A Novel Polymorphic Allele of Human Arylacetamide Deacetylase Leads to Diminished Enzyme Activity

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**Abbreviations:** AADAC, arylacetamide deacetylase; CES, carboxylesterase; CYP, Cytochrome P450; FLU-1, 4-nitro-3-(trifluoromethyl)phenylamine; HLM, human liver microsomes; PNPA, p-nitrophenyl acetate; RT-PCR, reverse transcription-polymerase chain reaction; SNP, single-nucleotide polymorphism; UTR, untranslated region.
Abstract

Human arylacetamide deacetylase (AADAC) is responsible for the hydrolysis of clinical drugs such as flutamide, phenacetin, and rifamycins. Our recent studies have suggested that human AADAC is a relevant enzyme in pharmacology and toxicology. Until now, the genetic polymorphisms that affect enzyme activity in AADAC have been unknown. In this study, we found single-nucleotide polymorphisms in the human AADAC gene in a liver sample showing remarkably low flutamide hydrolase activity. Among them, g.13651G>A (V281I) and g.14008T>C (X400Q) were nonsynonymous. The latter would be predicted to cause C-terminal 1 amino acid (glutamine) extension. The AADAC*2 allele (g.13651G>A) was found in all populations investigated in this study (European-American, African-American, Koreans, and Japanese) at allele frequencies of 52.6% ~ 63.5%, whereas the AADAC*3 allele (g.13651G>A/g.14008T>C) was found in European-Americans (1.3%) and African-Americans (2.0%). COS7 cells expressing AADAC.1 (wild-type) showed flutamide, phenacetin, and rifampicin hydrolase activities with $CL_{\text{int}}$ values of $1.31 \pm 0.06 \mu L/min/unit$, $1.00 \pm 0.02 \mu L/min/unit$, and $0.39 \pm 0.02 \mu L/min/unit$, respectively. AADAC.2, which is a protein produced from the AADAC*2 allele, showed moderately lower or similar $CL_{\text{int}}$ values to AADAC.1, but AADAC.3 showed substantially lower $CL_{\text{int}}$ values ($0.21 \pm 0.02 \mu L/min/unit$, $0.12 \pm 0.00 \mu L/min/unit$, and $0.03 \pm 0.01 \mu L/min/unit$, respectively). Microsomes from a liver sample genotyped as AADAC*3/AADAC*3 showed diminished enzyme activities compared to those genotyped as AADAC*1/AADAC*1, AADAC*1/AADAC*2, and AADAC*2/AADAC*2. In conclusion, we first found an AADAC allele causing diminished enzyme activity. This study would provide useful information about interindividual variation in AADAC enzyme activity.
Introduction

Human arylacetamide deacetylase (AADAC) is a member of the serine esterase superfamily and is mainly expressed in the liver and gastrointestinal tracts (Watanabe et al., 2009). Human AADAC was first identified as an enzyme that catalyzes the deacetylation of 2-acetylaminofluorene, which is associated with carcinogenicity (Probst et al., 1991). A later study reported that human AADAC was capable of hydrolyzing cholesterol ester when expressed in yeast (Tiwari et al., 2007). The active site domain of AADAC shares high homology with hormone-sensitive lipase (Probst et al, 1994; Trickett et al, 2001), suggesting that AADAC can mediate the hydrolysis of diacylglycerol (Lo et al, 2010).

Recently, we demonstrated that human AADAC is involved in the metabolism of clinical drugs such as flutamide, phenacetin, and rifamycins (rifampicin, rifabutin, and rifapentine) (Watanabe et al., 2009; Watanabe et al., 2010; Nakajima et al., 2011). Flutamide is a nonsteroidal antiandrogen drug used for the treatment of prostate cancer. The hydrolyzed metabolite of flutamide, 4-nitro-3-(trifluoromethyl)phenylamine (FLU-1), was further metabolized to N-hydroxyl FLU-1, which has been suggested to be associated with hepatotoxicity (Goda et al., 2006). Phenacetin had been widely used as an analgesic antipyretic, but it was withdrawn from the market after causing renal failure (Sicardi et al., 1991; Gago-Dominguez et al., 1999). The hydrolyzed metabolite of phenacetin, p-phenetidine, is considered to be further metabolized to N-hydroxyphenetidine, which is a possible causal factor of nephrotoxicity and hematotoxicity (Shudo et al., 1978; Wirth et al., 1982; Jensen and Jollow, 1991). Rifamycins such as rifampicin, rifabutin, and rifapentine have been used as antituberculosis drugs (Jamis-Dow et al., 1997). Rifampicin is largely considered to exacerbate hepatotoxicity induced by isoniazid and other antitubercular drugs in human. Our recent study demonstrated that the hydrolyzed metabolite of rifamycins (25-desacetylated forms) showed low cytotoxicity and induction potency of CYP3A4 (Nakajima et al., 2011). Thus, AADAC plays various roles in the metabolism of exogenous substrates.
Drug-metabolizing enzymes are often subject to genetic polymorphisms that alter the protein expression or catalytic activity. In general, genetic factors are estimated to account for 15 - 30% of interindividual differences in drug metabolism and response (Evans and McLeod, 2003; Evans and Relling, 2004). Single nucleotide polymorphisms (SNPs), single base mutations in the genetic sequence, are the most simple form and most common source of genetic polymorphism in the human genome. There are many reports about genetic polymorphisms of drug-metabolizing enzymes including human esterases. In carboxylesterase (CES) 1, the variant-type with G143Q showed dramatically decreased catalytic efficiency in the hydrolysis of methylphenidate (Zhu et al., 2008). In paraoxonase (PON) 1, Q192R polymorphism affects the hydrolase activities of various substrates (Davies et al., 1996; Billecke et al., 2000; Hioki et al., 2011). For example, paraoxon and pilocarpine hydrolase activities by PON1 192R are higher than those by PON1 192Q, but soman and sarin are efficiently hydrolyzed by PON1 192 Q rather than by PON1 192R. In acetylcholinesterase and butyrylcholinesterase, their genetic polymorphisms were suggested to be associated with Alzheimer’s disease (Scacchi et al., 2009; Cook et al., 2005), because both of them appeared to be associated with β-amyloid plaques and tangles (Darvesh et al., 2003). Therefore, the genetic polymorphisms of esterases affect the drug efficacy and are sometimes associated with diseases. However, until now there have been no reports about genetic polymorphisms of AADAC that affect enzyme activity.

Our recent study found that AADAC and CES2 are involved in the hydrolysis of flutamide in human liver at high and low concentrations, respectively (Kobayashi et al., submitted). When the flutamide hydrolase activity was analyzed using 6 individual human liver samples in that study, we found a human liver sample showing extremely low flutamide hydrolase activity at a high concentration of 200 µM, but showing moderate activity at a low concentration of 5 µM. This result implied that the human liver sample showed extremely low AADAC enzyme activity. In this study, to examine whether the extremely low enzyme activity was due to the genetic polymorphisms of AADAC, the sequences of the AADAC gene
in this human liver sample were analyzed. Furthermore, we investigated the association of \textit{AADAC} genetic polymorphisms with the interindividual variability in the enzyme activity.
Materials and Methods

Chemicals and Reagents. Flutamide, 4-nitro-3-(trifluoromethyl)phenylamine (FLU-1), \( p \)-nitrophenol (PNP), phenacetin, and rifampicin were purchased from Wako Pure Chemical Industries (Osaka, Japan). \( p \)-Phenetidine and \( p \)-nitrophenyl acetate (PNPA) were purchased from Sigma-Aldrich (St. Louis, MO). 25-Desacetylrifampicin was purchased from Toronto Research Chemicals (Toronto, Canada). Primers were commercially synthesized at Hokkaido System Sciences (Sapporo, Japan). The random hexamer and SYBR Premix Ex Taq were from Takara (Shiga, Japan). RevaTra Ace (Mononey Murine Leukemia Virus Reverse Transcriptase RNaseH Minus) and Blend TaqDNA polymerase were obtained from Toyobo (Tokyo, Japan). Taq polymerase was obtained from Greiner Japan (Tokyo, Japan). All other chemicals used in this study were of analytical or the highest quality commercially available.

Genomic DNA. Genomic DNA samples were extracted from 50 human livers and human blood samples using a Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN). Human liver samples from 27 donors (18 Caucasians, 6 Hispanic, 3 Blacks) were supplied by National Disease Research Interchange (Philadelphia, PA) through the Human and Animal Bridging Research Organization (Chiba, Japan), and those from 23 Japanese were obtained from autopsy materials that were discarded after pathological investigation. The blood samples were from 184 European-American, 177 African–American, 212 Korean, and 117 Japanese healthy participants who provided written informed consent. The use of the human livers and genomic DNA was approved by the Ethics Committees of Kanazawa University (Kanazawa, Japan), Iwate Medical University (Morioka, Japan), Soonchunhyang University Hospital (Chonan, Korea), and the Human Studies Committee of Washington University School of Medicine (St Louis, MO).

Sequence Analysis of the \( \text{AADAC} \) Gene. Sequence analysis was performed to examine the
nucleotide sequences of exons, exon-intron junctions, and 5'- and 3'-untranslated regions in the *AADAC* gene of a human liver sample showing extremely low flutamide hydrolase activity (Kobayashi et al., submitted). The PCR mixture contained genomic DNA (100 ng), 1 × PCR buffer, 0.2 mM deoxynucleoside-5'-triphosphates (dNTPs), 0.4 µM primers, and 0.5 U of Blend *Taq* DNA polymerase in a final volume of 25 µl. Primers used in this analysis are shown in Table 1 and Fig. 1. After an initial denaturation at 94°C for 3 min, the amplification was performed by denaturation at 94°C for 25 s, annealing at 57°C for 25 s, and extension at 72°C for 1 min/1 kbp for 40 cycles, followed by a final extension at 72°C for 5 min. The PCR product was submitted to DNA sequencing by use of a Thermo Sequenase Cy5.5 Dye Terminator Cycle Sequencing kit (GE Healthcare Bio-Science, Buckinghamshire, UK) with a Long-Read Tower DNA sequencer (GE Healthcare Bio-Science).

**RNA Preparation from Human Tissues and Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analyses.** Total RNA samples were extracted from the human livers using RNAiso. Real-time RT-PCR was performed for quantitative determination of AADAC mRNA using an MX3000P real-time PCR system (Stratagene, La Jolla, CA) as described previously (Watanabe et al., 2009). Human glyceraldehyde 3- phosphate dehydrogenase (hGAPDH) mRNA was also quantified to normalize the amount of total transcripts in each sample according to a method described previously (Tsuchiya et al., 2004). The copy numbers were calculated using standard amplification curves.

**Genotyping Assays of AADAC*2 and AADAC*3 Alleles.** For genotyping of the AADAC*2 allele concerning g.13651G>A, an allele specific PCR (AS-PCR) was performed. The primers using in this analysis were AADAC g.13651-wild or AADAC g.13651-mutant, and AADAC g.14307AS (Table 1). The PCR mixture contained genomic DNA (100 ng), 1 × PCR buffer [67 mmol/l Tris–HCl, pH 8.8, 16.6 mmol/l (NH₄)₂SO₄, 0.45% Triton X-100, 0.02% gelatin], 1.5 mmol/l MgCl₂, 0.2 mmol/l dNTPs, 0.4 mmol/l each primer, and 0.5 U of *Taq* polymerase.
in a final volume of 25 ml. After an initial denaturation at 94°C for 3 min, the amplification was performed by denaturation at 94°C for 25 s, annealing at 54°C for 25 s, and extension at 72°C for 50 s for 30 cycles, followed by a final extension at 72°C for 5 min. The PCR products were determined by electrophoresis in a 2% agarose gel.

For genotyping of the AADAC*3 allele concerning g.14008T>C, PCR-restriction fragment length polymorphism (RFLP) was performed. The primers used in this analysis were AADAC ex5 (Acc I) and AADAC UTRAS (Table 1). The PCR mixture was the same as described above except for the primers. After an initial denaturation at 94°C for 3 min, the amplification was performed by denaturation at 94°C for 25 s, annealing at 52°C for 25 s, and extension at 72°C for 30 s for 45 cycles, followed by a final extension at 72°C for 5 min. The PCR products were digested with Acc I. The digestion patterns were determined by electrophoresis in a 3% agarose gel. AADAC*1 (wild type) yielded 181 bp fragment and AADAC*3 yielded 158 and 23 bp fragments.

**Construction of Plasmid Expressing Human AADAC Variants.** The expression plasmids of AADAC.1 and AADAC.2, which are proteins produced from AADAC*1 and AADAC*2 alleles (c.841G>A), respectively, were constructed in our previous study (Watanabe et al., 2009). To construct the expression plasmid of AADAC.3, which is a protein produced from the AADAC*3 allele (c.841G>A /c.1288T>C), the mutation of c.1288T>C was introduced into the expression plasmid of AADAC.2 by site-directed mutagenesis (SDM) using a Quick Change II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The primers used are AADAC SDMS and AADAC SDMAS (Table 1). Nucleotide sequences were confirmed by DNA sequence analysis using primers of T7F and pTargeT-AS (Table 1).

**Expression of Human AADAC in COS7 cells.** African green monkey kidney cells, COS7 cells, were obtained from American Type Culture Collection (Manassas, VA). The COS7 cells were grown in Dulbecco’s modified Eagle’s medium containing 4.5 g/l glucose and 10% fetal
bovine serum with 5% CO₂ at 37°C. The cells were transfected in 10-cm dishes (1 × 10⁶ cells/well) with 7.5 µg of each expression plasmid using Lipofectamine (Invitrogen, Carlsbad, CA). After incubation for 48 h, the cells were harvested and suspended in a small amount of TGE buffer (10 mM Tris-HCl, 20% glycerol, 1 mM EDTA, pH 7.4) and disrupted by freeze-thawing three times. Each protein expression level was determined by immunoblot analysis as described below.

**Immunoblot Analysis.** SDS-polyacrylamide gel electrophoresis and immunoblot analysis for human AADAC were performed according to our previous report (Watanabe et al., 2009). Enzyme sources (30 µg) were separated on 10% polyacrylamide gels and electrotransferred onto polyvinylidene difluoride membrane, Immobilon-P (Millipore Corporation, Billerica, MA). The membranes were probed with monoclonal mouse anti-human AADAC (Abnova, Taipei City, Taiwan), and the corresponding fluorescent dye-conjugated second antibody and an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE) were used for the detection. The expression level was quantified using ImageQuant TL Image Analysis software (GE Healthcare). This analysis was performed in the linear range of band intensity with respect to the amount of protein. The relative expression levels of recombinant AADAC wild- and variant-types were estimated by comparing these band intensities.

**PNPA Hydrolase Activity.** The hydrolase activity of PNPA, a general esterase substrate, was determined using COS7 cell homogenates expressing AADAC and individual human liver microsomes (HLM). The PNPA hydrolase activity was determined as follows: a typical incubation mixture (final volume of 0.2 ml) contained 100 mM potassium phosphate buffer (pH 7.4) and various enzyme sources (human microsomal protein: 0.1 mg/ml). PNPA was dissolved in DMSO, and the final concentration of DMSO in the incubation mixture was 1.0%. The reaction was initiated by the addition of 500 µM PNPA after 2-min preincubation
at 37°C. After 1-min incubation at 37°C, the reaction was terminated by the addition of 100 µl of ice-cold methanol. The produced PNP, a metabolite of PNPA hydrolysis, was measured by the absorbance at 405 nm using a Biotrak II plate reader (GE Healthcare). The quantification of PNP was performed by comparing the absorbance with that of an authentic standard. Because PNPA was non-enzymatically converted to PNP at 37°C to some extent, the content of PNP in the mixture incubated without the enzyme was subtracted from that with the enzyme to correct the activity.

**Flutamide, Phenacetin, and Rifampicin Hydrolase Activities.** The flutamide, phenacetin, and rifampicin hydrolase activities were determined according to our previous studies (Watanabe et al., 2009; Watanabe et al., 2010; Nakajima et al., 2011). Microsomal proteins and COS7 cell homogenates expressing AADAC (flutamide and phenacetin hydrolase activities: 0.4 mg/ml; rifampicin hydrolase activity: 0.5 mg/ml) were used as enzyme sources.

**Statistical Analysis.** The distribution of genotype frequencies was compared to the Hardy-Weinberg equilibrium model using the Fisher’s exact test using the computer program Instat 2 (GraphPad Software, San Diego, CA). Kinetic parameters were estimated from the fitted curve using a computer program designed for nonlinear regression analysis (KaleidaGraph, Synergy Software, Reading PA). The kinetic parameters $K_m$, $V_{max}$, and $K_i$ were calculated by Michaelis-Menten equation (1) for flutamide and phenacetin hydrolase activity, or substrate inhibition equation (2) for rifampicin hydrolase activity. The $CL_{int}$ value was calculated by equation (3).

\[
V = \frac{V_{max} \cdot S}{K_m + S} \quad (1)
\]
\[
V = \frac{V_{max} \cdot S}{K_m + S + S^2/K_i} \quad (2)
\]
\[
CL_{int} = \frac{V_{max}}{K_m} \quad (3)
\]
Statistical significance between multiple groups was determined by ANOVA followed by Dunnet or Tukey test using the computer program Instat 2. A value of $P < 0.05$ was considered statistically significant.
Results

Sequence Analysis of Human AADAC Gene. Sequence analysis of the AADAC gene (Reference genomic sequence: NC_000003.11) was performed using a human liver sample that showed extremely low flutamide hydrolase activity in our previous study (Kobayashi et al., submitted). Seven SNPs were present in a homozygous state (Fig. 1). Two SNPs (g.-1507G>T and g. -425TA>X) were located at the 5’-flanking region, and 5 SNPs were located at exon 1 and 5. Among them, g.13651G>A and g.14008T>C located at exon 5, were nonsynonymous SNPs. The g.13651G>A leads to an amino acid change from valine to isoleucin at the 281 position (V281I). The g.14008T>C leads to an amino acid change from a stop codon to glutamine at the 400 position (X400Q), resulting in 1 amino acid extension at the C-terminal, because the codon (TAA) after 400Q indicates the stop codon.

Expression of AADAC mRNA in Human Liver. The expression level of AADAC mRNA in human liver was determined by real-time RT-PCR analysis. In this analysis, 6 human liver samples, which were analyzed in our previous study (Kobayashi et al., submitted), were employed. The expression level of AADAC mRNA in a liver sample showing extremely low flutamide hydrolase activity (flutamide hydrolase activity at 500 µM: 0.02 ± 0.01 nmol/min/mg, AADAC mRNA/GAPDH mRNA: 0.04 ± 0.00) was comparable with those in others (flutamide hydrolase activity at 500 µM: 0.15 ± 0.02 ~ 0.40 ± 0.04 nmol/min/mg, AADAC mRNA/GAPDH mRNA: 0.09 ± 0.01 ~ 0.39 ± 0.01). This result suggested that the SNPs in the 5’-flanking region of AADAC gene could not affect the transcriptional regulation.

Allele Frequencies of AADAC*2 and AADAC*3. Genotyping assays of g.13651G>A and g.14008T>C, which are nonsynonymous mutations, were performed using genomic DNA samples in 200 European-Americans, 178 African-Americans, 212 Koreans, and 140 Japanese (Table 2). It was confirmed by subcloning using genomic DNA samples genotyped as
homozygotes or heterozygotes of g.14008T>C that the allele with g.14008T>C concurrently possesses g.13651G>A. In this study, the AADAC alleles were designated in line with the recommendations of Wain et al. (2002). AADAC wild-type and the alleles with g.13651G>A, and g.13651G>A/g.14008T>C were termed AADAC*1, AADAC*2, and AADAC*3, respectively. The AADAC*2 allele was found in all populations, with allele frequencies of 52.6% ~ 63.5%, whereas the AADAC*3 allele was found in European-Americans (1.3%) and African-Americans (2.0%), but not in Japanese and Koreans. The allele frequencies of the AADAC*2 and AADAC*3 were in accordance with the Hardy-Weinberg equation. Thus, there were ethnic differences in the allele frequency of AADAC*3.

Kinetic Analyses of Flutamide, Phenacetin, and Rifampicin Hydrolase Activities by Recombinant Human AADAC Wild-type and Variants. To compare the flutamide, phenacetin, and rifampicin hydrolase activities among human AADAC wild-type and variants, they were transiently expressed in COS7 cells. To compare the expression levels among wild and variant AADAC, immunoblot analysis was performed using cell homogenates (Fig. 2A). Given that the expression level of recombinant AADAC.1 (wild-type) was 1.0 unit, the expression levels of AADAC.2 and AADAC.3 were 0.9 ± 0.0 units and 0.5 ± 0.0 units, respectively. In the subsequent study, the activities in the expression systems were normalized using these units.

In both AADAC wild-type and variants, data for the flutamide and phenacetin hydrolase activities were fitted to the Michaelis-Menten kinetics, and data for the rifampicin desacetylase activity were fitted to the substrate inhibition kinetics (Figs. 2B, C, and D). AADAC.1 (wild-type) showed flutamide, phenacetin, and rifampicin hydrolase activities with $CL_{\text{int}}$ values of 1.31 ± 0.06 µL/min/unit, 1.00 ± 0.02 µL/min/unit, and 0.39 ± 0.02 µL/min/unit, respectively (Table 3). AADAC.2 showed flutamide and rifampicin hydrolase activities with similar $CL_{\text{int}}$ values (0.95 ± 0.05 µL/min/unit and 0.51 ± 0.00 µL/min/unit, respectively) to AADAC wild type. AADAC.2 showed a lower $CL_{\text{int}}$ value for phenacetin
hydrolase activity (0.61 ± 0.01 µL/min/unit) than the AADAC wild type, although statistical significance was not reached (P > 0.05). AADAC.3 showed significantly lower CL_int values for all activities (0.21 ± 0.02 µL/min/unit, 0.12 ± 0.00 µL/min/unit, and 0.03 ± 0.01 µL/min/unit, respectively) due to the diminished Vmax values. The PNPA hydrolase activity by AADAC.3 at a concentration of 500 µM (0.21 ± 0.03 µmol/min/unit) was also substantially lower than those by AADAC.1 (1.47 ± 0.03 µmol/min/unit) and AADAC.2 (1.23 ± 0.11 µmol/min/unit). These results suggested that 1 amino acid extension at the C-terminal in AADAC.3 caused the diminished catalytic efficiency of the AADAC enzyme activity.

PNPA, Flutamide, Phenacetin, and Rifampicin Hydrolase Activity and AADAC protein levels in Individual Human Liver Microsomes. To further investigate the effects of AADAC genetic polymorphisms on enzyme activities, the hydrolase activities of PNPA (500 µM), flutamide (500 µM), phenacetin (1 mM), and rifampicin (50 µM) were measured in 24 individual human liver microsomes (Figs. 3A, B, C, and D). PNPA is a general esterase substrate, whereas phenacetin and rifampicin are specific AADAC substrates. Flutamide is hydrolyzed by AADAC with a high contribution at a concentration of 500 µM (Kobayashi et al., submitted). The numbers of HLM samples from subjects genotyped as AADAC*1/AADAC*1, AADAC*1/AADAC*2, AADAC*2/AADAC*2, and AADAC*3/AADAC*3 were 1, 13, 9, and 1, respectively. HLM from a subject genotyped as AADAC*3/AADAC*3 showed PNPA hydrolase activities (4.18 µmol/min/mg) similar to those genotyped as AADAC*1/AADAC*1, AADAC*1/AADAC*2, and AADAC*2/AADAC*2 (1.73 µmol/min/mg, 3.34 ± 1.01 µmol/min/mg, and 2.42 ± 0.95 µmol/min/mg, respectively) (Fig. 4A). In contrast, flutamide, phenacetin, and rifampicin hydrolase activities in HLM from a subject genotyped as AADAC*3/AADAC*3 (0.02 nmol/min/mg, 0.07 nmol/min/mg, and 0.16 pmol/min/mg, respectively) were substantially lower than those in others (Figs. 3A, B, and C).

The AADAC protein expression levels in HLM samples were analyzed by
immunoblotting (Fig. 3E). The AADAC protein expression levels appeared to be correlated with the AADAC enzyme activities measured using flutamide, phenacetin, and rifampicin as substrates, and HLM from a subject genotyped as AADAC*3/AADAC*3 showed the lowest expression of AADAC protein. This result suggested that AADAC*3 might cause decreased protein expression as well as decreased enzyme activity, although there was only 1 HLM sample with AADAC*3/AADAC*3.
Discussion

Our previous study found that human AADAC is responsible for the hydrolysis of clinical drugs such as flutamide, phenacetin, and rifamycins (Watanabe et al., 2009; Watanabe et al., 2010; Nakajima et al., 2011). The hydrolyzed metabolite of flutamide, FLU-1, is further metabolized to N-hydroxyl FLU-1, which has been suggested to be associated with hepatotoxicity (Goda et al., 2006). The hydrolyzed metabolite of phenacetin is suggested to be associated with nephrotoxicity and hematotoxicity (Shudo et al., 1978; Wirth et al., 1982; Jensen and Jollow, 1991). Because human AADAC is involved in their hydrolysis, its potency may be important for the incidence of flutamide- and phenacetin-induced toxicities. Our recent study using HepG2 cells found that the hydrolyzed metabolites of rifamycins, showed low cytotoxicity and induction potency of CYP3A4 (Nakajima et al., 2011). Based on these reports, it is suggested that AADAC is involved in their detoxification and drug interactions. Therefore, the interindividual variability of AADAC enzyme activity would be pharmacologically and toxicologically relevant. Our recent study found a human liver sample that demonstrated remarkably low flutamide hydrolase activity at 500 µM (Kobayashi et al., submitted). Because AADAC is mainly involved in the flutamide hydrolysis at high concentrations, the sample was expected to show extremely low AADAC enzyme activity. In the present study, we analyzed the nucleotide sequences of the AADAC gene with the sample and found a novel allele of the AADAC gene that caused the diminished enzyme activity.

The AADAC gene sequences in a human liver sample that showed extremely low flutamide hydrolase activity were analyzed, and 7 SNPs were found in a homozygous state (Fig. 1). Two SNPs were present in the 5’-flanking region of the AADAC gene, suggesting the altered expression of AADAC mRNA. In a search for transcription factor binding sites predicted using MATCH (http://www.gene-regulation.com/cgi-bin/pub/programs/pmatch/bin/p-match.cgi), TFSERCH (http://www.cbrc.jp/research/db/TFSEARCHJ.html), and MOTIF
(http://www.genome.jp/tools/motif/), there were no candidate transcription factors associated with the SNPs in the 5'-flanking region. In fact, the human liver sample showing extremely low flutamide hydrolase activity had a moderate expression level of AADAC mRNA. Therefore, we focused on 2 nonsynonymous SNPs (g.13651G>A, c.841G>A, V281I; g.14008T>C, c.1198T>C, X400Q) in the coding regions as a cause of the low flutamide hydrolase activity. The g.14008T>C was predicted to cause the C-terminal 1 amino acid (glutamine) extension.

The AADAC*2 allele (g.13651G>A) was found in all populations investigated in this study, with allele frequencies of 49.5% ~ 63.5%. This result was in accordance with the dbSNP database in the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?searchType=adhoc_search&type=rs&rs=rs1803155). The AADAC*3 allele was also described in the dbSNP database, but the allele frequency was unknown. In this study, the AADAC*3 allele (g.13651G>A/g.14008T>C) was found only in European-Americans (1.3%) and African-Americans (2.0%). Thus, there were ethnic differences in the allele frequency of AADAC*3.

For kinetic analyses of the flutamide, phenacetin, and rifampicin hydrolase activities, wild and variant types of AADAC were expressed in COS-7 cells. The homogenates of COS7 cells expressing AADAC.2 and AADAC.3 showed a single band with a similar apparent molecular mass as AADAC.1 (Fig. 2A). This result suggested that the glycosylation modifications of AADAC variant-types were normal. The expression level of AADAC.3 protein was lower than those of AADAC.1 (wild-type) and AADAC.2. We analyzed the transfection efficacy between wild- and variant-types by measuring the AADAC mRNA expression level, and found that AADAC*3 showed similar AADAC mRNA expression as AADAC*1 and AADAC*2 (data not shown). Furthermore, the rate of AADAC.3 protein degradation showed no difference from those of AADAC.1 and AADAC.2 (Supplement Fig. 1). AADAC is located on the lumen side of the endoplasmic reticulum, and its N-terminal domain is a membrane-spanning region (Frick et al., 2004). Although there is no evidence
about the conformational importance of the C-terminal domain of the AADAC protein, the subtle change in the C-terminal region might cause low AADAC protein expression.

The enzyme activities of each AADAC protein were evaluated by normalizing them with their expression level as a unit (Figs. 2B, C, and D). AADAC.2 showed lower or similar CLint values compared with AADAC.1, although statistical significance was not reached. Our previous report revealed that AADAC.2 (V281I) did not alter the flutamide hydrolase activity (AADAC.1 CLint values: 0.8 ± 0.0 µmol/min/mg; AADAC.2 CLint values: 0.9 ± 0.0 µmol/min/mg) (Watanabe et al., 2009). In a previous study, the flutamide hydrolase activity was evaluated without normalizing the AADAC protein expression level in COS7 cells. This may have caused a slight difference in the measured effect of AADAC.2 between previous and present studies. On the other hand, AADAC.3 showed substantially lower CLint values for all enzyme activities. There have been some reports about genetic polymorphisms leading to C-terminal extensions that alter the protein function. For example, in apolipoprotein AII (ApoAII), the stop codon mutation (X78G), which causes a C-terminal 21 amino acid extension, was found in the patients with hereditary systemic amyloidosis (Benson et al., 2001). The protein structure at the C-terminal region of ApoAII is important for the lipid binding, thus the protein extension is predicted to decrease the lipid binding. In our previous study, we constructed recombinant AADAC with 5 histidines tandemly ligated at the C-terminus (Kobayashi et al., submitted), but this also showed no enzyme activity (data not shown). The C-terminal extension in the AADAC protein might alter the protein structure leading to decreased enzyme activity. In phenacetin hydrolase activity, AADAC.3 showed the similar Km value to AADAC.1, whereas AADAC.3 showed the lower Km values than AADAC.1 in flutamide and rifampicin hydrolase activities. Although we cannot clearly account for the difference, it may be due to the altered AADAC protein structure. To clarify them, further study should be performed in the near future.

The effects of AADAC genetic polymorphisms on the enzyme activities were investigated using 24 HLM samples. HLM from subjects genotyped as AADAC*1/AADAC*2
and AADAC*2/AADAC*2 tended to show similar flutamide, phenacetin, and rifampicin hydrolase activities as those genotyped as AADAC*1/AADAC*1. However, because only 1 sample with AADAC*1/AADAC*1 was detected among the HLM samples in this study, the effect of the AADAC*2 allele on the enzyme activity could not be correctly evaluated. It was obvious that an HLM sample with AADAC*3/AADAC*3 showed substantially lower activities (Figs. 3B, C, and D). The AADAC protein expression in this HLM sample was also lowest (Fig. 3E). This observation was in accordance with the result that the AADAC.3 protein expression level was low compared with that of AADAC.1 when it was expressed in COS7 cells (Fig. 2A). Thus, it was possible that the C-terminal 1 amino acid extension in AADAC.3 would affect the protein expression level in addition to the decreased enzyme activity. However, because there was only 1 sample with AADAC*3/AADAC*3 in this study, further study with more samples will be needed to verify the association of AADAC*3 allele with the protein expression level. Nevertheless, it would be reasonable based on the data of recombinant AADAC.3 that the AADAC*3 allele causes diminished AADAC enzyme activity (Figs. 2A, B, and C).

In conclusion, this study firstly found a novel polymorphic allele of AADAC leading to diminished enzyme activity. There were ethnic differences in the allele frequencies of AADAC*3. Human AADAC is involved in the metabolism of some clinical drugs. Information about interindividual variations in AADAC enzyme activity would be important for clinical drug therapy.
Acknowledgments

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Authorship Contributions

Participated in research design: Shimizu, Fukami, Nakajima, and Yokoi

Conducted experiments: Shimizu and Kobayashi

Contributed new reagents or analytical tools: Shimizu, Kobayashi, Takamiya, and Aoki

Performed data analysis: Shimizu and Fukami

Wrote or contributed to the writing of manuscript: Shimizu, Fukami, and Yokoi
References


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Footnotes

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Figure legends

Fig. 1. Schematic representation of the human AADAC gene (Reference genomic sequence: NC_000003.11) in the liver sample showing extremely low flutamide hydrolase activity. The nucleotide positions on the genomic DNA are indicated. The cDNA and amino acid change are in parentheses. Open and solid boxes indicate untranslated regions and coding exons, respectively. Lines indicate introns. Horizontal arrows indicate location and direction of the primer pairs for PCR amplification and for sequence analyses. Broken lines represent the amplified regions by the PCR.

Fig. 2. Effects of AADAC variants on flutamide, phenacetin, and rifampicin hydrolase activities. (A) Immunoblot analysis of recombinant human AADAC expressed in COS7 cells. Total cell homogenates from COS7 cells (30 μg) were separated by electrophoresis using 10 % SDS-polyacrylamide gel. Kinetic analyses of (B) flutamide, (C) phenacetin, and (D) rifampicin hydrolase activities by homogenates of COS7 cells expressing human AADAC wild- and variant-types. The homogenates of COS7 cells expressing these enzymes were incubated with 100 ~ 750 μM flutamide, 50 ~ 4000 μM phenacetin, and 5 ~ 1000 μM rifampicin, respectively. The activities by the expression systems were normalized with each unit measured by immunoblot analysis (AADAC.1: AADAC.2: AADAC.3 = 1.0 ± 0.0 units: 0.9 ± 0.0 units: 0.5 ± 0.0 units). Each data point represents the mean ± SD of triplicate determinations. *P < 0.05 compared with AADAC.1 (wild-type).

Fig. 3. AADAC enzyme activities and protein levels among 24 individual HLM samples with different AADAC genotypes. (A) PNPA, (B) flutamide, (C) phenacetin, and (D) rifampicin hydrolase activities in 24 individual HLM. HLM were incubated with 500 μM PNPA, 500 μM flutamide, 1 mM phenacetin, and 50 μM rifampicin. (E) Expression level of AADAC protein in 24 individual HLM samples. HLM samples (50 μg) were separated by electrophoresis
using 10 % SDS-polyacrylamide gel. The AADAC protein expression levels are represented as relative to that of the sample with AADAC*3/AADAC*3.
Table 1. Sequence of primers used in the present study.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tr>
<td>For sequence analysis of the AADAC gene</td>
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<tr>
<td>AADAC -2085S</td>
<td>5’-CATTTTACCCATAGCAGCTGG-3’</td>
</tr>
<tr>
<td>AADAC -1447AS</td>
<td>5’-GGCAAGACAGTATATATTTCC-3’</td>
</tr>
<tr>
<td>AADAC -1014AS</td>
<td>5’-GCTGGGAAGACATCTTTTGA-3’</td>
</tr>
<tr>
<td>AADAC -536AS</td>
<td>5’-AAGTAGTTGGTTGAGGGGTG-3’</td>
</tr>
<tr>
<td>AADAC -97AS</td>
<td>5’-GATACAGTCATGTAAAGTG-3’</td>
</tr>
<tr>
<td>AADAC -21AS</td>
<td>5’-GTGAACGTCGCCGCTTCTTGG-3’</td>
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<td>AADAC -79S</td>
<td>5’-CAGATAAAAATTGGCGCTTA-3’</td>
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<td>AADAC int1S</td>
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<td>AADAC int2AS</td>
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<td>For RT-PCR analysis</td>
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<td>AADAC exon 2F</td>
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<td>AADAC exon 3R</td>
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<tr>
<td>hGAPDHAS</td>
<td>5’-GCTCCCCCCTGCAATGA-3’</td>
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<td>AADAC g.13651-wild</td>
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<tr>
<td>pTargeT-AS</td>
<td>5’-ATTTAGGTCAGCGATAGAATA-3’</td>
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### Table 2. Allele frequencies of AADAC*2 and AADAC*3 in four populations.

<table>
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<tr>
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<th>Number of subjects</th>
<th>AADAC genotypes (%)</th>
<th>Allele frequencies (%)</th>
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<tr>
<td></td>
<td></td>
<td>AADAC*/1/ AADAC*/2</td>
<td>AADAC*/2/ AADAC*/3/ AADAC*/3/</td>
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<tr>
<td>European-Americans</td>
<td>200</td>
<td>26.5 21.5</td>
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<td>African-Americans</td>
<td>178</td>
<td>20.8 25.8</td>
<td>49.4 2.3 2.3</td>
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<td>Japanese</td>
<td>140</td>
<td>22.1 34.3</td>
<td>43.6 0.0 0.0</td>
</tr>
<tr>
<td>Koreans</td>
<td>212</td>
<td>28.7 37.3</td>
<td>34.0 0.0 0.0</td>
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Table 3. Kinetic parameters of flutamide, phenacetin, and rifampicin hydrolase activities.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_m$</th>
<th>$V_{max}$</th>
<th>$CL_{int}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flutamide hydrolase activity</td>
<td>µM</td>
<td>pmol/min/unit</td>
<td>µL/min/unit</td>
</tr>
<tr>
<td>AADAC.1</td>
<td>472 ± 15.9</td>
<td>617 ± 28.9</td>
<td>1.31 ± 0.06</td>
</tr>
<tr>
<td>AADAC.2</td>
<td>348 ± 27.5</td>
<td>331 ± 9.7</td>
<td>0.95 ± 0.05</td>
</tr>
<tr>
<td>AADAC.3</td>
<td>124 ± 9.31*</td>
<td>261 ± 1.4*</td>
<td>0.21 ± 0.02*</td>
</tr>
</tbody>
</table>

| Phenacetin hydrolase activity| mM     | pmol/min/unit | µL/min/unit |
| AADAC.1                     | 1.42 ± 0.03 | 1420 ± 8.8 | 1.00 ± 0.02  |
| AADAC.2                     | 1.52 ± 0.09 | 921 ± 36.2 | 0.61 ± 0.01  |
| AADAC.3                     | 1.56 ± 0.01*| 186 ± 8.0* | 0.12 ± 0.00* |

| Rifampicin hydrolase activity| µM     | pmol/min/unit | µL/min/unit | µM       |
| AADAC.1                     | 154 ± 7.27 | 60.2 ± 1.19 | 0.39 ± 0.02 | 237 ± 29.5 |
| AADAC.2                     | 129 ± 18.2 | 66.0 ± 8.67 | 0.51 ± 0.00 | 249 ± 71.6 |
| AADAC.3                     | 75.9 ± 14.2*| 2.41 ± 0.14*| 0.03 ± 0.01*| 242 ± 50.7 |

*P < 0.05 compared with AADAC.1.
Fig. 1.

AADAC -79S → AADAC int1 AS → AADAC -97AS → AADAC -536AS → AADAC -1014AS → AADAC -1447AS
AADAC -2085S → AADAC int2S → AADAC int3AS → AADAC int4AS → AADAC int5S → AADAC 3'-flankingAS

Exon 1 2 3 4 5 3'

5'

-8.43TA > X
-8.44C > T
8.39C > A
8.150TG > T
8.1265IG > A
8.1408T > C
8.1409IG > A
8.1598T > C
8.44AG > A
8.1186T > C

Wild type 14000 · · AAAATCTATAG TAAACATG · · 14019

N  L stop codon

Mutant type 14000 · · AAAATCTACAC TAAACATG · · 14019

N  L ↓Q stop codon
Fig. 2.