NOTE *Pharmacology*

Expression of Two Novel Cytochrome P450 3A131 and 3A132 in Liver and Small Intestine of Domestic Cats

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ABSTRACT. Cytochrome P450 3A (CYP3A) is the major subfamily of CYP, one of the most important metabolizing enzymes for drugs in humans and other mammals. We found two novel CYP3A genes, CYP3A131 and CYP3A132 in domestic cats (*Felis catus*). Both feline CYP3A proteins consist of 504 deduced amino acids and show high identity with canine CYP3A homologues and those of some artiodactyls. CYP3A131 transcripts were expressed predominantly in liver and small intestine, and to a negligible extent in other tissues, including brain, heart, kidney and lung. CYP3A132 expression was only detected in liver with much lesser amount. These results suggest the possible major role of CYP3A131 in xenobiotic metabolism including first-pass effects in domestic cats.

KEY WORDS: carnivore, cytochrome P450, domestic cat, felid, xenobiotics.

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Cytochrome P450 (CYP) monooxygenase is one of the most important enzymes for metabolism of chemicals to inactive hydrophilic substances for excretion, while active intermediates could be generated to cause toxic responses in some cases. CYP3A is constitutively expressed as the most abundant CYP enzymes in liver and is the only CYP subfamily present in substantive amounts in the enteric mucosa of gastrointestinal tract [9]. CYP3A is of major importance in the biotransformation of broad clinical therapeutics including calcium channel blockers, benzodiazepines, immunosuppressants and others in humans. It is well known that CYP3A is inducible by some chemicals like rifampicin and corticosteroids as one of the factors responsible for variable drug effectiveness [3].

Knowledge of CYP involved in the biotransformation of drugs and other xenobiotics in cats is mostly lacking. In general, they are very sensitive to chemicals. This is partly because of deficiency of enzymes that participate in phenol glucuronidation [2]. Enzymatic activities of CYP3A-like protein, as well as CYP1A, 2B, 2C, 2D and 2E, were characterized in hepatic microsomes of domestic cats, using substrates specific for humans or fluorescent substrates with combination of human prototypical inhibitors to CYP species [11, 16]. CYP2A-like and other CYP activities in cat liver microsomes have been also studied with substrates specific for humans to be compared with CYP activities in other mammals [1, 10]. Molecular structures and enzymatic properties of CYP1A1, CYP1A2, CYP2E1 and CYP2E2 heterologously expressed in yeast have been also studied in domestic cats [13, 14]. Recently, tissue distribution of feline CYP2D6 was characterized as a major metabolizing enzyme in liver [4]. However, there is no other information available so far, even in whole felids.

In the present study, we found two novel CYP3A homologues, CYP3A131 and CYP3A132 from liver of domestic cats. We determined the complete cDNA sequence of the open reading frame with 5'- and 3'-ends and deduced amino acid sequences. Expression of CYP3A131 and CYP3A132 was quantified in liver and in some other tissues such as small intestine.

Mongrel cats (short-haired type) of both sex (5 males and 6 females) received humane care under the guidelines of Sapporo Medical University for laboratory animals and the experimental protocol was approved by the Committee for Animal Experiments at Rakuno Gakuen University. They were allowed food and drinking water *ad libitum* and were maintained at 25 ± 1°C under a standard 12 hr light–dark cycle. Fresh liver, brain (cerebrum), heart, lung, small intestine and kidney (cortex) were removed from cats euthanized after bleeding from carotid artery under anesthesia with intravenous administration of pentobarbital sodium (50 mg/kg body weight). Total RNA was extracted from the liver using a conventional acid guanidine–phenol–chloroform method (Trizol; Invitrogen, Carlsbad, CA, U.S.A.) [4]. cDNA was synthesized from total RNA using oligo dT primer (Invitrogen) and reverse transcriptase (SuperScript III; Invitrogen). The feline CYP3A fragments were amplified by reverse transcription-PCR (RT-PCR) with degenerate primers, and 3'- or 5'-rapid amplification of cDNA ends (3'- and 5'-RACE). Degenerate oligonucleotide primers used for PCR were based on the nucleotide sequences of CYP3A from several mammalian species (forward 5'-TACAGCATRGAYGTGATYAC-3': reverse 5'-GGYTKGAAGGARAAGTTCTG-3'). 3'-RACE and 5'-RACE were conducted using 3'-Full RACE and 5'-Full RACE Core Sets (Takara, Otsu, Japan) according to the

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manufacturer's instructions. PCR products were subcloned into T-vectors (Promega, Madison, WI, U.S.A.). The nucleotide sequences of these clones were determined using an automated DNA sequencer (ABI 310 Genetic Analyzer; Applied Biosystems, Foster City, CA, U.S.A.) after reactions using a BigDye terminator cycle sequencing kit (Applied Biosystems). Sequence alignment and phylogenetic analysis were carried out using Clustal W software (neighbor-joining method, Kimura's 2-parameter model) found at the DNA Data Bank of Japan (DDBJ) homepage. We repeated these procedures from RNA extraction with different 2 female cat livers.

In order to examine expression levels of feline CYP3A in some tissues, quantitative RT-PCR analysis was performed with a real-time PCR detector (Chromo4; Bio-Rad, Hercules, CA, U.S.A.) using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen), as described previously [15].

Using degenerate RT-PCR procedures, we succeeded in obtaining two different fragments of feline CYP3A with a translation initiation codon and with both 5'- and 3'-ends. These feline sequences cloned did not show conserved synteny with CYP3As in other species. They appear to be paralogs of the human CYP3A gene cluster with four genes. A similar CYP3A cluster that consists of two genes exists also in dogs. However, what is orthologous to feline sequences cannot be determined. Thus these were regarded as novel CYP3A homologues and were named as CYP3A131 (DDBJ Accession No. AB558978) and CYP3A132 (AB558979) by P450 Nomenclature Committee [7].

Open reading frames of both feline CYP3As were 1,512 bp and the deduced amino acid sequences were 504. The 3'-UTR region contained 303 and 434 nucleotides before the poly (A) tail for CYP3A131 and CYP3A132, respectively. Heme-binding cysteine was located at position 446, and the sequence of the HR2 region around the cysteine was well conserved for both CYP3As.

Percent identities of CYP3A131 with CYP3A132 were 94.4 for open reading frame of cDNA and 90.9 for deduced amino acids. The coding sequence for the feline CYP3A131 shared 74.8%–91.1% nucleotide similarity and 65.3%–88.3% amino acid identity with other mammalian CYP3As. Similarly, the feline CYP3A132 shared 74.0%–90.7% nucleotide similarity and 64.5%–88.7% amino acid identity with other mammalian CYP3As. Both feline CYP3As showed the highest homology with CYP3A12 of domestic dog, the only other carnivore that can be compared. Phylogenetic analysis demonstrates the feline CYP3A genes are in a monophyletic group with canine CYP3As and far from rodent CYP3As, but nearer to other mammalian CYP3A clusters including those of some higher primates and artiodactyls (Fig. 1).

With the use of primers designed based on the 2 feline

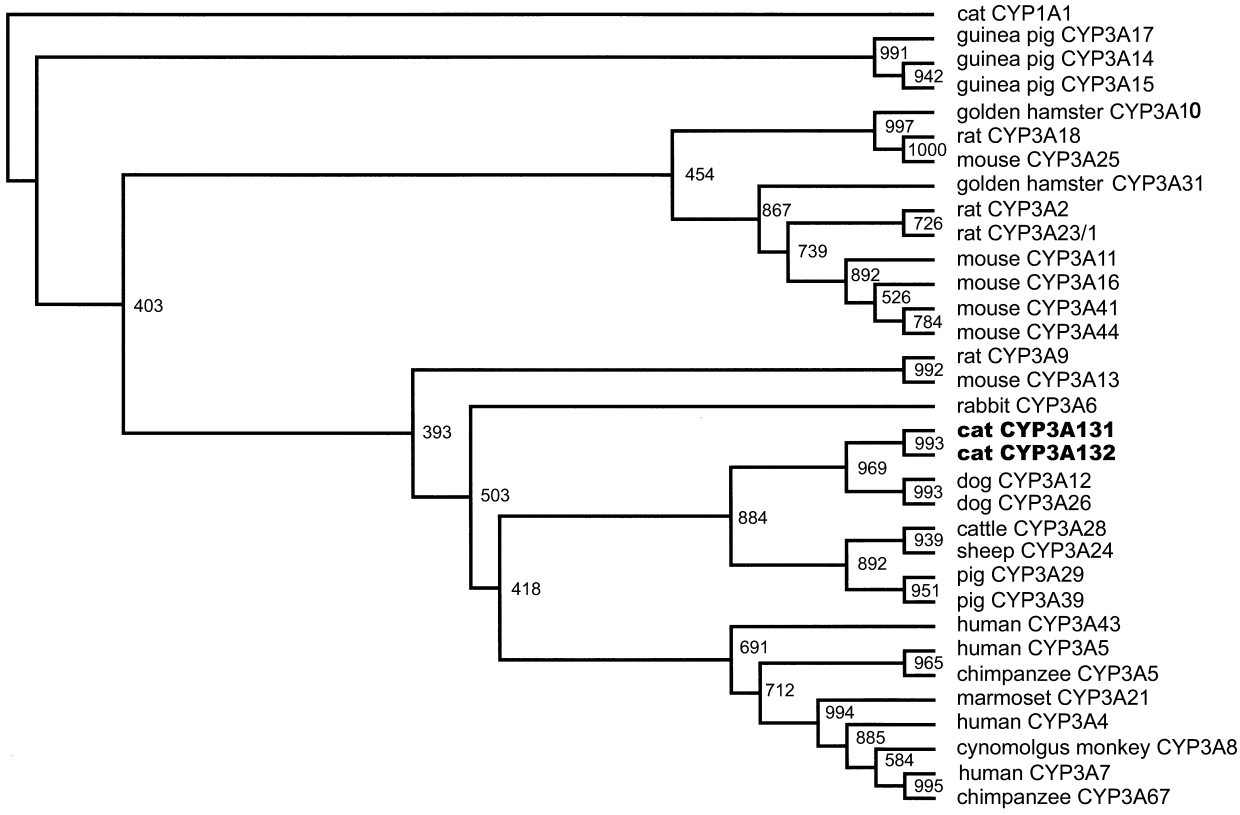

Fig. 1. Phylogenetic tree of CYP3As. The neighbor-joining method was used, based on the entire deduced amino acid sequence with feline CYP1A1 as an outgroup. The numbers on trees represent the local bootstrap values from 1,000 replicates.

CYP3A sequences determined above, we studied mRNA expression in various tissues by quantitative real-time RT-PCR. As 2 feline CYP3As identified showed high homology for each other, we used a primer set for amplification refractory mutation system (ARMS) PCR to obtain their specific PCR products [8]. Sense primer was common for both CYP3As and the sequence was 5–GAGATTGATGCAACTTTCCC-3'. 5'-GCAAGTTTCA TGTTCATGAC-3' for CYP3A131 and 5'-AAGGAGAA GTTCTGCAGCAC-3' for CYP3A132 were used as antisense primers. Specificity of these primer pairs was confirmed by sequencing clones of these PCR products. Additionally, restriction enzyme test (AlwI) with a single band obtained with primer sets for CYP3A131 and CYP3A132 confirmed the specificity of these determinations (data not shown). Serially diluted PCR products with the corresponding primer pairs for CYP3A131 and CYP3A132 described above were prepared as standard for quantitative analysis of the mRNA expression. Expression levels of both CYP3A transcripts were normalized per μg total RNA used for reverse transcription. The significance of differences between the values was determined at $P<0.05$ using Student's t-test for single comparisons or Kruskal-Wallis test followed by Steel-Dwass test for multiple comparisons.

Messenger RNA expression of both feline CYP3As was determined in the tissues, including brain (cerebrum), heart, lung, liver, small intestine, and kidney (Fig. 2). Among six tissues addressed, feline CYP3A131 was expressed predominantly in liver and small intestine, while much lower expression was detected in brain. It was poorly expressed in other tissues. Statistical difference of CYP3A131 expression of liver and small intestine from brain, heart, lung and kidney was confirmed by Kruskal-Wallis test followed by Steel-Dwass test. There was no significant difference of CYP3A131 expression in liver to that in small intestine. CYP3A131 expression in brain and lung were significantly higher than that in heart and kidney (Fig. 2). Expression of CYP3A132 was slightly observed only in liver (0.29 ± 0.14, n=11: arbitrary unit/μg total RNA). Amplification efficiency of CYP3A132 (90.2%) was almost same as CYP3A131 (92.3%) in our quantitative PCR study. Thus, it is suggested that expression of CYP3A132 could be much lower than that of CYP3A131. There was no clear sex-difference in the hepatic expression of CYP3A131 [male 21.85 ± 7.50 (n=5), female 38.45 ± 6.47 (n=6): arbitrary unit/μg total RNA]. In contrast, hepatic microsomal CYP3A-like activities tend to be higher in female cats than in male cats [11]. CYP3A132 expression in male liver was also not significantly different from that in female liver [male 0.15 ± 0.03 (5), female 0.41 ± 0.27 (6): arbitrary unit/μg total RNA]. This issue should be further studied with greater number of samples as well as those from other breeds of cats before a final conclusion is drawn.

Expression of four genes of CYP3A, including 3A4, 3A5, 3A7 and 3A43 has been determined in humans so far. CYP3A4 is predominant and occupies about 30% of CYP proteins in liver [12] and about 80% in small intestine of humans [9]. CYP3A26 is the dominant CYP3A gene in dog liver, while CYP3A12 expression is greater than CYP3A26 in small intestine [6]. In rat, CYP3A1 and CYP3A18 are major CYP3A genes in liver, while three genes of CYP3A including CYP3A62, CYP3A18 and CYP3A9 are abundantly expressed in rat small intestine [5]. Overall, intestinal CYP3A expression is much lower than the hepatic expression in dog and rat, compared to humans [5, 6, 9, 12]. Thus, the pattern of tissue distribution of feline CYP3A131 was similar to human CYP3A4.

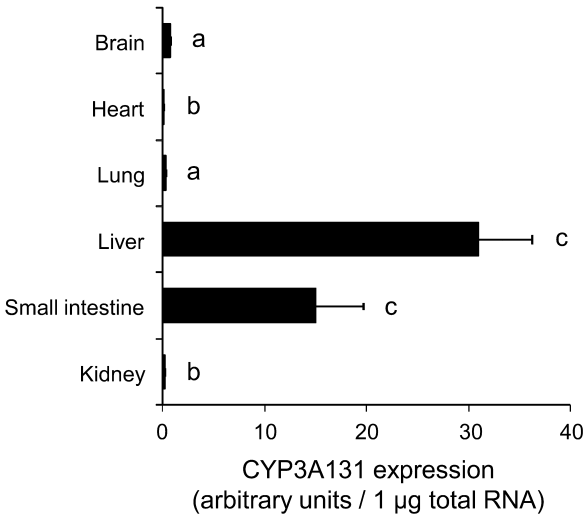

Fig. 2. Expression of feline CYP3A131 in six tissues. Tissues addressed were brain (cerebrum), heart, lung, liver, small intestine and kidney (cortex). Results are expressed as mean ± standard error, by arbitrary unit per 1 μg total RNA used for reverse transcription. Eleven cats (5 males and 6 females) were used for each tissue except small intestine (n=5: 2 males and 3 females). Values with different letters are significantly ($P<0.05$) different.

In this study, two feline CYP3A homologues, CYP3A131 and CYP3A132, were identified as novel CYP3A genes. Predominant expression of CYP3A131 in liver and small intestine suggests the possible major role of this CYP in metabolism of drugs and other chemicals, including first-pass effect of drugs. Functional studies of feline CYP3As with the expressed enzymes are required to clarify the selectivity of this enzyme for drugs and other chemicals in cats, because the relationship between molecular structure and metabolic activity so far remains largely unknown. The present results represent an important step toward the systematic understanding of pharmacokinetics of drugs to be useful for effective treatments without side effects in domestic cats.

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