

Isolation and characterization of arylacetamide deacetylase in cynomolgus macaques

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ABSTRACT. Arylacetamide deacetylase (AADAC), a microsomal serine esterase, hydrolyzes drugs, such as flutamide, phenacetin and rifampicin. Because AADAC has not been fully investigated at molecular levels in cynomolgus macaques, the non-human primate species widely used in drug metabolism studies, cynomolgus AADAC cDNA was isolated and characterized. The deduced amino acid sequence, highly homologous (92%) to human AADAC, was more closely clustered with human AADAC than the dog, rat or mouse ortholog in a phylogenetic tree. *AADAC* was flanked by *AADACL2* and *SUCNRI* in the cynomolgus and human genomes. Moreover, relatively abundant expression of AADAC mRNA was found in liver and jejunum, the drug-metabolizing organs, in cynomolgus macaques, similar to humans. The results suggest molecular similarities of AADAC between cynomolgus macaques and humans.

KEY WORDS: arylacetamide deacetylase, cynomolgus monkey, drug metabolism, genome, tissue expression

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Arylacetamide deacetylase (AADAC) comprises the serine esterase superfamily, along with carboxylesterase (CES) [4], and is responsible for the hydrolysis of various xenobiotics, including clinically important drugs, such as flutamide, phenacetin and rifampicin, in humans [2]. AADAC is involved in the occurrence of renal failure and hematotoxicity in some patients who take flutamide or phenacetin and thus is an important drug-metabolizing enzyme [2]. AADAC partly shares the substrate selectivity with CES enzymes probably due to their structural and functional similarities; for example, flutamide is hydrolyzed by AADAC and CES2 in human liver [6]. Human AADAC, rat *Aadac* and mouse *Aadac* mRNAs are expressed in liver and small intestine, but human AADAC mRNA is not expressed in kidney whereas rat and mouse *Aadac* mRNAs are [5, 8, 11]. Moreover, human AADAC hydrolyzes flutamide, phenacetin and rifampicin, whereas rat and mouse *Aadac* enzymes hydrolyze flutamide and phenacetin, but not rifampicin [2]. Therefore, species differences are evident for AADAC-dependent drug metabolism between rodents and humans.

Cynomolgus macaques are frequently used in drug metabolism and toxicity studies due to their evolutionary closeness to humans. Our and other groups have identified and characterized a number of drug-metabolizing enzymes, including cytochromes P450, flavin-containing monooxygenases and glutathione *S*-transferases, and found generally

similar molecular characteristics of these enzymes between cynomolgus macaques and humans [14, 16, 17, 20]. Despite the importance of cynomolgus macaques in drug metabolism studies, AADAC has not been fully investigated partly due to the lack of molecular information on the cynomolgus AADAC. In this study, therefore, cynomolgus AADAC cDNA was characterized by analysis of genomic organization, gene structure, the primary sequence structure, phylogeny and tissue expression pattern.

Cynomolgus AADAC cDNA was originally isolated as an expressed sequence tag, which was generated from cynomolgus liver [21]. Cynomolgus AADAC cDNA sequence identified in this study was deposited to GenBank under accession number KJ922601. Sequence analyses were carried out using DNASIS Pro (Hitachi Software, Tokyo, Japan) and Genetyx system (Software Development, Tokyo, Japan). Multiple alignment of amino acid sequences was carried out using the ClustalW program, and a phylogenetic tree was created by the neighbor-joining method. A homology search was performed using BLAST (National Center for Biotechnology Information, Bethesda, MD, U.S.A.). The human and cynomolgus macaque genome data were analyzed using BLAT (UCSC Genome Bioinformatics) and Sequence Viewer (National Center for Biotechnology Information), respectively. Amino acid sequences used were from GenBank, including human AADAC (NP_001077), human CES1c (NP_001257), dog AADAC (XP_534309), rat *Aadac* (NP_065413) and mouse *Aadac* (NP_075872). Human AADAC cDNA sequence (NM_001086) used was also from GenBank. In addition, cynomolgus AADAC cDNA sequence from this study and the deduced amino acid sequence were also used for the analyses.

Sequence analysis revealed that cynomolgus AADAC had 399 amino acid residues containing the primary sequence structures characteristic of AADAC, such as the His-Gly-Gly-Gly box and the active site motif (Gly-X-Ser-X-Gly)

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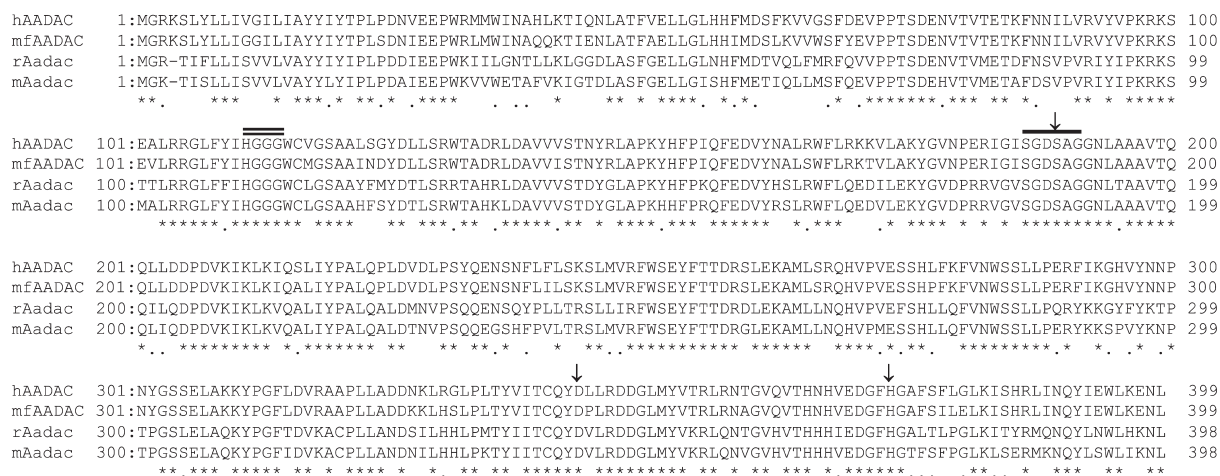


Fig. 1. Multiple alignment of cynomolgus AADAC. AADAC amino acid sequences of human (h), cynomolgus (mf), rat (r) and mouse (m) were aligned as described in text. Single and double solid lines above the sequences indicate the His-Gly-Gly-Gly box and the active site motif (Gly-X-Ser-X-Gly), respectively, characteristic of AADAC. Arrows indicate the residues of the catalytic triad. Asterisks and dots under the sequences show identical and conservatively changed amino acids, respectively.

[11], similar to human AADAC, rat Aadac and mouse Aadac (Fig. 1). Together with 343Asp and 373His, Ser189 of the active site motif forms the catalytic triad essential for the catalytic process, and these three residues are also conserved in cynomolgus AADAC, suggesting that cynomolgus AADAC is most likely functional. Cynomolgus AADAC had high sequence identities of amino acid (92%) and cDNA (96%) to human AADAC, substantially higher than dog AADAC, rat Aadac or mouse Aadac (Table 1). A phylogenetic tree of AADAC amino acid sequences showed that cynomolgus AADAC was most closely clustered with human AADAC as compared with dog AADAC, rat Aadac or mouse Aadac (Fig. 2). These results suggest the evolutionary closeness of AADAC between cynomolgus macaques and humans.

The analysis of the genome data found a single *AADAC* gene, flanked by *AADACL2* and *SUCNRI*, in the cynomolgus and human genomes (data not shown). Therefore, cynomolgus *AADAC* was located in the genomic regions corresponding to the human ortholog. Cynomolgus *AADAC* (~16.2 kb) contained 5 coding exons, and the size of each of these *AADAC* exons was the same between cynomolgus macaques and humans; 138, 223, 70, 172 and 597 bp for exons 1–5, respectively (Fig. 3). All the cynomolgus and human *AADAC* introns begin with the dinucleotide GU and end with AG, consistent with the consensus sequences for splice junctions in eukaryotic genes. These results indicated similar genome and gene structures of *AADAC* in cynomolgus macaques and humans.

Single *AADAC* gene in the cynomolgus and human genomes is in contrast to the multiple *CES1* and *CES2* genes which have been identified in the genomes of various species [3]. The cytochrome P450 family of drug-metabolizing enzymes has evolved by gene duplication and gene loss during evolution [10]. Similarly, the multiple *CES1* and *CES2* genes might have evolved by gene duplication to increase their abilities to detoxify diverse xenobiotics taken. In contrast, a single gene of *AADAC* in various species might reflect the roles of

Table 1. Sequence identity of cynomolgus AADAC cDNA and amino acids as compared to the human, dog, rat or mouse ortholog

| | cDNA (%) | Amino acid (%) |
|-------------|----------|----------------|
| Human AADAC | 96 | 92 |
| Dog AADAC | 81 | 69 |
| Rat Aadac | 74 | 67 |
| Mouse Aadac | 74 | 68 |

Sequence identity was determined in the homologous region of amino acids using BLAST.

this enzyme to metabolize mainly endogenous substrates.

To determine tissue expression patterns of cynomolgus AADAC mRNA, total RNA was extracted from brain, lung, heart, liver, kidney, adrenal gland, jejunum, testis, ovary and uterus tissues, which were collected from six cynomolgus macaques (three males and three females from Indochina, 4–5 years of age, 3–5 kg) as described previously [13]. Pooled samples of these six animals were used to measure mRNA expression. The study was reviewed and approved by the Institutional Animal Care and Use Committee (Shin Nippon Biomedical Laboratories, Ltd., Tokyo, Japan). Quantitative polymerase chain reaction (qPCR) was performed as described previously [13] with the following modifications. PCR was carried out in a total volume of 25 μ l using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, U.S.A.) with the ABI Prism 7500 sequence detection system (Applied Biosystems) according to the manufacturer's protocol. PCR reactions contained a twenty-fifth volume of each reverse transcription product with amplification efficiency of 97% for AADAC mRNA. The primers were used at a final concentration of 600 nM, including mf&hCES5 (5qrt1) 5'-TGCAGGAGGGAATTTAGCTG-3' and mf&hCES5 (3qrt1) 5'-AGAGGCTGAAGGGCAG-GATA-3'. The relative expression level was determined by

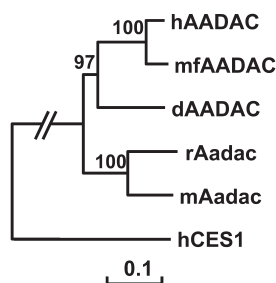


Fig. 2. Phylogenetic analysis of cynomolgus AADAC. AADAC amino acid sequences of humans (h), cynomolgus macaques (mf), dogs (d), rats (r) and mice (m) were used to create a phylogenetic tree as described in text. Human CES1 was used as the outgroup. Cynomolgus AADAC was most closely clustered with human AADAC. Bootstrap values are indicated above branches. The scale bar indicates 0.1 amino acid substitutions per site for distance measurement.

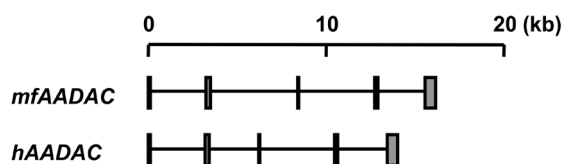


Fig. 3. Gene structures of cynomolgus and human AADAC. The coding region of AADAC cDNA sequence was aligned with the AADAC gene sequence for cynomolgus macaques (mf) and humans (h) as described in text. AADAC gene structure was similar in cynomolgus macaques and humans.

normalization of the raw data to the 18S ribosomal RNA level based on three independent amplifications, as described previously [13].

The analysis showed that among these tissue types, cynomolgus AADAC mRNA was most abundantly expressed in liver, followed by jejunum (Fig. 4), important organs for drug metabolism, similar to human AADAC mRNA [5]. Similarly, human AADAC protein is also expressed in liver and small intestine [8, 23]. In contrast, rodent Aadac mRNA is expressed in liver, jejunum and kidney, but human AADAC mRNA is not expressed in kidney [5]. The results indicated expression of cynomolgus AADAC mRNAs in liver and jejunum, important organs for drug metabolism, and the tissue expression pattern of cynomolgus AADAC mRNA is more similar to that of human AADAC mRNA compared with that of rodent Aadac mRNA.

AADAC is homologous to CES enzymes to some extent and contained the primary sequence structures important for the enzyme function, including active site triad residues and the His-Gly-Gly-Gly box [11] (Fig. 1). AADAC metabolizes the substrates that contain a large alcohol group and small acyl group, such as flutamide, similar to CES2 [6], indicating that AADAC partly has the function similar to CES enzymes. However, unlike CES enzymes, AADAC is retained on the lumen side of the endoplasmic reticulum, because the signal anchor sequence in the N-terminus is not cleaved [1].

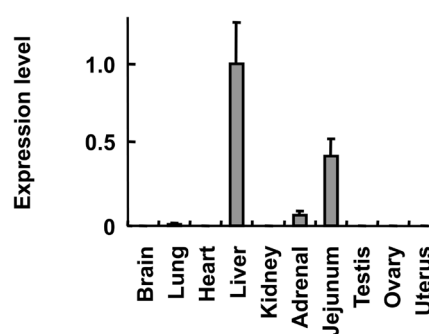


Fig. 4. Tissue distribution of cynomolgus AADAC mRNA expression. Quantitative PCR was conducted in brain, lung, heart, liver, kidney, adrenal gland, jejunum, testis, ovary and uterus. For each tissue, total RNAs from six cynomolgus macaques (three males and three females) were pooled and used for qPCR. Expression level of cynomolgus AADAC mRNA was normalized to 18S rRNA level. Values represent the average \pm S.D. from three independent amplifications, and the highest expression level (liver) was adjusted to 1.

Human AADAC enzyme hydrolyzes flutamide, phenacetin and rifampicin, whereas rat and mouse Aadac enzymes hydrolyze flutamide and phenacetin, but not rifampicin [5]. If substrate specificity of cynomolgus AADAC is similar to that of human AADAC, together with the similar tissue expression pattern of AADAC mRNA as described earlier, cynomolgus macaques might serve as a better animal species in an AADAC-dependent drug metabolism investigation. It is of great importance to investigate hydrolysis properties of cynomolgus AADAC to assess its functional similarity to human AADAC.

In human AADAC, several genetic variants have been identified, including AADAC*3, the enzyme of which shows substantially lower intrinsic clearance of flutamide, phenacetin and rifampicin [9]. Genetic polymorphisms have not been investigated in cynomolgus AADAC. However, cynomolgus macaques possess a heterogeneous genetic background, similar to humans, and thus are expected to have genetic variants. Indeed, numerous genetic variants have been identified in cynomolgus macaques, including the genes encoding drug-metabolizing enzymes, such as cytochromes P450 [12, 15, 18, 19, 22]. The genetic variants would alter enzyme activity of AADAC, possibly resulting in the inter-individual variations of an AADAC-dependent drug metabolism. Metabolic polymorphisms, most likely mediated by AADAC, have been reported for rhesus macaques [7], closely related to cynomolgus macaques. The high- and low-affinity enzymes were purified from extensive metabolizer while only the low-affinity enzyme was from poor metabolizer, and the high-affinity enzyme appeared to account for metabolic polymorphisms. It is of great interest to investigate genetic variants in cynomolgus AADAC.

In conclusion, cynomolgus AADAC, highly homologous to human AADAC, was characterized in this study. Sequence analysis showed the sequence similarity of cynomolgus AADAC to the human ortholog more than that

of the dog, rat or mouse ortholog. Cynomolgus *AADAC* had similar gene structure and genomic organization to human *AADAC*. Moreover, cynomolgus *AADAC* mRNA was relatively abundantly expressed in liver and jejunum, similar to human *AADAC* mRNA, likely reflecting the roles as drug-metabolizing enzymes. The results suggest overall molecular similarities of *AADAC* in cynomolgus macaques and humans and the potential roles of cynomolgus *AADAC* for drug metabolism and toxicity.

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