational polymorphism analysis, followed by direct sequencing of these products from 25 Japanese subjects (Kawajiri et al., 1995). The functional effect of this variation was marginal \textit{in vitro} TCDD-induced \textit{CYP1A1} mRNA expression (Wong et al., 2001a). However, another study suggested that 3-methylcholanthrene-induced CYP1A1 activity in lymphocytes was significantly higher in Lys-554 Caucasian subjects than in Arg-554 ones (Smart et al., 2000). Furthermore, a less frequent variation found in African populations, Val570Ile, is linked with Arg554Lys, and this haplotype shows an abrogated TCDD-induced \textit{CYP1A1} mRNA expression (Wong et al., 2001b). Thus, it is suggested that the genetic polymorphisms in \textit{AHR} at least partly contribute to the interindividual differences in CYP1A1 expression. However, there are few reports on AhR variants altering the CYP1A1 inducibility, especially in Asian populations.

We had recently reported the four novel variations K17T, K401R, N487D, and I514T in a Japanese population (Fukushima-Uesaka et al., 2004). In this study, functional properties of the four haplotype-based naturally occurring variants (K401R, N487D, I514T, and K17T/R554K) of human AhR were examined.
Materials and Methods

Haplotype analysis

The haplotypes regarding novel and known nonsynonymous variations were analyzed. K401R and N487D were found as single heterozygous SNPs. K17T or I514T was found together with a known variation R554K in the same subject. To determine a linkage between these variations, the amplified genomic fragments from the subjects were cloned into the pENTR/D-TOPO vector (Invitrogen Corp., Carlsbad, CA) and sequenced with an ABI Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and ABI Prism 3730 DNA Analyzer (Applied Biosystems). The linkage between K17T and R554K was determined by linkage between K17T and −742C>T that was linked with R554K. To determine the linkage between I514T and R554K, the genomic fragments containing the codons 514 and 554 were sequenced. Ethical review boards of the National Cancer Center and the National Institute of Health Sciences approved this study.

Plasmids

hAhR cDNA was cloned into the pcDNA3.2-DEST vector (Invitrogen Corp.) and the resulting expression plasmid (pcDNA3.2-DEST-hAhR) was verified according to the reference sequence (Genbank accession number NM_001621). Similarly, hArnt cDNA was cloned into pcDNA3.2-DEST and the resulting expression plasmid (pcDNA3.2-DEST-hArnt) was verified according to the reference sequence (Genbank accession number NM_001668). Six expression plasmids encoding the variant hAhR (K17T, K401R, N487D, I514T, R554K, and K17T/R554K) were prepared with a QuickChange Site-Directed Mutagenesis Kit (Stratagene Co., La Jolla, CA). The empty vector plasmid used as a control was prepared by removing the Gateway recombinational region between SacI and ApaI fragment from pcDNA3.2-DEST. A human CYPIA1 promoter region (-1566 to +73; Genbank accession number AC091230.23) was subcloned into the HindIII-digested pGL3 basic vector (Promega, Madison, WI) as described previously (Morel and Barouki, 1998). phRL-TK (Promega) encoding Renilla (sea pansy) luciferase was used for cotransfection and normalization of transfection efficiency.
Cell culture

HeLa cells were obtained from JCRB (National Institute of Health Sciences) and grown in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% FBS, and 100 U/ml penicillin and streptomycin under an atmosphere of 5% CO₂ at 37°C.

AhR activators

Two AhR ligands, β-naphthoflavone (BNF) and 3-methylcholanthrene (3MC), were purchased from Sigma-Aldrich Co. (Sigma-Aldrich Co., St. Louis, MO). An AhR activator omeprazole (OME) was also purchased from Sigma-Aldrich Co.

TaqMan real-time RT-PCR

HeLa cells (1.4 x 10⁵ cells) were transfected with 600 ng of the wild-type AhR expression plasmid or each of the variant AhR expression plasmids together with 600 ng of the hArnt expression plasmid by using the FuGENE 6 Transfection Reagent (Roche Diagnostics Corp., Indianapolis, IN). Four hours after transfection, the cell culture media was replaced and the cells were cultured for an additional 48 h. Then, the cells were harvested, and total RNA was extracted with an RNeasy kit (Qiagen) in combination with the RNase-free DNase-treatment to minimize plasmid contamination of samples. Each RNA sample (500 ng) was reverse-transcribed using an oligo d(T)₁₆ primer with TaqMan Reverse Transcription Reagents (Applied Biosystems, Inc.) according to the manufacturer’s instructions. PCR reactions were performed with hAhR specific primers and TaqMan probe (Assay ID: Hs00169233_m1, Applied Biosystems, Inc.) using TaqMan Universal PCR Master Mix (Applied Biosystems, Inc.) with an ABI7700 PCR thermal cycler. β-Actin mRNA expression levels were used as an internal control. All the AhR mRNA expression levels were normalized according to the β-Actin mRNA levels. The expression levels were shown as averages of three separate transfection experiments.

Western blot analysis

HeLa cells (1.4 x 10⁶ cells) were cotransfected with 6 µg of expression plasmids for the wild type or variant hAhRs together with 6 µg of the hArnt expression plasmid using the FuGENE 6 Transfection Reagent (Roche Diagnostics Corp.). Four hours after
transfection, the cell culture media was replaced with a fresh one and the cells were then cultured for 48 h. Next, the cells were harvested, and the cell pellets were boiled in a protein sample buffer. Total cellular proteins were separated on a 8% SDS-polyacrylamide gel (Tefuco Co., Tokyo, Japan) and transferred onto a PVDF membrane (Bio-Rad Lab., Hercules, CA). The membrane was blocked with 3% bovine serum albumin (Nakalai Tesque Inc., Kyoto, Japan), and incubated with polyclonal rabbit anti-AhR antibody (BIOMOL, Plymouth Meeting, PA) and then goat anti-rabbit IgG conjugated with horseradish peroxidase (Amersham Biosciences, Buckinghamshire, UK) for the secondary antibody. AhR proteins were visualized with the WestFemto maximum sensitivity substrate (Pierce Biotechnology Inc., Rockford, IL) according to the manufacturer’s instructions. Equal amounts of protein (100 µg) were used for Western blot analysis from each expression plasmid-transfected cell lysates. The expressed levels were obtained from three separate transfection experiments.

**MG-132 treatment**

HeLa cells (1.4 x 10⁵ cells) were cotransfected with 600 ng of the wild type, the K401R or N487D hAhR expression plasmid together with 600 ng of the hArnt expression plasmid using the FuGENE 6 Transfection Reagent. Four hours after transfection, the cell culture media was replaced and the cells were continuously cultured for 24 h. Then, the cells were treated with vehicle (DMSO) or 10 µM of a proteasome inhibitor MG-132 (CALBIOCHEM, San Diego, CA) for 8 hours. Next, the cells were harvested, and the cell pellets were subjected to Western blotting as described above.

**Luciferase reporter assay**

HeLa cells (2.8 x 10⁴ cells) were cotransfected with 100 ng of the wild-type hAhR expression plasmid pcDNA 3.2-DEST hAhR or the variant hAhR expression plasmid together with 100 ng of the hArnt expression plasmid, 30 ng of pGL3-hCYP1A1 promoter plasmid, and 10 ng of phRL-TK plasmid as an internal control. Four hours after transfection, the cells were treated with a vehicle (DMSO) or various concentrations of BNF, 3MC, or OME, and then cultured for an additional 48 h. The cells were washed with PBS, and the lysates were prepared using the Dual Luciferase Reporter Assay
System (Promega) described previously (Koyano et al., 2003; Koyano et al., 2004). All transfection efficiencies were normalized according to the Renilla luciferase activity. Quantification of the luciferase activity was done by three independent transfections. The values of basal luciferase activity with the empty vector were subtracted from those with the AhR expression plasmids.

**Immunocytochemistry**

Immunocytochemistry was performed as described previously (Ikuta et al., 1998; Koyano et al., 2003). Briefly, HeLa cells (1.4 x 10^5 cells) were transfected with 600 ng of the expression plasmid for empty, wild type, or variant AhR together with 600 ng of the Arnt expression plasmid, and then cultured for 4 h. The cells were treated with a vehicle (DMSO) or 1 µM 3MC for 48 h. Transfected HeLa cells were washed twice with PBS, fixed with 3.7% formaldehyde in PBS for 15 min at room temperature, and then permeabilized with 0.5% Triton X-100 for 15 min. The fixed cells were blocked by incubation with PBS containing 10% goat serum and 100 ng/ml of DAPI (Santa Cruz Biotechnology, Inc.) for 30 min at room temperature, and then washed with PBS containing 0.05% Tween-20. Next, the cells were incubated overnight at 4°C with rabbit polyclonal anti-AhR antibody (BIOMOL) diluted in PBS containing 10% goat serum. They were washed 5 times with PBS containing 0.05% Tween-20, and incubated for 1 h at room temperature with goat anti-rabbit IgG conjugated with Alexa 594 (FUNAKOSHI, Co., Tokyo, Japan) diluted in PBS containing 10% goat serum. Immunoreactive AhR and the nuclei stained with DAPI were visualized by fluorescent microscopy.

**Statistical analysis**

The data of the TaqMan real-time RT-PCR assay, Western blot analysis, and reporter assays were assessed for their statistical significance by one-way analysis of variance, followed by Fisher's PLSD method or Student t-test using StatView software (SAS Institute Inc., Cary, NC).
Results

Haplotype analysis for the non-synonymous AhR variations

Four novel variations (K17T, K401R, N487D, and I514T) were found in previous sequence analysis of the AhR gene from 242 Japanese individuals (Fukushima-Uesaka et al., 2004). We also detected a common SNP, R554K at a 0.444 frequency (Fukushima-Uesaka et al., 2004). K401R and N487D were found as individual heterozygous SNPs. K17T or I514T was found together with R554K in the same subject. By genomic cloning and subsequent sequencing, K17T was shown to be linked to the R554K, while I514T was not. Thus, functional properties of the variants K17T/R554K, K401R, N487D, and I514T were examined in transiently transfected HeLa cells. In addition, functional analysis for the single variants, K17T and R554K, was also performed.

mRNA expression levels of variant AhR in the transfected HeLa cells

To determine the expression levels of variant AhR mRNAs, the TaqMan real-time RT-PCR assay was performed using total RNA from HeLa cells transfected with the wild type (WT) or variant expression plasmids (n=3) (Fig. 1). By the RT-PCR assay, the AhR mRNA expression levels of K17T, K401R, N487D, I514T, R554K, and K17T/R554K variants were estimated at 122.7±33.8, 106.3±12.6, 101.7±17.2, 118.8±17.9, 119.9±9.1, and 118.9±9.1, respectively, when the expression level of WT AhR was defined as 100. Thus, the mRNA expression level of each AhR variant was comparable to that of WT.

Protein expression levels of variant AhR in total cell lysates

In order to quantify the expression levels of the variant AhR proteins, we performed immunoblotting for whole cell lysates obtained from the transfected HeLa cells (n=3). Although we detected a low level of AhR protein in HeLa cells, which is consistent with a previous report (Pimental et al., 1993 and data not shown), it was negligible as compared with the overexpressed AhR proteins (Fig. 2). By densitometric quantification, the protein expression levels of the K17T, K401R, N487D, I514T, R554K, and K17T/R554K AhR variants were estimated at 101.6±7.0, 52.2±5.4 (p<0.0001), 47.4±6.3 (p<0.0001), 105.5±5.7, 104.9±6.1, and 95.6±6.4, respectively, when the WT expression level was...
defined as 100. Thus, no significant difference in the expressed protein levels was found among WT, K17T, I514T, R554K, and K17T/R554K (Fig. 2). However, K401R and N487D expressions were significantly reduced compared to that of the WT (Fig. 2). Taken together with the results of the mRNA expression levels, it is likely that the reduction of protein levels seen in K401R and N487D were caused by protein instability or ineffective translation in HeLa cells.

**Effect of MG-132 on the protein levels of the K401R and N487D variants**

To clarify whether the reduced protein levels in the K401R and N487D variants are caused by accelerated protein degradation by proteasomes, transfected cells were treated with or without a proteasome inhibitor MG-132. The wild type AhR protein levels slightly increased to 128.2±6.7% when the cells were treated with MG-132 (Fig. 3). In contrast, the protein levels of the K401R and N487D variants were dramatically restored by addition of MG-132 (313.6±20.6% and 270.2±23.8%, p<0.0005 and p<0.001 by the Student t-test, respectively) (Fig. 3). This suggests that the reduced K401R or N487D levels are caused by the accelerated degradation through a proteasomal pathway.

**Transcriptional activity of the variant AhRs**

To investigate the transcriptional activity of the variant AhRs, HeLa cells were transfected with the WT or variant AhR expression plasmid together with a plasmid containing the human \(CYP1A1\) promoter-luciferase reporter construct (pGL3-h\(CYP1A1\) promoter). The effects of AhR ligands or activator were also determined. The results of the reporter gene experiments are shown in Figs. 4-6 (n=3). Transfection of HeLa cells with the empty vector plasmid alone showed a significant but very weak luciferase activity, even in the presence of BNF, and this basal activity was subtracted from the luciferase activity in AhR-transfected cells. BNF (0-20 nM) increased the WT luciferase activity in a dose-dependent manner. Similarly, the K17T, I514T, R554K, and K17T/R554K variants showed luciferase activities comparable to those of WT at all the BNF concentrations used (0-20 nM) (Fig. 4). The K401R variant, however, showed significant decreases in the BNF-induced activities (approximately 50.1±4.2% (p<0.0005) and 39.6±11.9% (p<0.0005) of the WT levels for 2 and 20 nM BNF, respectively) (Fig. 4). Similarly, N487D also had a reduced induction of the luciferase activity.
activity (approximately 50.7±3.4% \((p<0.0005)\) and 57.8±7.6% \((p<0.005)\) of the WT levels for 2 and 20 nM BNF, respectively).

As for 3MC, the luciferase activities of K17T, I514T, R554K, and K17T/R554K showed only marginal differences compared with those of the WT. However, K401R and N487D showed significantly reduced 3MC-dependent activities when the cells were treated with 10 and 100 nM 3MC (55.9±14.1% (K401R at 10 nM; \(p<0.05\)), 40.0±9.3% (K401R at 100 nM; \(p<0.0005\)), 63.3±14.7% (N487D at 10 nM; \(p<0.05\)), and 50.7±6.4% (N487D at 100 nM; \(p<0.0005\)) (Fig. 5).

Furthermore, in the cells treated with OME, the luciferase activities of the K17T, I514T, R554K, and K17T/R554K variants showed transcriptional activities similar to those of the WT, whereas K401R and N487D showed significant decreased OME-dependent activities compared to WT at 2 µM OME (55.7±9.4% \((p<0.005)\) for K401R and 74.1±7.0% \((p<0.05)\) for N487D) (Fig. 6).

**Subcellular localization of the variant AhR**

AhR in the cytoplasm exists as a ligand-free form (Ikuta et al., 1998). In order to investigate the subcellular localization of each variant AhR, immunostaining of HeLa cells transfected with the WT or variant AhR plasmids was performed using 3MC as a ligand (data not shown). The localization patterns of all the six variant proteins were similar to that of the WT (data not shown). Thus, all the variants are active in terms of protein nuclear localization although K401R and N487D show apparently reduced ligand-dependent and OME-induced transcriptional activities for the *CYP1A1* promoter reporter.
Discussion

Aryl hydrocarbon receptor (AhR) is an important transcriptional regulator involved in the inductions of CYP1A1, CYP1A2, CYP1B1, UGT1A1, and UGT1A6. In order to identify the single nucleotide polymorphisms (SNPs) of the \textit{AHR} gene that affect the expression of these drug metabolizing enzymes, we surveyed \textit{AHR} SNPs and found four novel heterozygous SNPs, K17T, K401R, N487D, and I514T. The allele frequencies of K17T, K401R, N487D, and I514T were 0.002, 0.010, 0.002, and 0.002, respectively (Fukushima-Uesaka et al., 2004). The common SNP R554K was also detected at a frequency of 0.444.

Previous reports have shown that multiple amino acid substitutions exert different (or rather counteracting) effects on the protein expression levels and/or functions. For example, an additional R139K variation in CYP2C8 restores the reduced protein expression caused by K399R alone (both variants form CYP2C8*3) (Soyama et al., 2001). As for AhR, Wong \textit{et al.} has reported that V570I is linked with the common SNP R554K, and that this haplotype shows abrogated TCDD-induced \textit{CYP1A1} mRNA expression (Wong et al., 2001b). Therefore, the linkage between these four SNPs and R554K was analyzed. Our data indicated that K17T was linked to R554K, whereas SNPs K401R, N487D, and I514T were not linked. Next, functional properties of K17T/R554K, K401R, N487D, and I514T were assessed in transiently transfected HeLa cells. The results showed that K401R and N487D were partially defective variants.

Successive deletions of the C-terminal domain showed that the region containing K401 and N487 was important for both ligand binding and ligand-independent DNA binding (Dolwick et al., 1993b). The expression of the K401R and N487D variant proteins were reduced to approximately 52% and 47% of the WT level, respectively, without any changes in their mRNA expression levels (Figs. 1 and 2). The reductions were restored by addition of a proteasome inhibitor MG-132. Thus, these reduced expressions are assumed to be caused by accelerated protein degradation by proteasomes (Fig. 3). Furthermore, our present data showed that the luciferase activities of the K401R and N487D variants were reduced to 40-58% of those of WT when BNF and 3MC were used as the ligands (Figs. 4 and 5). Furthermore, the OME-induced activity was also reduced to 56% and 74% of those of the WT for K401R and N487D, respectively (Fig. 6).
BNF and 3MC are well-known classical ligands for AhR (Song et al., 2002; Denison et al., 2003), but OME is thought not to bind to AhR (Dzeletovic et al., 1997; Backlund and Ingelman-Sundberg, 2004). Meanwhile, Y320F substitution of the AhR was found to be resistant to activation by OME but not by TCDD (Backlund and Ingelman-Sundberg, 2004). Thus, the Tyr-320 was important for AhR activation, and OME mediate AhR activation via the ligand-independent mechanism probably through a tyrosine kinase-dependent signal transduction pathway, leading to conformational changes of AhR similar to those caused by ligand binding (Backlund and Ingelman-Sundberg, 2004; Lemaire et al., 2004). The immunostaining data revealed that, similar to the WT protein, the K401R and N487D variant proteins were localized in the nuclei of cells treated with 3MC (data not shown). These data suggested that the K401R and N487D variants showed reduced ligand-dependent and OME-induced AhR activities probably through reduced protein levels. This was confirmed by the following experiment. HeLa cells transfected with WT, K401R or N487D together with the CYP1A1 reporter were treated with BNF, 3MC or OME in the presence or absence of MG-132. The results showed that MG-132 restored the luciferase activities for K401R and N487D with BNF, 3MC or OME to the levels comparable to those for the respective WT (data not shown). The findings suggested that the intrinsic transcriptional activities of the K401R and N487D variants are comparable to that of WT, and that their decreased absolute transcriptional activities in the absence of MG-132 are most likely due to their reduced protein expression levels.

Alterations in inducing activity of a transcriptional factor often reflect changes in its intrinsic activity. As for 3MC and BNF (but not OME), K401R and N487D showed significantly reduced fold-inducibilities compared to WT ($p<0.01$ and $p<0.05$ for 3MC, $p<0.05$ and $p<0.05$ for BNF, respectively), apparently suggesting that K401R and N487D might have reduced intrinsic transcriptional activities. However, this possibility is ruled out from the findings on restoration of the luciferase activities for K401R and N487D by MG-132 (described above). A previous report indicated that a ligand TCDD accelerated degradation of AhR through the ubiquitin-proteasome pathway (Ma and Baldwin, 2000). Thus, it is likely that the ligands 3MC and BNF also induce the increased degradation of AhR, and that K401R and N487D are more sensitive to the ligand-induced degradation than WT. Further studies are necessary to clarify these points.
The region including Ile-514 was required for ligand-dependent binding to DNA (Dolwick et al., 1993b). The I514T variant showed no apparent effect on the mRNA level, protein stability, transcriptional activities, and subcellular localization compared to the WT AhR (Figs. 1, 2, 4-6; and data not shown).

Lys-17 is located within the nuclear localization signal. Previous mutational analysis revealed that the substitution of alanine for lysine does not affect its nuclear translocation (Ikuta et al., 1998). The K17T variant also showed the transcriptional activity and nuclear localization similar to those of the WT. The K17T/R554K double variant showed no significant differences in the luciferase activity from that of the WT. The functional effect of the R554K substitution was reported to be marginal in the in vitro TCDD-induced CYP1A1 mRNA expression (Wong et al., 2001a). Our data also showed that the K17T, R554K and K17T/R554K variations did not affect the mRNA levels, protein levels, transactivation properties with BNF, 3MC or OME, and the cellular localizations of AhR (Figs. 1, 2, 4-6; and data not shown).

AhR is thought to be involved in the chemical carcinogenesis, and thus a few studies tried to find the associations between R554K variation and the risks of lung and bladder cancers, but failed (Kawajiri et al., 1995; Zhang et al., 2002). However, very recent report showed significant and adverse association of R554K with survival for soft tissue sarcoma (Berwick et al., 2004). Because K401R and N487D variants showed reduced transcriptional activities in response to the ligands and OME, it might be interesting to assess their relation to the cancer risks.

In conclusion, the AhR variants, K401R and N487D, had reduced ligand-induced transcriptional activities with reduced protein expression levels. The reduced activities were also observed in case of the OME-induced activation. Therefore, K401R and N487D show their apparently reduced ligand- and OME-dependent transcriptional activities probably through reduced protein expression. Thus, the two genetic variations may decrease the drug metabolizing rate through the reduced induction of CYP1A1 or other metabolizing enzymes such as CYP1A2, CYP1B1, UGT1A1 and UGT1A6.
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References


Wong JM, Okey AB and Harper PA (2001b) Human aryl hydrocarbon receptor polymorphisms that result in loss of CYP1A1 induction. Biochem Biophys Res Commun 288: 990-996.


Footnotes

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Legends for figures

Fig. 1. Quantification of mRNA levels of the wild type (WT) and variant AhRs by TaqMan real-time RT-PCR. HeLa cells were transfected with the empty vector, WT, or each variant-expression plasmid, together with the Arnt-expression plasmid. AhR mRNA expression levels after 48 hours were normalized with β-actin mRNA levels. Bars represent the mean±SD (n=3).

Fig. 2. Western blot analysis of the wild type (WT) and variant AhR proteins. Whole cell lysates were prepared from HeLa cells, which were transfected with the empty vector, WT, or each variant AhR-expression plasmid, together with the Arnt-expression plasmid. The lysates were obtained after 48 hours, then were subjected to electrophoresis, blotted, and stained as described in the Materials and Methods. One representative result from three independent experiments is shown.

Fig. 3. Effects of MG-132 on the levels of the wild type (WT), K401R, and N487D AhR proteins. (a) HeLa cells were transfected with the WT, K401R, or N487D expression plasmid. Twenty-four hours after transfection, the cells were treated with vehicle (-) or 10 μM MG-132 (+) for 8 hours. Then, whole cell lysates were subjected to Western blot analysis. One of the representative results from three independent experiments is shown. (b) Densitometric quantification of the bands in (a). The protein expression level of WT without MG-132 was defined as 100. Bars represent the mean±SD (n=3).

Fig. 4. Transcriptional activity of the wild type (WT) and variant AhRs with β-naphthoflavone (BNF). HeLa cells were transfected with an AhR variant expression plasmid, hArnt expression plasmid, pGL3-CYP1A1 reporter, and phRL-TK plasmid. The cells were cultured with 0, 0.02, 0.2, 2, and 20 nM BNF (from left to right for each transfectant) for 48 hours. Relative luciferase activity, shown as the activity of the WT without BNF, was set as 100. Bars represent the mean±SD (n=3). **p<0.005, ***p<0.0005 by Fisher’s PLSD Method.

Fig. 5. Transcriptional activity of the wild type (WT) and variant AhRs with
3-methylcholanthrene (3MC). HeLa cells were transfected with an AhR variant expression, hArnt expression, pGL3-CYP1A1 reporter, and phRL-TK plasmids. The cells were cultured with vehicle (none), 10 nM, or 100 nM 3MC for 48 hours. Relative luciferase activity, shown as the activity of the WT without 3MC, was set as 100. Bars represent the mean±SD (n=3). *p<0.05, **p<0.005 by Fisher’s PLSD Method.

Fig. 6. Transcriptional activity of the wild type (WT) and variant AhRs with omeprazole (OME). HeLa cells were transfected with an AhR variant expression, hArnt expression, pGL3-CYP1A1 reporter, and phRL-TK plasmids. The cells were treated with vehicle (none), 200 nM, and 2 µM OME for 48 hours. Relative luciferase activity, shown as the activity of the WT without OME, was set as 100. Bars represent the mean±SD (n=3). *p<0.05, **p<0.005 by Fisher’s PLSD Method.
Fig. 1
Fig. 2
Fig. 4

Relative Luciferase Activity

WT  K17T  K401R  N487D  I514T  R554K  K17T/R554K

BNF
Fig. 5