

A Novel Polymorphic Allele of Human Arylacetamide Deacetylase Leads to Decreased Enzyme Activity^S

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ABSTRACT:

Human arylacetamide deacetylase (AADAC) is responsible for the hydrolysis of clinically used drugs such as flutamide, phenacetin, and rifamycins. Our recent studies suggested that human AADAC is a relevant enzyme pharmacologically and toxicologically. To date, 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 that showed 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 a C-terminal one-amino acid (glutamine) extension. The AADAC*2 allele (g.13651G>A) was found in all populations investigated in this study (European American, African American, Korean, and Japanese), at allelic frequencies of 52.6 to 63.5%, whereas the AADAC*3 allele (g.13651G>A/g.14008T>C) was found in European American (1.3%) and African American (2.0%) samples. COS7 cells express-

ing AADAC.1 (wild-type) exhibited flutamide, phenacetin, and rifampicin hydrolase activities with intrinsic clearance (CL_{int}) values of 1.31 ± 0.06, 1.00 ± 0.02, and 0.39 ± 0.02 μl · min⁻¹ · unit⁻¹, respectively. AADAC.2, which is a protein produced from the AADAC*2 allele, showed moderately lower or similar CL_{int} values, compared with AADAC.1, but AADAC.3 showed substantially lower CL_{int} values (flutamide hydrolase, 0.21 ± 0.02 μl · min⁻¹ · unit⁻¹; phenacetin hydrolase, 0.12 ± 0.00 μl · min⁻¹ · unit⁻¹; rifampicin hydrolase, 0.03 ± 0.01 μl · min⁻¹ · unit⁻¹, respectively). Microsomes from a liver sample genotyped as AADAC*3/AADAC*3 showed decreased enzyme activities, compared with those genotyped as AADAC*1/AADAC*1, AADAC*1/AADAC*2, and AADAC*2/AADAC*2. In conclusion, we found an AADAC allele that yielded decreased enzyme activity. This study should provide useful information on interindividual variations in AADAC enzyme activity.

Introduction

Human arylacetamide deacetylase (AADAC) is a member of the serine esterase superfamily and is expressed mainly in the liver and gastrointestinal tract (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), which suggests that AADAC can mediate the hydrolysis of diacylglycerol (Lo et al., 2010).

We demonstrated that human AADAC is involved in the metabolism of clinical drugs such as flutamide, phenacetin, and rifamycins

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(rifampicin, rifabutin, and rifapentine) (Watanabe et al., 2009, 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 in 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 antituberculosis drugs in humans. Our recent study demonstrated that the hydrolyzed metabolite of rifamycins (25-desacetylated forms) showed low levels of cytotoxicity and induction potency for CYP3A4 (Nakajima et al., 2011). Therefore, AADAC plays various roles in the metabolism of exogenous substrates.

ABBREVIATIONS: AADAC, arylacetamide deacetylase; FLU-1, 4-nitro-3-(trifluoromethyl)phenylamine; HLMS, human liver microsomes; PNP, *p*-nitrophenol; PNPA, *p*-nitrophenyl acetate; RT, reverse transcription; PCR, polymerase chain reaction; SNP, single-nucleotide polymorphism; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PON1, paraoxonase 1; CL_{int}, intrinsic clearance.

Drug-metabolizing enzymes are often subject to genetic polymorphisms that alter protein expression or catalytic activity. In general, genetic factors are estimated to account for 15 to 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 simplest form and most common source of genetic polymorphism in the human genome. There are many reports on genetic polymorphisms of drug-metabolizing enzymes, including human esterases. For carboxylesterase 1, the variant type with G143Q showed dramatically decreased catalytic efficiency in the hydrolysis of methylphenidate (Zhu et al., 2008). For paraoxonase 1 (PON1), 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 of PON1 192R are higher than those of PON1 192Q, but soman and sarin are efficiently hydrolyzed by PON1 192Q rather than by PON1 192R. For acetylcholinesterase and butyrylcholinesterase, genetic polymorphisms were suggested to be associated with Alzheimer's disease (Cook et al., 2005; Scacchi et al., 2009), because both seemed to be associated with β -amyloid plaques and tangles (Darvesh et al., 2003). The genetic polymorphisms of esterases affect drug efficacy and are sometimes associated with diseases. To date, however, there have been no reports of genetic polymorphisms of *AADAC* that affect enzyme activity.

Our recent study found that *AADAC* and carboxylesterase 2 are involved in the hydrolysis of flutamide in human liver at high and low concentrations, respectively (Kobayashi et al., 2012). When the flutamide hydrolase activity was analyzed with six individual human liver samples in that study, we found a human liver sample that showed extremely low flutamide hydrolase activity at a high concentration of 200 μ M but moderate activity at a low concentration of 5 μ M. This result indicated that the human liver sample showed extremely low *AADAC* enzyme activity. In this study, to examine whether the extremely low enzyme activity was attributable to genetic polymorphisms of *AADAC*, the sequences of the *AADAC* gene in this human liver sample were analyzed. Furthermore, we investigated the association of *AADAC* genetic polymorphisms with the interindividual variability in enzyme activity.

Materials and Methods

Chemicals and Reagents. Flutamide, FLU-1, *p*-nitrophenol (PNP), phenacetin, and rifampicin were purchased from Wako Pure Chemicals (Osaka, Japan). *p*-Phenetidine and *p*-nitrophenyl acetate (PNPA) were purchased from Sigma-Aldrich (St. Louis, MO). 25-Desacetylriofampicin was purchased from Toronto Research Chemicals Inc. (North York, ON, 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 (Moloney murine leukemia virus reverse transcriptase RNaseH minus) and Blend Taq DNA polymerase were obtained from Toyobo (Tokyo, Japan). *Taq* polymerase was obtained from Greiner Bio-One (Tokyo, Japan). All other chemicals used in this study were of analytical grade or the highest quality commercially available.

Genomic DNA. Genomic DNA samples were extracted from 50 human livers and human blood samples by using a Puregene DNA isolation kit (Gentra Systems, Inc., Minneapolis, MN). Human liver samples from 27 donors (18 white, 6 Hispanic, and 3 black donors) were supplied by the National Disease Research Interchange (Philadelphia, PA) through the Human and Animal Bridging Research Organization (Chiba, Japan), and those from 23 Japanese individuals were obtained from autopsy materials that were discarded after pathological investigations. 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 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 *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 that showed extremely low flutamide hydrolase activity (Kobayashi et al., 2012). The PCR mixture contained genomic DNA (100 ng), 1 \times PCR buffer, 0.2 mM dNTPs, 0.4 μ M primers, and 0.5 U of Blend Taq DNA polymerase, in a final volume of 25 μ l. Primers used in this analysis are shown in Table 1 and Fig. 1. After an initial denaturation at 94°C for 3 min, amplification was performed with denaturation at 94°C for 25 s, annealing at 57°C for 25 s, and extension at 72°C for 1 min per 1 kilobase pair for 40 cycles, followed by a final extension at 72°C for 5 min. The PCR product was subjected to DNA sequencing with the use of a Thermo Sequenase Cy5.5 dye terminator cycle sequencing kit (GE Healthcare Bio-Science, Little Chalfont, Buckinghamshire, UK) with a Long-Read Tower DNA sequencer (GE Healthcare Bio-Science).

RNA Preparation from Human Tissues and Reverse Transcription-Polymerase Chain Reaction Analyses. Total RNA samples were extracted from the human livers by using RNAiso (Takara, Shiga, Japan). Real-time reverse transcription (RT)-PCR was performed for quantitative determination of *AADAC* mRNA levels by using an MX3000P real-time PCR system (Stratagene, La Jolla, CA), as described previously (Watanabe et al., 2009). Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were 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 by using standard amplification curves.

Genotyping Assays of *AADAC2 and *AADAC**3 Alleles.** For genotyping of the *AADAC**2 allele concerning g.13651G>A, an allele-specific PCR analysis was performed. The primers used 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 \times PCR buffer [67 mM Tris-HCl, pH 8.8, 16.6 mM (NH₄)₂SO₄, 0.45% Triton X-100, 0.02% gelatin], 1.5 mM MgCl₂, 0.2 mM dNTPs, 0.4 mM levels of each primer, and 0.5 U of *Taq* polymerase, in a final volume of 25 μ l. After an initial denaturation at 94°C for 3 min, the amplification was performed with 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 with electrophoresis in a 2% agarose gel. For genotyping of the *AADAC**3 allele concerning g.14008T>C, PCR-restriction fragment length polymorphism analysis 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 with 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 AccI. The digestion patterns were determined with electrophoresis in a 3% agarose gel. *AADAC**1 (wild-type) yielded a 181-base pair fragment, and *AADAC**3 yielded 158- and 23-base pair fragments.

Construction of Plasmids Expressing Human *AADAC* Variants. The expression plasmids for *AADAC*.1 and *AADAC*.2, which are proteins produced from *AADAC**1 and *AADAC**2 (c.841G>A) alleles, respectively, were constructed in our previous study (Watanabe et al., 2009). To construct the expression plasmid for *AADAC*.3, which is a protein produced from the *AADAC**3 allele (c.841G>A/c.1288T>C), the c.1288T>C mutation was introduced into the expression plasmid of *AADAC*.2 through site-directed mutagenesis with a Quick Change II XL site-directed mutagenesis kit (Stratagene). The primers used were *AADAC* SDMS and *AADAC* SDMAS (Table 1). Nucleotide sequences were confirmed with DNA sequence analysis by using primers 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 \times 10⁶ cells per well) with 7.5 μ g of each expression plasmid, by 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 were disrupted with freeze-thawing three times. The

TABLE 1
Sequences of primers used in present study

Primer	Sequence
For sequence analysis of AADAC gene	
AADAC -2085S	5'-CATTTTACCCATAGCACTGG-3'
AADAC -1447AS	5'-GGCAAGACAGTATATATTCC-3'
AADAC -1014AS	5'-GCTGGGAAGACATCTTTTGA-3'
AADAC -536AS	5'-AAGTAGTTTGTGTTGAGGGTG-3'
AADAC -97AS	5'-GATACAGTCATGTTAAGTGC-3'
AADAC -21AS	5'-GTGAACGTCCCCTTCTTGG-3'
AADAC -79S	5'-CAGATAAAATTTTGGCCTTA-3'
AADAC int1AS	5'-AGCCACAGCAGTTATTTTA-3'
AADAC int1S	5'-CAGATGCAAATCACCTCCAC-3'
AADAC int2AS	5'-GTAAATGTCTGCGAACTACC-3'
AADAC int2S	5'-GCCAGAACGTGAAAGAAGTG-3'
AADAC int3AS	5'-TGAGAATCACTTGAAACCGG-3'
AADAC int3S	5'-CTGGGATGCGGGCAATGTTA-3'
AADAC int4AS	5'-CTATGACAGAAGCAGGGATTG-3'
AADAC int4S	5'-AGATAGATAATCTTATTAGG-3'
AADAC 3'-flankingAS	5'-TGGCACTTAATTCATATCTG-3'
For RT-PCR analysis	
AADAC exon 2F	5'-TTGTGGAGCTCCTGGGACTT-3'
AADAC exon 3R	5'-TCTGTCTGCTGTCCATCTTG-3'
hGAPDH	5'-CCAGGGCTGCTTTTAACTC-3'
hGAPDHAS	5'-GCTCCCCCTGCAAATGA-3'
For genotyping of AADAC*2 and AADAC*3 alleles	
AADAC g.13651-wild	5'-AAGTCATCTCTTCAAATTTG-3'
AADAC g.13651-mutant	5'-AAGTCATCTCTTCAAATTTA-3'
AADAC g.14307AS	5'-GGCAATATTTGCTCACATTT-3'
AADAC ex5 (Acc I)	5'-TTGAGTGGCTAAAGGAAAGTCTA-3'
AADAC UTRAS	5'-AATGAAATCCCACAGTAAGA-3'
For construction of plasmid expressing human AADAC variants	
AADAC SDMS	5'-GAGTGGCTAAAGGAAAATCTACAGTAAACATG-3'
AADAC SDMAS	5'-CATGTTTACTGTAGATTTTCCTTAGCCACTC-3'
T7F	5'-TAATACGACTCACTATAGGG-3'
pTarget-AS	5'-ATTTAGGTGACACGATAGAATA-3'

expression level of each protein was determined with 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 membranes (Immobilon-P; Millipore Corporation, Billerica, MA). The membranes were probed with monoclonal mouse anti-human AADAC antibody (Abnova, Taipei City, Taiwan), and the corresponding fluorescent dye-conjugated secondary antibody and an Odyssey IR imaging system (LI-COR Biosciences, Lincoln, NE) were used for detection. The expression level was quantified by using ImageQuant TL image analysis software (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). This analysis was performed in the

linear range of band intensity with respect to the amount of protein. The relative expression levels of recombinant wild-type and variant-type AADAC were estimated by comparing band intensities.

PNPA Hydrolase Activity Assay. The hydrolase activity of PNPA, a general esterase substrate, was determined by using COS7 cell homogenates expressing AADAC and individual human liver microsomes (HLMs). The PNPA hydrolase activity was determined as follows. A typical incubation mixture (final volume, 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 dimethylsulfoxide, and the final concentration of dimethylsulfoxide in the incubation mixture was 1.0%. The reaction was initiated with the addition of 500 μM PNPA after a 2-min preincubation at 37°C. After a 1-min incubation at 37°C, the reaction was terminated with the

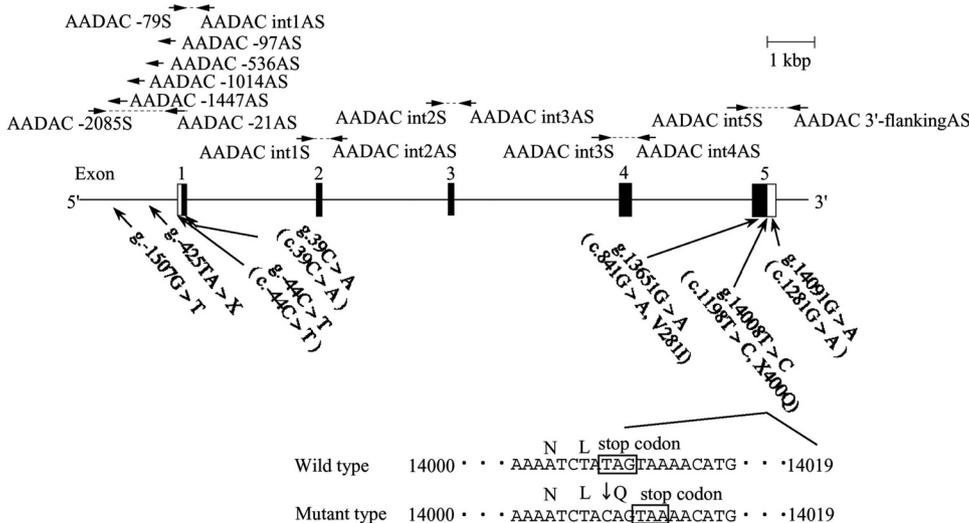


Fig. 1. Schematic representation of the human AADAC gene (reference genomic sequence, NC_000003.11) in the liver sample that showed extremely low flutamide hydrolase activity. The nucleotide positions on the genomic DNA are indicated. The cDNA and amino acid changes are in parentheses. Open boxes, untranslated regions. Solid boxes, coding exons. Solid lines, introns. Horizontal arrows, locations and directions of primer pairs for PCR amplification and sequence analyses. Dashed lines, regions amplified with PCR.

addition of 100 μ l of ice-cold methanol. The produced PNP, a metabolite of PNPA hydrolysis, was measured on the basis of the absorbance at 405 nm, by 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 nonenzymatically converted to PNP at 37°C to some extent, the content of PNP in a mixture incubated without the enzyme was subtracted from that in a mixture with the enzyme, to correct activity values.

Flutamide, Phenacetin, and Rifampicin Hydrolase Activity Assays. The flutamide, phenacetin, and rifampicin hydrolase activities were determined according to our previous reports (Watanabe et al., 2009, 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 with the Hardy-Weinberg equilibrium model by using Fisher's exact test with the computer program InStat 2 (GraphPad Software, San Diego, CA). Kinetic parameters were estimated from the fitted curve by 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 with the Michaelis-Menten equation, $V = V_{max}S/(K_m + S)$, for flutamide and phenacetin hydrolase activity or the substrate inhibition equation, $V = V_{max}S/(K_m + S + S^2/K_i)$, for rifampicin hydrolase activity. The CL_{int} value was calculated as follows: $CL_{int} = V_{max}/K_m$. Statistical significance between multiple groups was determined through analysis of variance, followed by the Dunnett or Tukey test, by using InStat 2. A P value of <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; the National Center for Biotechnology Information, <http://www.ncbi.nlm.nih.gov/gene/13>) was performed by using a human liver sample that showed extremely low flutamide hydrolase activity in our previous study (Kobayashi et al., 2012). 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 five SNPs were located at exons 1 and 5. Among them, g.13651G>A and g.14008T>C, located at exon 5, were nonsynonymous SNPs. The g.13651G>A SNP leads to an amino acid change from valine to isoleucine at the 281 position (V281I). The g.14008T>C SNP leads to an amino acid change from a stop codon to glutamine at the 400 position (X400Q), which results in a one-amino acid extension at the C terminus, because the codon (TAA) after Glu400 indicates the stop codon.

Expression of AADAC mRNA in Human Liver. The expression level of AADAC mRNA in human liver was determined with real-time RT-PCR analysis. In this analysis, six human liver samples that were analyzed in our previous study (Kobayashi et al., 2012) were used. The expression level of AADAC mRNA in a liver sample that showed extremely low flutamide hydrolase activity (flutamide hydrolase activity at 500 μ M, 0.02 ± 0.01 nmol \cdot min $^{-1}$ \cdot mg $^{-1}$; AADAC mRNA/GAPDH mRNA, 0.04 ± 0.00) was comparable to those in other samples (flutamide hydrolase activity at 500 μ M, 0.15 ± 0.02

to 0.40 ± 0.04 nmol \cdot min $^{-1}$ \cdot mg $^{-1}$; AADAC mRNA/GAPDH mRNA, 0.09 ± 0.01 to 0.39 ± 0.01). This result suggested that the SNPs in the 5'-flanking region of the AADAC gene could not affect transcriptional regulation.

Allelic Frequencies of AADAC*2 and AADAC*3. Genotyping assays of g.13651G>A and g.14008T>C, which are nonsynonymous mutations, were performed by using genomic DNA samples from 200 European American, 178 African American, 212 Korean, and 140 Japanese individuals (Table 2). It was confirmed, through 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). Wild-type AADAC 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 allelic frequencies of 52.6 to 63.5%, whereas the AADAC*3 allele was found in European American (1.3%) and African American (2.0%) but not Japanese and Korean individuals. The allelic frequencies of AADAC*2 and AADAC*3 were in accordance with the Hardy-Weinberg equation. Therefore, there were ethnic differences in the allelic frequency of AADAC*3.

Kinetic Analyses of Flutamide, Phenacetin, and Rifampicin Hydrolase Activities of Recombinant Human Wild-Type AADAC and Variants. For comparison of the flutamide, phenacetin, and rifampicin hydrolase activities among human wild-type AADAC and variants, they were transiently expressed in COS7 cells. To compare the expression levels among wild-type and variant AADAC, immunoblot analysis was performed by 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 and 0.5 ± 0.0 units, respectively. In the subsequent study, the activities in the expression systems were normalized by using these units. For both wild-type AADAC and variants, data for the flutamide and phenacetin hydrolase activities were fitted to Michaelis-Menten kinetics, and data for the rifampicin desacetylase activity were fitted to substrate inhibition kinetics (Fig. 2, B–D). AADAC.1 (wild-type) showed flutamide, phenacetin, and rifampicin hydrolase activities with CL_{int} values of 1.31 ± 0.06 , 1.00 ± 0.02 , and 0.39 ± 0.02 μ l \cdot min $^{-1}$ \cdot unit $^{-1}$, respectively (Table 3). AADAC.2 showed flutamide and rifampicin hydrolase activities with similar CL_{int} values (0.95 ± 0.05 and 0.51 ± 0.00 μ l \cdot min $^{-1}$ \cdot unit $^{-1}$, respectively), compared with wild-type AADAC. AADAC.2 showed a lower CL_{int} value for phenacetin hydrolase activity (0.61 ± 0.01 μ l \cdot min $^{-1}$ \cdot unit $^{-1}$) than did wild-type AADAC, although statistical significance was not reached ($P > 0.05$). AADAC.3 showed significantly lower CL_{int} values for all activities (flutamide hydrolase, 0.21 ± 0.02 μ l \cdot min $^{-1}$ \cdot unit $^{-1}$; phenacetin hydrolase, 0.12 ± 0.00 μ l \cdot min $^{-1}$ \cdot unit $^{-1}$; rifampicin hydrolase, 0.03 ± 0.01 μ l \cdot min $^{-1}$ \cdot unit $^{-1}$) because of the decreased V_{max} values. The PNPA hydrolase activity of AADAC.3 at

TABLE 2
Allele frequencies of AADAC*2 and AADAC*3 in four populations

No. of Subjects	AADAC Genotypes						Allelic Frequencies			
	AADAC*1/ AADAC*1	AADAC*1/ AADAC*2	AADAC*2/ AADAC*2	AADAC*1/ AADAC*3	AADAC*2/ AADAC*3	AADAC*3/ AADAC*3	AADAC*1	AADAC*2	AADAC*3	
	%						%			
European American	200	26.5	21.5	50.0	0.0	1.5	0.5	37.20	61.50	1.3
African American	178	20.8	25.8	49.4	2.3	2.3	0.0	34.50	63.50	2.0
Japanese	140	22.1	34.3	43.6	0.0	0.0	0.0	39.3	60.7	0.0
Korean	212	28.7	37.3	34.0	0.0	0.0	0.0	47.40	52.60	0.0

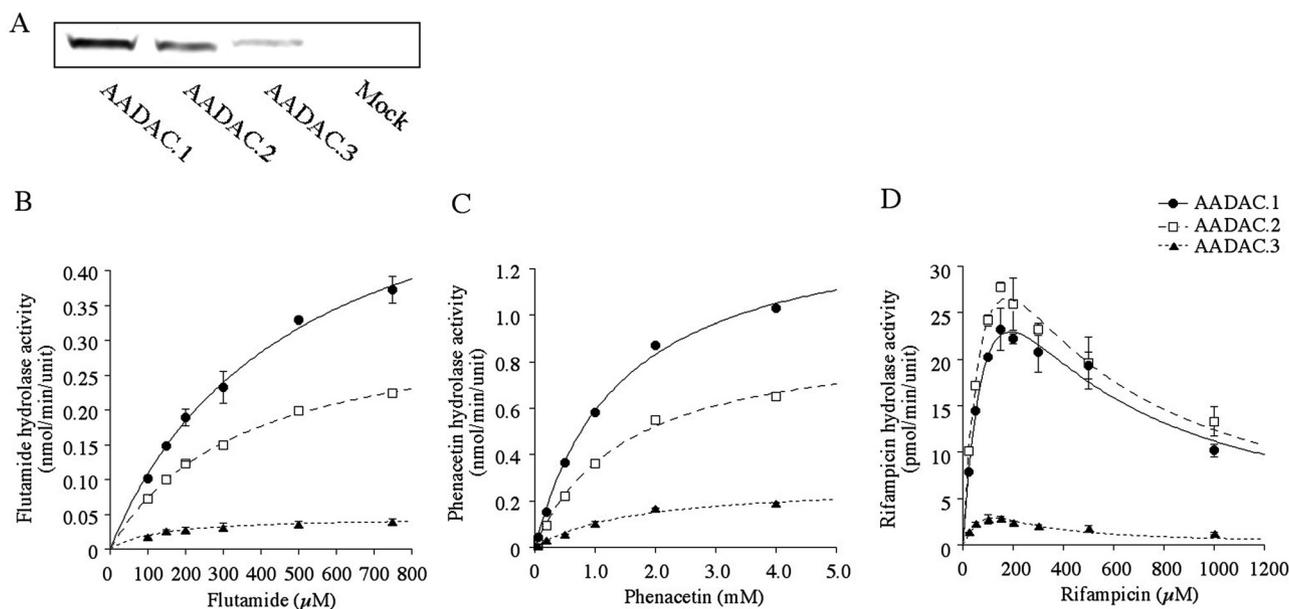


FIG. 2. Effects of AADAC variants on flutamide, phenacetin, and rifampicin hydrolase activities. A, immunoblot analysis of recombinant human AADAC expressed in COS7 cells. Total cell homogenates from COS7 cells (30 μg) were separated through electrophoresis with a 10% SDS-polyacrylamide gel. B to D, kinetic analyses of flutamide (B), phenacetin (C), and rifampicin (D) hydrolase activities of homogenates of COS7 cells expressing human wild-type AADAC and variant types. The homogenates of COS7 cells expressing these enzymes were incubated with 100 to 750 μM flutamide, 50 to 4000 μM phenacetin, and 5 to 1000 μM rifampicin, respectively. The activities of the expression systems were normalized with respect to the units measured in immunoblot analyses (AADAC.1, 1.0 ± 0.0 units; AADAC.2, 0.9 ± 0.0 units; AADAC.3, 0.5 ± 0.0 units). Each data point represents the mean \pm S.D. of triplicate determinations. *, $P < 0.05$, compared with AADAC.1 (wild-type).

a concentration of 500 μM ($0.21 \pm 0.03 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{unit}^{-1}$) was also substantially lower than those of AADAC.1 ($1.47 \pm 0.03 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{unit}^{-1}$) and AADAC.2 ($1.23 \pm 0.11 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{unit}^{-1}$). These results suggested that the one-amino acid extension at the C terminus in AADAC.3 caused the decreased catalytic efficiency of the AADAC enzyme.

PNPA, Flutamide, Phenacetin, and Rifampicin Hydrolase Activities and AADAC Protein Levels in Individual HLM Samples.

To investigate further the effects of AADAC genetic polymorphisms on enzyme activities, the hydrolase activities for PNPA (500 μM), flutamide (500 μM), phenacetin (1 mM), and rifampicin (50 μM) were measured in 24 individual HLM samples (Fig. 3). PNPA is a general esterase substrate, whereas phenacetin and rifampicin are specific AADAC substrates. Flutamide is hydrolyzed by AADAC with a large contribution at a concentration of 500 μM (Kobayashi et al., 2012). 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. HLMs from a

subject genotyped as AADAC*3/AADAC*3 showed PNPA hydrolase activity ($4.18 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$) similar to that of samples genotyped as AADAC*1/AADAC*1, AADAC*1/AADAC*2, and AADAC*2/AADAC*2 (1.73, 3.34 ± 1.01 , and $2.42 \pm 0.95 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$, respectively) (Fig. 3A). In contrast, flutamide, phenacetin, and rifampicin hydrolase activities in HLMs from a subject genotyped as AADAC*3/AADAC*3 ($0.02 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$, $0.07 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$, and $0.16 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$, respectively) were substantially lower than those in other samples (Fig. 3, A–C). The AADAC protein expression levels in HLM samples were analyzed through immunoblotting (Fig. 3E). The AADAC protein expression levels seemed to be correlated with the AADAC enzyme activities measured by using flutamide, phenacetin, and rifampicin as substrates, and HLMs from a subject genotyped as AADAC*3/AADAC*3 showed the lowest levels of 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 one HLM sample with AADAC*3/AADAC*3.

TABLE 3

Kinetic parameters for flutamide, phenacetin, and rifampicin hydrolase activities

Enzyme Activity	K_m	V_{max}	CL_{int}	K_i
		$\text{pmol} \cdot \text{min}^{-1} \cdot \text{unit}^{-1}$	$\mu\text{l} \cdot \text{min}^{-1} \cdot \text{unit}^{-1}$	μM
Flutamide hydrolase				
AADAC.1	$472 \pm 15.9 \mu\text{M}$	617 ± 28.9	1.31 ± 0.06	
AADAC.2	$348 \pm 27.5 \mu\text{M}$	331 ± 9.7	0.95 ± 0.05	
AADAC.3	$124 \pm 9.31 \mu\text{M}^*$	$261 \pm 1.4^*$	$0.21 \pm 0.02^*$	
Phenacetin hydrolase				
AADAC.1	$1.42 \pm 0.03 \text{ mM}$	1420 ± 8.8	1.00 ± 0.02	
AADAC.2	$1.52 \pm 0.09 \text{ mM}$	921 ± 36.2	0.61 ± 0.01	
AADAC.3	$1.56 \pm 0.01 \text{ mM}^*$	$186 \pm 8.0^*$	$0.12 \pm 0.00^*$	
Rifampicin hydrolase				
AADAC.1	$154.0 \pm 7.27 \mu\text{M}$	60.2 ± 1.19	0.39 ± 0.02	237 ± 29.5
AADAC.2	$129.0 \pm 18.2 \mu\text{M}$	66.0 ± 8.67	0.51 ± 0.00	249 ± 71.6
AADAC.3	$75.9 \pm 14.2 \mu\text{M}^*$	$2.41 \pm 0.14^*$	$0.03 \pm 0.01^*$	242 ± 50.7

* $P < 0.05$, compared with AADAC.1.

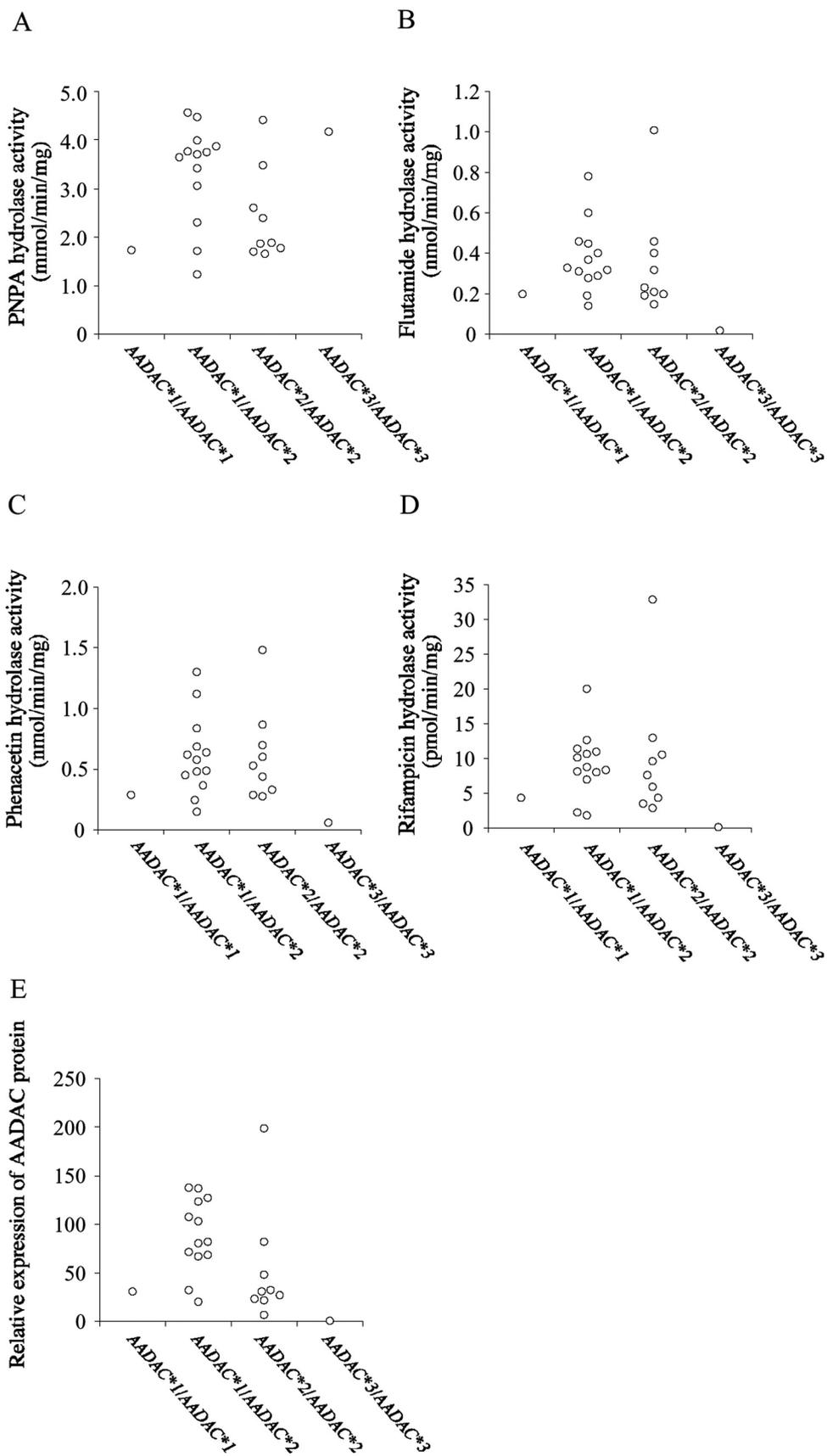


FIG. 3. AADAC enzyme activities and protein levels for 24 individual HLM samples with different AADAC genotypes. A to D, PNPA (A), flutamide (B), phenacetin (C), and rifampicin (D) hydrolase activities in 24 individual HLM samples. HLMs were incubated with 500 μ M PNPA, 500 μ M flutamide, 1 mM phenacetin, and 50 μ M rifampicin, respectively. E, expression levels for AADAC protein in 24 individual HLM samples. HLM proteins (50 μ g) were separated through electrophoresis with a 10% SDS-polyacrylamide gel. The AADAC protein expression levels are represented relative to the level of a AADAC*3/AADAC*3 sample.

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, 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 for CYP3A4 (Nakajima et al., 2011). On the basis of those reports, it was suggested that AADAC is involved in their detoxification and drug interactions. Therefore, interindividual variability in 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 μ M (Kobayashi et al., 2012). Because AADAC is mainly involved in 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 in the sample and found a novel allele of the *AADAC* gene that yielded decreased enzyme activity.

The *AADAC* gene sequences in a human liver sample that showed extremely low flutamide hydrolase activity were analyzed, and seven SNPs were found in a homozygous state (Fig. 1). Two SNPs were present in the 5'-flanking region of the *AADAC* gene, which suggests altered expression of AADAC mRNA. In a search for transcription factor binding sites predicted by 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 that showed extremely low flutamide hydrolase activity had a moderate level of expression of AADAC mRNA. Therefore, we focused on two nonsynonymous SNPs (g.13651G>A, c.841G>A, V281I; and g.14008T>C, c.1198T>C, X400Q) in the coding regions as a cause of the low flutamide hydrolase activity. The g.14008T>C form was predicted to cause the C-terminal one-amino acid (glutamine) extension.

The *AADAC*2* allele (g.13651G>A) was found in all populations investigated in this study, with allelic frequencies of 49.5% to 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=ad hoc_search&type=rs&rs=rs1803155). The *AADAC*3* allele was also described in the dbSNP database, but the allelic frequency was unknown. In this study, the *AADAC*3* allele (g.13651G>A/g.14008T>C) was found only in European American (1.3%) and African American (2.0%) samples. Therefore, there were ethnic differences in the allelic frequency of *AADAC*3*.

For kinetic analyses of flutamide, phenacetin, and rifampicin hydrolase activities, wild-type and variant types of AADAC were expressed in COS7 cells. The homogenates of COS7 cells expressing AADAC.2 and AADAC.3 showed a single band with a similar apparent molecular mass, compared with 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 of wild-type and variant forms by

measuring AADAC mRNA expression levels, and we found that *AADAC*3* showed similar AADAC mRNA expression, compared with *AADAC*1* and *AADAC*2* (data not shown). Furthermore, the rate of AADAC.3 protein degradation showed no difference from the rates for AADAC.1 and AADAC.2 (Supplemental Fig. 1). AADAC is located on the luminal side of the endoplasmic reticulum, and its N-terminal domain is a membrane-spanning region (Frick et al., 2004). Although there is no evidence regarding the conformational importance of the C-terminal domain of the AADAC protein, the subtle change in the C-terminal region might cause low levels of AADAC protein expression.

The enzyme activities of each AADAC protein were evaluated by normalizing the data to expression levels as a unit (Fig. 2, B–D). AADAC.2 showed lower or similar CL_{int} values, compared with AADAC.1, although statistical significance was not reached. Our previous report revealed that AADAC.2 (V281I) did not exhibit altered flutamide hydrolase activity (AADAC.1 CL_{int} , $0.8 \pm 0.0 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$; AADAC.2 CL_{int} , $0.9 \pm 0.0 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$) (Watanabe et al., 2009). In a previous study, the flutamide hydrolase activity was evaluated without normalization to the AADAC protein expression level in COS7 cells. This might have caused a slight difference in the measured effect of AADAC.2 between previous and present studies. However, AADAC.3 showed substantially lower CL_{int} values for all enzyme activities. There have been some reports on genetic polymorphisms leading to C-terminal extensions that alter the protein function. For example, in apolipoprotein AII, the stop codon mutation (X78G), which causes a C-terminal, 21-amino acid extension, was found in patients with hereditary systemic amyloidosis (Benson et al., 2001). The protein structure at the C-terminal region of apolipoprotein AII is important for lipid binding; therefore, the protein extension is predicted to decrease lipid binding. In our previous study, we constructed a recombinant AADAC with five histidines tandemly ligated at the C terminus (Kobayashi et al., 2012), 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 a K_m value similar to that of AADAC.1, whereas AADAC.3 showed lower K_m values than did AADAC.1 for flutamide and rifampicin hydrolase activities. Although we cannot clearly account for the difference, it may be attributable to the altered AADAC protein structure. To clarify this, further study should be performed.

The effects of AADAC genetic polymorphisms on the enzyme activities were investigated by using 24 HLM samples. HLMs from subjects genotyped as *AADAC*1/AADAC*2* and *AADAC*2/AADAC*2* tended to show similar flutamide, phenacetin, and rifampicin hydrolase activities, compared with those genotyped as *AADAC*1/AADAC*1*. However, because only one sample with *AADAC*1/AADAC*1* was detected among the HLM samples in this study, the effect of the *AADAC*2* allele on enzyme activity could not be evaluated correctly. It was obvious that a HLM sample with *AADAC*3/AADAC*3* showed substantially lower activities (Fig. 3, B–D). The level of AADAC protein expression in that 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 expressed in COS7 cells (Fig. 2A). Therefore, it was possible that the C-terminal, one-amino acid extension in AADAC.3 affected the protein expression level in addition to the decrease in enzyme activity. However, because there was only one sample with *AADAC*3/AADAC*3* in this study, additional study with more samples will be needed to verify the association of the *AADAC*3* allele with the protein expression level. It would be rea-

sonable to suggest, on the basis of the data for recombinant AADAC.3, that the AADAC*3 allele yields decreased AADAC enzyme activity (Fig. 2, A–C).

In conclusion, this study found a novel polymorphic allele of AADAC that yielded decreased enzyme activity. There were ethnic differences in the allelic frequencies of AADAC*3. Human AADAC is involved in the metabolism of some clinical drugs. Information on interindividual variations in AADAC enzyme activity would be important for clinical drug therapy.

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Authorship Contributions

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

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