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Genetic diversity of cytochrome P450 3A with different metabolic activity in domestic cats

Souta SUGIYAMA[1)], Yasuhiro UNO[2)], Tomoko AMANO[3)], Takio KITAZAWA[1)] and Hiroki TERAOKA[1)]*

[1)]School of Veterinary Medicine, Rakuno Gakuen University, Ebetsu, Hokkaido 069-8501, Japan
[2)]Shin Nippon Biomedical Laboratories, Ltd., Kainan, Wakayama 642-0017, Japan
[3)]College of Agriculture, Food and Environment Sciences, Rakuno Gakuen University, Ebetsu, Hokkaido 069-8501, Japan

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ABSTRACT. Knowledge on genetic polymorphisms of metabolising enzymes including cytochrome P450 (CYP) is very limited in cats. We investigated polymorphisms in CYP3A131, one of the major CYP isoforms in the feline liver and small intestine. Eight non-synonymous variants and one synonymous variant of feline CYP3A131 were identified in 29 cats. A major non-synonymous type was not observed. Metabolic parameters (Km and Vmax) of dibenzylfluorescein hydroxylation were ranged within about 2 times for the identified non-synonymous variants by using a heterologous coexpression system of CYP3A131 and feline cytochrome P450 reductase in *Escherichia coli*. The results confirmed the polymorphic nature of CYP3A131 as a basis for effective application of medicines and prevention of adverse reactions in the treatment of domestic cats.

KEY WORDS: cytochrome P450, domestic cat, polymorphism, xenobiotic

Cytochrome P450 monooxygenase (CYP or P450) is the most important enzyme involved in the biotransformation of a broad range of endogenous and exogenous chemicals including drugs. There have been reports from many years ago that there is considerable interindividual variability in response to drugs in humans and possibly in animals including dogs [1]. Extensive studies on genetic polymorphism of CYPs have been carried out in human, because that is the main factor responsible for variation in sensitivity to medical drugs and toxicants [9]. CYP3A is constitutively expressed as the most abundant CYP enzymes in the liver and is the only CYP subfamily present in substantive amounts in the enteric mucosa of gastrointestinal tract [7]. CYP3A is the most important CYP isoform in the biotransformation of broad clinical therapeutics [2]. At least 18 allelic variants of CYP3A4, the most important CYP3A isoform in human have been identified so far with substitutions of amino acids in the coding region [4].

Drugs approved for humans, even in developed countries (Animal Medicinal Drug Use Clarification Act of 1994 in U.S.A.; Pharmaceuticals and Medical Devices Law in Japan), can be prescribed for off-label use for veterinary medicine at the discretion of a veterinarian. Thus, they are often also used in companion animals, of which the domestic cat, *Felis catus*, is one of the most common. However, little is known about the CYPs that are involved in the biotransformation of xenobiotics in cats despite their remarkable species differences in drug metabolism. CYP3A131 is one of the major CYP isoforms, following CYP2E2, CYP2A13 and CYP2E1 in the feline liver, while CYP3A131 transcript was predominant in the small intestine [3, 5]. Metabolic activities of CYP3A131 and the interactions with 17 compounds have been determined using 7-benzyloxy-4-(trifluoromethyl)-coumarin (BFC), a fluorogenic substrate [5].

There is few reports on genetic polymorphism of CYP isoforms in the cat. Therefore, we determined genetic polymorphisms and their effects on metabolic activity of CYP3A131.

Gonad samples were collected from 18 male (testis) and 11 female (ovary) cats (*Felis catus*) that were obtained in spay surgery in local animal hospitals in Japan from 2010 to 2016 with permissions from the animal owners (Supplemental Table S1). Following the manufacturer's instructions, genomic DNA and total RNA were extracted from gonads with TRI Reagent (Molecular Research Centre, Cincinnati, OH, U.S.A.) and cDNA was prepared from total RNA with PrimeScript Reverse Transcriptase (Takara, Kusatsu, Japan). In order to address SNPs (single nucleotide polymorphisms), direct DNA sequencing of PCR products of CYP3A131 variants with genomic DNA (BigDye Terminator v3.1 Cycle Sequencing Kit, Thermo Fisher, Waltham, MI, U.S.A.) was repeated using ABI310 sequencer (Thermo Fisher) from the PCR step (KOD-Plus-Neo, Toyobo, Osaka, Japan). Heterologous

*Correspondence to: Teraoka, H.: hteraoka@rakuno.ac.jp

Table 1. Identified variants of CYP3A131 in cats

	Position	Nucleotide change	Amino acid change	Allele frequency	Comments
*1A	-	-	-	23/50	
*1B	63 (Exon1)	GTGCT (T>C) CTCTA	-	11/50	Synonymous
*2	322, 337 (Exon5)	CT (C>T) TT ; GA (C>T) TT	L108F ; L113F	8/50	SRS1
*3	337 (Exon5)	GGTCT (C>T) TTGGT	L113F	2/50	SRS1
*4	445 (Exon6)	CCATC (G >A) TTGGC	V149I	8/50	
*5	702 (Exon8)	TTTGA (A>C) CTATT	E234D	19/50	
*6	1,030 (Exon11)	AGGCA (C>T) CTCCC	P344S	18/50	
*7	1,243 (Exon11)	AGTTC (C>T) ATCCT	H415Y	8/50	
*8	1,480 (Exon13)	AGGTT (G>T) AGCTG	E494X	2/50	STOP

For each variant, single polymorphic nucleotide with the number from translational start codon was indicated different to CYP3A131*1A sequence recorded in DDBJ (LC457700). Mutation sites of CYP3A131*2 and *3 are located in SRS1 (possible substrate-recognition site 1). CYP3A131*8 contains a stop codon in the last part of the open reading frame.

Table 2. Apparent kinetic parameters for metabolism of DBF by CYP3A131 variants

	Km	Vmax	Vmax/Km
*1A	$6.3 \pm 0.6^{a)}$	$73.3 \pm 3.3^{a)}$	$11.9 \pm 0.7^{a,b)}$
*2	$9.0 \pm 0.9^{a,b)}$	$89.5 \pm 4.5^{a,b)}$	$10.0 \pm 0.4^{a)}$
*3	$8.6 \pm 1.0^{a)}$	$83.2 \pm 4.7^{a,b)}$	$9.6 \pm 0.5^{a)}$
*4	$8.3 \pm 0.6^{a)}$	$116.2 \pm 3.9^{b)}$	$13.9 \pm 0.3^{b)}$
*5	$14.6 \pm 2.2^{b)}$	$161.8 \pm 14.6^{c)}$	$11.8 \pm 0.7^{a)}$
*6	$8.6 \pm 0.8^{a)}$	$96.4 \pm 4.5^{a,b)}$	$11.2 \pm 0.1^{a)}$
*7	$8.0 \pm 0.8^{a)}$	$82.0 \pm 4.2^{a,b)}$	$10.3 \pm 0.4^{a)}$
*8	$8.1 \pm 0.6^{a)}$	$79.8 \pm 3.1^{a,b)}$	$9.9 \pm 0.4^{a)}$

Four different bactosomes were used for each determination (N=4). Values with different letters are significantly different ($P<0.05$).

coexpression of CYP3A131 isoforms in *E. coli* was carried out as previously described [5]. Feline cytochrome P450 reductase (POR) (DDBJ Accession No. LC416293) was coexpressed. For obtaining variants of CYP3A131 and POR, PCR products that were prepared with cDNA were modified using a KOD-Plus Mutagenesis Kit (Toyobo). Because we noticed that water-solubility of BFC was not enough in the preliminary experiments, metabolic activity of recombinant CYP3A131 was measured with dibenzylfluorescein (DBF) as described previously [6]. DBF is a specific substrate for CYP3A4 and CYP2C in human [6]. Primers used in PCR, DNA sequencing and mutagenesis are listed in Supplemental Tables 2 and 3. Results are presented as means ± SEM. Significance of differences among groups was determined by one-way ANOVA followed by the Tukey-Kramer test ($P<0.05$). Kinetic parameters were determined by nonlinear regression fitting of the concentration velocity curve with the Michaelis-Menten equation, using GraphPad Prism version 7 (GraphPad Software, San Diego, CA, U.S.A.).

Overall, nine CYP3A131 variants including eight non-synonymous variants and one synonymous variant were identified in 29 animals (Table 1). Surprisingly, a major genetic type was not found in this study. Thus, we named the most common type CYP3A131*1A. The only synonymous type that was identified was named CYP3A131*1B. Although CYP3A131*1A is the most common type (23/50 alleles), frequencies of CYP3A131*5 (19/50 alleles) and *6 (18/50 alleles) were very close to that of CYP3A131*1A. CYP3A131*2 and *3 contained non-synonymous variants in the possible substrate-recognition site (SRS). *CYP3A131**8 contained the stop codon near the end of the coding region.

Using a heterologous coexpression system of CYP3A131 in *E. coli*, hydroxylation activity of DBF (8 concentrations between 0.3125 and 15 μM) by CYP3A131 variants was determined. We used feline POR instead of human POR, which was used in previous experiments [5]. We used wild-type POR (POR*1) among two variants that we identified in this study (Supplemental Table S4). Based on Michaelis-Menten plots, Vmax and Km for each CYP3A131 variant were determined (Table 2). Significant differences were observed for some variants in both Vmax and Km. The range was almost 2 times (maximum rates of 2.2 for Vmax and 2.3 for Km) and Vmax/Km was smaller (Table 2). Activities of CYP3A131*2, *3 and *7 were not notably different to activities of the other types (Table 2).

This is the first study to confirm the presence of a polymorphism of the CYP isoform that could affect metabolism of drugs and other chemicals in the liver and small intestine of cats, using a heterologous coexpression system. In three major variants, frequencies of the second three major variants were almost half of those of the first major variants. Thus, the distribution of CYP3A131 varied greatly in the cat population. Although eight non-synonymous variants were identified in this study, only one synonymous variant was identified. This very high ratio of non-synonymous to synonymous mutations (ds/dn) suggests the necessity of many types of CYP3A131 for adaptation to various environments, although more extensive analysis using a much larger number of samples from cats obtained worldwide is needed. It was reported that human CYP3A4, a major enzyme catalysing

the metabolism of both endogenous and exogenous agents, may play a role in the etiology of carcinogenesis and early puberty [9]. Thus, it is interesting to study the role of CYP3A131 in survival other than xenobiotic metabolism in cats.

When DBF was used as a substrate in the heterologous expression system in *E. coli*, the variation of enzymatic activity of variants ranged around 2 times (Table 2). This situation is similar to that of human CYP3A4. Of the 26 non-synonymous variants known for human CYP3A4, only seven showed different metabolic activity [8]. There are few significant SNPs that have remarkable metabolic effects in human CYP3A4; only two variants without protein products including a frameshift mutation (CYP3A4*20) and a nonsense mutation (CYP3A4*26) have been reported [8]. Further study is required for feline CYP3A131 to determine the metabolic activity with other substrates and to find more variants.

In summary, the present study confirmed the polymorphic nature of CYP3A131 with varied hydroxylation activities in cats. This is the first study on polymorphism in xenobiotic metabolising enzymes in cats. The ratio of non-synonymous to synonymous variants was very high, suggesting the influence of powerful selection pressure. Although none of the eight non-synonymous variants identified in this study showed a great difference in their hydroxylation activity, critical CYP3A131 variants that are important for drug treatment might be found in the future.

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