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Cat Red Blood Cell Thiopurine S-Methyltransferase:

Companion Animal Pharmacogenetics

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Abstract

A common genetic polymorphism for thiopurine S-methyltransferase (TPMT) is a major factor responsible for individual variation in the toxicity and therapeutic efficacy of thiopurine drugs in humans. We set out to determine whether inheritance might also influence the level of TPMT activity in the domestic cat (*Felis domesticus*). As a first step, red blood cell (RBC) TPMT activity was measured in blood samples from 104 cats. The average level of cat RBC TPMT activity was lower than that observed in humans and was not related to either age or sex of the animal. We then cloned and characterized the *Felis domesticus* TPMT cDNA and gene. Genotype-phenotype correlation analysis was performed by resequencing the cat TPMT gene using DNA samples from 12 animals with high and 12 with low levels of RBC TPMT activity. Thirty-one single nucleotide polymorphisms (SNPs) were observed in these 24 DNA samples, including 5 that altered the encoded amino acid – resulting in 9 allozymes (6 observed and 3 inferred). Twelve of the 31 feline ***TPMT*** SNPs were associated, collectively, with 56% of the variation in level of RBC TPMT activity in these 24 animals. When those 12 SNPs were assayed in all 89 cats for which DNA was available, 30% of the variation in level of RBC TPMT activity was associated with these 12 polymorphisms. After expression in COS-1 cells, 5 of the 8 variant cat allozymes displayed decreased levels of both TPMT activity and immunoreactive protein when compared with the wild type allozyme. These observations are compatible with the conclusion that inheritance is an important factor responsible for variation in levels of RBC TPMT activity in the cat. They also represent a step toward the application of pharmacogenetic principles to companion animal thiopurine drug therapy.

Thiopurine drugs such as 6-mercaptopurine (6-MP) and azathioprine – which is converted to 6-MP *in vivo* – are used to treat both humans and companion animals, including the domestic cat (*Felis domesticus*) (Paterson and Tidd, 1975; Lennard, 1992; White et al., 2000). In humans, these drugs are used as cytotoxic agents to treat neoplasia and as immune suppressants (Paterson et al., 1975; Lennard, 1992) – with a similar spectrum of therapeutic applications in companion animals (Beale, 1988). However, thiopurine drugs have a relatively narrow therapeutic index and are capable of causing life-threatening toxicity – most often myelosuppression (Paterson et al., 1975; Lennard, 1992). Thiopurines also provide an excellent example of the successful use of pharmacogenetics to individualize human drug therapy (Weinshilboum et al., 1999; Weinshilboum, 2001). That is true because a genetic polymorphism for thiopurine S-methyltransferase (TPMT) (Weinshilboum and Sladek, 1980), an enzyme that catalyzes the S-methylation of these drugs (Remy, 1967; Woodson and Weinshilboum, 1983), plays an important role in individual variation in their toxicity and therapeutic efficacy (Weinshilboum et al., 1999; Weinshilboum, 2001).

In the present study, we set out to test the hypothesis that the most popular companion animal, the domestic cat, might also display inherited variation in TPMT activity. As a first step, TPMT activity was measured in the cat red blood cell (RBC), an easily accessible cell that has been widely used in human TPMT pharmacogenetic studies (Weinshilboum and Sladek, 1980; Weinshilboum et al., 1999; Yan et al., 2000). There were large individual variations in level of feline RBC TPMT activity – but the average level of activity was much lower than that in humans or dogs, the two other species in which large population studies have been conducted (Weinshilboum and Sladek, 1980; Yan et al., 2000; Salavaggione et al., 2002). The cat TPMT cDNA and gene were then cloned and characterized, and that information was used to perform a genotype-phenotype correlation analysis by resequencing the feline TPMT gene using DNA from

animals selected for extreme RBC TPMT phenotype. A total of 31 SNPs were identified, and 12 "informative" polymorphisms were then assayed in all animals for which DNA was available. Those 12 SNPs were associated with approximately 30% of TPMT phenotypic variance. Finally, functional genomic studies were conducted with all of the feline TPMT allozymes identified – i.e., all variant cat TPMT amino acid sequences. Five of the 8 variant allozymes displayed reduced levels of both enzyme activity and immunoreactive protein when compared with the "wild type" sequence. These results enhance our understanding of TPMT comparative genomics. They also represent a step toward the application of pharmacogenetic principles to companion animal drug therapy.

Methods

Tissue Acquisition. Blood samples were obtained from randomly selected cats seen at the Veterinary Medicine Teaching Hospital, University of Wisconsin-Madison. The cats studied presented for routine care or for the treatment of a variety of minor or chronic disorders. These studies had been reviewed and approved by the University of Wisconsin-Madison Institutional Animal Care and Use Committee (IACUC), and blood samples were obtained only after owners of the animals had consented to their participation. Cat RBCs were "washed", and RBC lysates were prepared as described previously (Weinshilboum et al., 1978). Hematocrits of the washed RBCs were determined prior to cell lysis to make it possible to express enzyme activity per ml of packed RBCs. Buffy coats were also prepared for use in DNA isolation.

TPMT Enzyme Assay and Substrate Kinetic Studies. TPMT activity was measured with the radiochemical method of Weinshilboum et al. (1978) as modified by Szumlanski et al. (1992) to omit the Chelex-100 step. Other modifications included an extension of the incubation time for the RBC lysates to 2 hours and a decrease of the reaction pH to 6.5 from the 7.5 value used to assay human RBC TPMT activity (Weinshilboum et al., 1978). One unit of TPMT activity represented the formation of 1 nmole of 6-methylmercaptopurine per hour of incubation. This same enzyme assay was also used to perform substrate kinetic studies with a pooled cat RBC lysate and – with an incubation time of 30 minutes – with recombinant cat TPMT allozymes. For the substrate kinetic experiments, eight 6-MP concentrations that varied from 60 μM to 7.5 mM and eight AdoMet concentrations that ranged from 0.19 to 24.2 μM were studied.

Cat TPMT cDNA and Gene Cloning. The feline TPMT cDNA was cloned by use of the PCR and a cross-species sequence homology-based strategy. Since TPMT is highly expressed in the kidney, the template was cDNA synthesized using mRNA isolated from a cat CCL-94 renal cell line (ATCC, Manassas, VA). Sequences of the primers used to perform this amplification, as well as

the sequences of all primers used in subsequent experiments, are listed in **Table 1**. The numbering scheme used for primers and the locations of polymorphic nucleotides is described in the legend for **Table 1.** Specifically, 5′- and 3′-rapid amplification of cDNA ends (RACE) (Frohman et al., 1988) were used to obtain partial cat TPMT cDNA sequences, and the complete cDNA open reading frame (ORF) was then amplified using primers that hybridized to the cDNA 5′- and 3′-untranslated regions (UTRs). Knowledge of the cat cDNA sequence was then used to clone and characterize the feline TPMT gene.

The cat TPMT gene structure was partially determined by use of the PCR and exon-specific primers with cat genomic DNA as template. Those experiments were performed using DNA isolated from buffy coat preparations obtained from ten cats with the QIAamp Blood Kit (QIAGEN, Inc., Chatsworth, CA). Buffy coat DNA was also used in subsequent experiments in which ***TPMT*** genotypes were determined for individual cats. However, amplifications performed with genomic DNA were only partially successful in characterizing the gene structure. Therefore, the cat TPMT cDNA was used to probe a cat genomic DNA BAC library created by Dr. P.J. de Jong at the Oakland Children's Hospital. Three positive clones were identified (BACs 523M14, 457C19 and 398H2) and – on the basis of PCR amplification – all three appeared to include the full TPMT gene. BAC 523M14 was then selected to characterize the TPMT gene structure by direct sequencing using TPMT cDNA ORF-specific sequencing primers. Use of this approach made it possible to characterize exon-intron splice junctions and to obtain intron sequence that flanked each exon. That information, in turn, was used to design intron-based primers which were used both to resequence the cat gene and to verify partial intron sequences determined initially by exon-to-exon amplifications. The cat TPMT gene structure was also compared with those of the human, dog, rat and mouse genes. Specifically, we made comparisons with the dog gene structure described by Salavaggione et al. (2002) and the human TPMT gene structure described by Szumlanski et al.

(1996). The mouse and rat TPMT gene structures by performing database searches with mouse and rat TPMT cDNA sequences having GenBank accession numbers AF046887 and AF120100, respectively.

Cat TPMT Polymorphism Detection. DNA isolated from blood obtained from 24 cats selected for either high or low levels of RBC TPMT activity was used to resequence the cat TPMT gene in order to perform genotype-phenotype correlation analysis. DNA sequencing was performed in the Mayo Molecular Biology Core Facility with ABI 377 DNA sequencers using BigDye™ dye primer sequencing chemistry. Dye primer sequencing was used to enhance our ability to detect heterozygosity (Chadwick et al., 1996). All resequencing primers contained 18 additional M13 nucleotides at their 5′-ends to make it possible to use dye-primer sequencing chemistry (see **Table 1**). After DNA sequence analysis had been completed for these initial 24 cats, restriction fragment length polymorphism (RFLP) assays were designed for SNPs found to be associated with variations in level of RBC TPMT activity – with the exception of SNPs located in exons 2, 5 and 6. Those polymorphisms were assayed by the use of dye primer DNA sequencing. Specifically, the RFLP assay for the nucleotide (-30) polymorphism in intron 3 involved digestion of the variant sequence with *Apo*I (New England Biolabs, Beverly, MA). *Nla*III (New England Biolabs) digested the variant sequence for the exon 4 nucleotide 337 polymorphism. The wild type sequence for the intron 8 SNP at nucleotide 75 was digested by *Dra*I (New England Biolabs), and *Acc*I (New England Biolabs) digested the variant sequence for the nucleotide 698 polymorphism in exon 9. Primers for the exon 8 and 9 amplifications were the same as those used during the resequencing experiments. All RFLP assays included controls for both genotypes being analyzed. After restriction digestion, samples were analyzed by electrophoresis with a 3% agarose gel (Invitrogen Corporation, Carlsbad, CA).

Site directed mutagenesis and transient expression. Site-directed mutagenesis was then performed using a "circular PCR" approach to create expression constructs for 8 variant allozymes (5 observed and 3 inferred) that resulted from the 5 nonsynonymous cSNPs at cDNA nucleotides 20, 23, 43, 337 and 698 (see **Table 1** for primer sequences). These ORF sequences were then cloned into the eukaryotic expression vector p91023(b) (Kaufman, 1985), and the insert was sequenced in both directions to ensure that only the desired sequence change had been introduced during amplification. These expression constructs were then used to transfect COS-1 cells as described previously (Honchel et al., 1993). The cells were also cotransfected with pSV-β-galactosidase DNA (Promega, Madison, WI) to make it possible to correct for transfection efficiency.

TPMT antibodies and Western blot analysis. A peptide corresponding to cat TPMT amino acids 40-59, with an additional cysteine residue at the amino terminus, was synthesized for use in the generation of antibodies. This polypeptide sequence had been compared with those in GenBank, the EMBL database and the SWISS-PROT protein sequence database to avoid use of a peptide with high homology to non-TPMT proteins. The synthetic peptide was conjugated to keyhole limpet hemocyanin and was used to generate rabbit polyclonal antibodies (Cocalico Biologicals, Inc., Reamstown, PA). Those antibodies were used to perform quantitative Western blot analyses with recombinant cat TPMT allozymes expressed in COS-1 cells. In the course of those experiments, the quantity of COS-1 cytosol loaded on a 12% acrylamide gel was adjusted on the basis of β-galactosidase activity to correct for transfection efficiency. Bound antibody was detected by chemiluminescence using the ECL Western Blotting system (Amersham Pharmacia, Piscataway, NJ). The AMBIS Radioanalytic Imaging System, Quant Probe Version 4.31 (Ambis, Inc., San Diego, CA), was used to quantitate immunoreactive protein, and the data were expressed as a percentage of the intensity of the wild type cat TPMT band on the same gel.

Data Analysis. DNA sequences obtained during the gene resequencing studies were analyzed with the PolyPhred 3.0 (Nickerson, 1997) and Consed 8.0 (Gordon, 1998) programs. The University of Wisconsin GCG software package, Version 10, was also used to analyze nucleotide sequence data. Apparent K_m values were calculated with the method of Wilkinson (1961) using a computer program written by Cleland (1963). Points that deviated from linearity on double inverse plots as a result of substrate inhibition were not included in those calculations. Specifically, 4 concentrations of 6-MP, 0.24 to 1.9 mM, and 4 concentrations of AdoMet from 1.5 to 12.1 μM were used to calculate the final apparent K_m values. Analysis of variance (ANOVA) was used to compare TPMT activity levels among female, male, spayed and neutered animals. Correlation analyses were performed by calculating Spearman rank correlation coefficients. TPMT activity levels in healthy animals and cats with minor or chronic health complaints were compared by the use of student's t-test. The association between polymorphisms and RBC TPMT activity level was determined by the use of ANOVA, and R-squared values from the ANOVA models were used to summarize the proportion of variability in TPMT activity that could be explained by the polymorphisms. Linkage disequilibrium was explored by computing D′ values for all possible pairwise combinations of SNPs. D′ is a measure of linkage disequilibrium that is independent of polymorphism frequency (Hartl, 1997; Hedrick, 2000). Haplotype analyses were performed by the use of a program based on the E-M algorithm (Long et al., 1995; Excoffier and Slatkin., 1995; Schaid et al., 2002).

Results

These experiments began with a determination of optimal conditions for the assay of cat RBC TPMT activity. The RBC was chosen because it is an easily accessible cell and because the RBC has been the cell used most often to measure TPMT activity in humans (Weinshilboum and Sladek, 1980; Yan et al., 2000). RBC TPMT activity was then measured in blood from cats that presented to a veterinary medical teaching hospital. After observing large individual variations in level of feline RBC TPMT activity, the cat TPMT cDNA and gene were cloned and genotype-phenotype correlation analysis was performed by resequencing exons and splice junctions of the cat TPMT gene using DNA from animals with high or low levels of RBC enzyme activity. Thirty-one ***TPMT*** polymorphisms were present in these selected samples, and 12 of those SNPs were associated with approximately 30% of the total variance in level of enzyme activity when assayed in all 89 animals for which DNA was available. Finally, functional genomic studies were performed for all cat TPMT variant allozymes observed during the gene resequencing studies. Those experiments demonstrated that 5 of the 8 variant allozymes showed decreases in levels of both enzyme activity and quantity of immunoreactive protein.

TPMT Assay and Activity. Assay conditions for cat RBC TPMT activity were optimized for enzyme quantity, time of incubation, pH and concentrations of the two cosubstrates for TPMT – 6-MP and AdoMet. The final assay utilized 100 μl of RBC lysate and a 2 hour incubation. This incubation time was necessary because of the relatively low level RBC TPMT activity in the cat when compared with that present in human RBCs. Both the quantity of RBC lysate and incubation time were within the linear range (data not shown). The pH optimum for TPMT in cat RBC lysates was 6.5, different from the optimal value of 7.5 for human RBC TPMT (Weinshilboum et al., 1978). Apparent K_m values of the enzyme for the two cosubstrates for the reaction, 6-MP and AdoMet, determined with a pooled RBC lysate as an enzyme source, were 800 μM and 2.4 μM,

respectively, values very similar to those which will be described subsequently for recombinant cat TPMT. These figures can be compared with reported apparent K_m values of 320 μM and 1.7 μM for human RBC lysate TPMT (Weinshilboum et al., 1978) and 710 μM and 19.1 μM, respectively, for RBC lysate TPMT in the dog (Salavaggione et al., 2002).

These optimal assay conditions were then used to measure RBC TPMT activity in blood samples from 104 cats seen at the University of Wisconsin-Madison Veterinary Medical Teaching Hospital (**Figure 1**). Cat RBC TPMT activity varied from 1.36 to 13.0 units/ml packed RBCs, with a mean ± SD of 4.47 ± 1.82 units/ml RBCs – for a 9.8-fold variation within ± 2 SD of the mean. This mean value was only about 20% of those that have been reported for human or dog RBC TPMT (Weinshilboum and Sladek, 1980; Yan et al., 2002; Salavaggione et al., 2002). Cat RBC TPMT activity did not differ significantly among male and female, spayed or neutered cats (**Table 2**). Furthermore, level of RBC TPMT activity was not significantly correlated with age in the 75 animals for which age was known (r_s = -0.072, p = 0.54) (**Figure 2**). Clinical information was available for 85 of these animals. Eighteen suffered from disease, and the remaining 67 cats were considered "healthy". There was no a significant difference between these two groups in average level of RBC TPMT activity (data not shown). Information on "breed" was available for 99 of the animals studied, but 96 of the 99 were identified only as "mixed breed domestic short hair" cats. The presence of large individual variations in level of cat RBC TPMT activity that were not related to age or sex raised the possibility that genetic factors might contribute to this variation. Therefore, the next series of experiments was designed to determine whether inheritance – involving the cat TPMT gene – might be one factor responsible for the variation, as it is in both humans and dogs (Weinshilboum and Sladek, 1980; Weinshilboum, 2001; Salavaggione et al., 2002). In order to test that hypothesis, we first had to clone and characterize the cat TPMT cDNA and gene.

Cat TPMT cDNA and gene cloning. The cat TPMT cDNA was cloned by using a cross-species sequence homology-based strategy performed with cDNA synthesized from mRNA isolated from a cat renal cortical cell line. The cat TPMT cDNA ORF was 735 bp in length and encoded a protein 82.4% identical to the amino acid sequence encoded by the most common ***TPMT*** allele in humans (Honchel et al., 1993; Szumlanski et al., 1996). Seventy-six bp of 5′-UTR and 30 bp of cat TPMT cDNA 3′-UTR sequence were obtained by performing 5′- and 3′-RACE. The cDNA was then used to clone and characterize the cat TPMT gene by using both a PCR-based strategy with cat genomic DNA as template and by directly sequencing a cat genomic DNA BAC clone isolated by probing a cat genomic DNA BAC library with the cDNA. A comparison of the domestic cat TPMT gene structure with those for humans, dogs, mice and rats is shown in **Figure 3**. The lengths of internal exons that encoded protein and the locations of splice junctions within the ORF were identical in these 5 species (**Figure 3**). Because humans have a processed pseudogene for TPMT (Lee et al., 1995), we tested the hypothesis that the cat might also have a processed pseudogene by performing PCR amplifications using 3 forward primers that hybridized with cat exons 3 and 4 paired with 3 reverse primers for cat exons 8 and 9. No amplicons were produced during those amplifications, thus failing to provide evidence for a TPMT processed pseudogenes in the cat. Obviously, we cannot eliminate the possible existence of a feline processed pseudogenes lacking adequate sequence homology to hybridize with these six primers.

Cat TPMT genotype-phenotype correlation analysis. Knowledge of the cat TPMT gene structure and sequence, including knowledge of intron sequences that flanked each exon, was then used to perform a genotype-phenotype correlation analysis. The first step in that process involved resequencing the cat gene using DNA samples from animals selected to have either high or low levels of RBC TPMT activity. Specifically, 24 DNA samples, 12 from animals with low activity (2.2 ± 0.44 units/ml RBCs, mean ± SD) and 12 from animals with high activity (7.8 ± 2.1), were

selected for inclusion in this phase of the analysis. Each exon encoding protein, plus approximately 100 bp of the flanking introns, was amplified using these DNA samples as template, and amplicons were sequenced using dye primer chemistry. Thirty-one SNPs were observed in these 24 samples, including 5 nonsynonymous cSNPs (**Table 3**). No insertions or deletions were observed. Twenty-three of the SNPs had allele frequencies equal to or greater than 10% in these 24 DNA samples, including 4 of the 5 nonsynonymous cSNPs. All SNPs with a frequency adequate for analysis (> 10%) were in Hardy-Weinberg equilibrium. We also determined "nucleotide diversity", a quantitative measure of genetic variation, adjusted for the number of alleles studied. Two standard measures of nucleotide diversity are π, average heterozygosity per site, and θ, a population mutation measure theoretically equal to the neutral mutation parameter (Fullerton et al., 2000). In the 24 cat DNA samples that had been completely resequenced $\pi = 0.27 \pm 0.14 \times 10^{-4}$, while θ was $6.99 \pm 2.27 \times 10^{-4}$. These values can be compared with π and θ values of $0.30 \pm 0.18 \times 10^{-4}$ and $1.83 \pm 0.75 \times 10^{-4}$, respectively, for 39 dog DNA samples that were resequenced for the TPMT gene as well as $0.15 \pm 0.09 \times 10^{-4}$ and $2.77 \pm 0.91 \times 10^{-4}$ for 90 human DNA samples in which ***TPMT*** was resequenced (www.PharmGKB). It can be seen that, on the basis of θ values, cats appeared to have a higher degree of nucleotide diversity than either humans or dogs – with a p value for a species-dependent difference of < 0.004 in both cases.

We next analyzed the possible association of cat ***TPMT*** polymorphisms with level of RBC TPMT activity. That analysis demonstrated that 12 of the 31 SNPs were associated with 56% of phenotypic variance in these 24 samples (**Table 3**). A previous analysis of human RBC TPMT population data showed that the common polymorphism for the trait of level of RBC TPMT in humans accounted for approximately 60-66% of the total variance (Vuchetich et al., 1995). A combination of RFLP analysis and dye primer DNA sequencing was then used to assay these 12 informative SNPs in DNA samples from all 89 of the 104 animals for which DNA was available

(**Table 3**). Data from these 89 cats were then used to calculate the proportion of variance in level of RBC TPMT activity that was associated with these 12 polymorphisms in the larger population sample. A total of 30% of the variance could be explained (**Table 3**). Because several of the SNPs were tightly linked – as described in subsequent paragraphs – values for the proportion of variance associated with individual polymorphisms listed in **Table 3** are not additive, e.g., the information listed for the intron 8 (75) SNP and the exon 9 (698) SNP is "redundant". Unfortunately, the relatively high nucleotide diversity present in the cat TPMT gene – and, therefore, the relatively large number of polymorphisms – resulted in such a large number of haplotypes, as described subsequently, that it proved difficult to associate individual haplotypes with level of activity in a statistically meaningful way.

We next computed pairwise linkage disequilibrium for the 12 informative SNPs in these 89 DNA samples by calculating D′ values (**Table 4**). D′ values can range from (+1) when two polymorphisms are maximally linked to (-1) when they never occur together (Hartl and Clark, 1997; Hedrick, 2000). This analysis showed, for example, that the nonsynonymous cSNPs at exon 9 ORF nucleotide 698 was in positive linkage disequilibrium with the intron 8 (75) SNP, with a D′ value of 1.0 – as was mentioned previously (**Table 4**). Because we had observed 31 polymorphisms, there were theoretically up to (2^{30}) possible haplotypes for the cat TPMT gene – too many to analyze with even the relatively sophisticated programs and hardware available to us. The same problem occurred when only the 12 SNPs that were assayed in all animals were included in the analysis – because of their relatively high frequencies, and also because of linkage disequilibrium. When only the nonsynonymous cSNPs were considered, there were 6 unambiguous and 3 inferred allozymes – for a total of 8 variant amino acid sequences (**Table 5**). That information was of practical importance for the recombinant allozyme expression studies which will be described subsequently. We were able to determine complete haplotypes based on polymorphism data for all 12 of the SNPs

assayed for the 2 most common allozymes. There were 2 unambiguous and 4 inferred haplotypes for each of these allozymes with – not surprisingly – the unambiguous haplotypes being those with the highest frequencies (**Table 6**). The final series of experiments involved functional genomic studies of the variant allozymes encoded by the 5 nonsynonymous cSNPs.

Cat TPMT allozyme expression in COS-1 cells. Expression constructs were created for the 9 cat TPMT allozymes listed in Table 5 (6 observed and 3 inferred amino acid sequences), and those constructs were used to transfect COS-1 cells. Mammalian cells were used to perform these experiments to ensure appropriate post-translational modification as well as the presence of mammalian systems for protein degradation. Alteration in only a single amino acid as a result of a genetic polymorphism has been found to have significant functional implications for many enzymes, including human TPMT – most often as a result of a decrease in the quantity of protein (Szumlanski et al., 1996; Preuss et al., 1998; Thomae et al., 2001, 2003; Adjei et al., 2003; Shield et al., 2003). After expression in COS-1 cells, 5 of the recombinant cat allozymes had decreased levels of TPMT activity when compared with the wild type allozyme (**Figure 4A** and **Table 7**). The values shown in **Figure 4A** and **Table 7** are averages of 12 independent transfections for each allozyme and all values have been corrected for transfection efficiency.

One possible explanation for these decreases in level of activity would involve a change in substrate kinetics as a result of the alteration in amino acid sequence. Therefore, apparent K_m values for the two cosubstrates for the reaction, 6-MP and AdoMet, were determined for all 9 allozymes. Some differences in apparent K_m values were observed (**Table 7**). For example, although most of the recombinant allozymes had apparent K_m values for 6-MP of approximately 1 mM, three had higher values of 2-3 mM. As a result, for those constructs, the basal level of enzyme activity in the transfected COS-1 cell preparation was reassayed using 15 mM 6-MP, twice the concentration used to perform the "standard" assay, to assure that a saturating concentration of the

methyl acceptor substrate was present. Those assays showed that use of the higher 6-MP concentration failed to result in a significant increase in basal level of activity. Therefore, alterations in substrate kinetics were unable to explain the striking differences that we had observed in levels of allozyme activity (**Figure 4A**). A common mechanism by which inherited variation in amino acid sequence can result in a lower level of activity is a decrease in the quantity of the encoded protein (Szumlanski et al., 1996; Preuss et al., 1998; Adjei et al., 2003; Shield et al., 2003). Therefore, we next performed quantitative Western blot analysis to measure levels of TPMT immunoreactive protein for these recombinant cat allozymes.

Quantitative Western blot analysis was performed for each of the 9 cat TPMT allozymes. Average values for levels of TPMT protein based on 5 independent transfections for each construct are shown in **Figure 4B** and are listed in **Table 7**. A representative Western blot used to obtain these data is shown in **Figure 4C**. Variations in levels of immunoreactive protein for the 9 allozymes were significantly correlated with variations in levels of enzyme activity ($r_s = 0.987$, $p < 0.001$; **Figure 5**). These observations were compatible with a growing body of evidence which indicates that the change in only one or two encoded amino acids as a result of common genetic polymorphisms can result in significant alterations in levels of immunoreactive protein – most often a significant decrease (Szumlanski et al., 1996; Preuss et al., 1998; Thomae et al., 2002, 2003; Xu et al., 2002; Adjei et al., 2003; Shield et al., 2003). Because frequencies of alleles encoding the 5 allozymes with decreased levels of activity and immunoreactive protein were relatively low (see **Table 3**), no samples were either homozygous or compound heterozygotes for these alleles. However, 27 of the 89 samples genotyped were heterozygous for 1 of the 5 allozymes that displayed reductions in activity after the transfection of COS-1 cells, but the average level of activity in those 27 samples, 4.28 ± 0.32 units/ml packed RBC (mean ± SEM), was not statistically lower than that in the remaining 62 samples, 4.78 ± 0.24 ($p = 0.23$).

Discussion

The genetic polymorphism for TPMT in humans represents a striking example of the functional implications of a common genetic polymorphism and of the application of pharmacogenetics to individualize drug therapy (Weinshilboum et al., 1999; Weinshilboum, 2001). The thiopurine drugs that are metabolized by TPMT are also used to treat companion animals, including cats, and companion animals also display large individual variations in thiopurine efficacy and toxicity (Houston and Taylor, 1991; Rinkhardt and Kruth, 1996). Cats in particular have been reported to be especially sensitive to myelosuppression following azathioprine therapy (Beale et al., 1992), and some authors have even recommended that these drugs not be used to treat cats (White, 2000). If principles of individualized therapy based, in part, on pharmacogenetics are of value when applied to humans, it is reasonable to ask whether similar principles might also apply to companion animals. We set out both to test that possibility for the cat and – at the same time – to expand our understanding of TPMT comparative genomics.

Average levels of RBC TPMT activity in the cat were significantly lower than those in the two other species that have been studied in a similar fashion, humans and dogs (Weinshilboum and Sladek, 1980; Yan et al., 2000; Salavaggione et al., 2002). Two previous studies that included a much smaller number of cats also reported that levels of RBC TPMT activity in this species were only approximately one fifth of those observed in humans or dogs (White et al., 2000; Foster et al., 2000). If RBC TPMT activity reflects the level of this enzyme activity in other cat tissues, these relatively low levels of activity may help to explain the sensitivity of this species to thiopurine therapy (Beale et al., 1992). We also observed that cats, like humans, displayed large individual variations in level of RBC TPMT activity (**Figure 1**). To make it possible to perform genotype-phenotype correlation analysis to study the possible contribution of inheritance to this variation, we cloned the cat TPMT cDNA and gene.

Knowledge of the sequence and structure of the cat TPMT gene made it possible to resequence all coding exons and splice junctions of the cat TPMT gene using DNA from 24 animals selected on the basis of either low or high levels of RBC TPMT activity. Thirty-one SNPs were observed in these 24 DNA samples, including 5 nonsynonymous cSNP that resulted in Leu7Ser, Ile8Thr, Asp15Asn, Met113Leu and Asp233Val alterations in encoded amino acids (**Table 3**). Twelve of those 31 SNPs were associated, collectively, with 56% of the total variation in level of RBC TPMT activity in these 24 animals (**Table 3**). When those 12 SNPs were assayed in DNA from all 89 animals for which DNA was available, 30% of the variance in level of RBC TPMT in this population sample was associated with these 12 polymorphisms – polymorphisms present only within exons and splice junctions. Obviously, the present observations do not eliminate the possibility of additional functionally significant genetic variation located elsewhere in the gene (e.g., within introns) (**Table 3**). To place these observations in context, the well-defined and functionally well-characterized ORF-based polymorphisms for the human TPMT gene are associated with approximately 60-66% of the total variation in level of human RBC TPMT activity (Vuchetich et al., 1995). We were also able to identify 6 unequivocal, as well as 3 inferred cat TPMT allozymes (**Table 5**). Expression of all 9 of those allozymes showed that 5 displayed decreases in levels of TPMT enzyme activity measured under optimal conditions – with parallel decreases in levels of immunoreactive protein – and 3 of the allozymes displayed significant increases in both activity and protein levels (**Figures 4** and **5**). Common variant allozymes for TPMT and other genetically polymorphic enzymes in humans have often been associated with decreased levels of enzyme protein (Szumlanski et al., 1996; Preuss et al., 1998; Thomae et al., 2001, 2003; Adjei et al., 2003; Shield et al., 2003), and our observations in the cat serve to emphasize just how common that mechanism is. The decrease in level of protein for the most common TPMT variant allele in humans results from rapid degradation through a ubiquitin-

proteasome-mediated process – with the involvement of molecular chaperones such as hsp90 (Tai et al., 1997; 1999; Wang et al., 2003). Whether similar mechanisms might apply in other mammalian species remains to be determined. Finally, the nature of cellular mechanisms that result in elevated levels of protein – as seen in 3 of our samples – remains to be explored.

In summary, we set out to determine whether inheritance might influence RBC TPMT activity level in *Felis domesticus*, the domestic cat, as it does in humans and dogs (Weinshilboum and Sladek, 1980; Yan et al., 2000; Salavaggione et al., 2002). There was a wide range in level of feline RBC TPMT activity (**Figure 1**), and the cat TPMT gene appeared to be more polymorphic than those of the other two species that have been studied in depth – humans and dogs. A significant portion of the variation in level of feline RBC TPMT activity was associated with these polymorphisms – although we cannot eliminate associations between the SNPs that we studied and other functionally significant polymorphisms located elsewhere in the cat genome. It is possible that thiopurine drug therapy in the cat – like that in humans – might eventually benefit from the application of pharmacogenetic information to help "individualize" treatment with this class of drugs. As a result, the present studies have not only broadened our understanding of the comparative genomics of an important drug-metabolizing enzyme, but they may also represent a step toward the application of pharmacogenetic principles to an important companion animal species.

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Footnotes Page

* These two individuals contributed equally to the work reported in this manuscript.

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The GenBank accession number for the cat TPMT cDNA is AY324659, and those for the cat TPMT gene are AY324660 to AY324667.

Figure Legend

Figure 1. Cat red blood cell (RBC) TPMT activity. The figure shows the frequency distribution of RBC TPMT activity in 104 cats.

Figure 2. Correlation of cat RBC TPMT with age. The figure shows the relationship between cat RBC TPMT activity and age for the 75 animals for which those data were available

Figure 3. TPMT gene structures. The figure show a comparison of the structures of ***TPMT*** in the cat with those of four other mammalian species. Black rectangles represent exons encoding the ORF, and white rectangles represent exons encoding UTR sequences. Exon lengths in bp and intron lengths in kb are also listed. The numbers in parentheses are lengths of the portions of the initial and terminal exons which encode protein.

Figure 4. Cat recombinant TPMT allozyme activity and immunoreactive protein levels. (A) Cat TPMT allozyme levels of activity after expression in COS-1 cells are shown. Activities (mean ± SEM, N=12) are expressed relative to the activity of the WT allozyme, after correction for transfection efficiency. (*) = $p < 0.001$ when compared with cells transfected with the WT construct. (B) Cat allozyme TPMT immunoreactive protein after transient expression in COS-1 cells (mean ± SEM, N=5), corrected for transfection efficiency. (*) = $p < 0.001$ when compared with cells transfected with the WT construct. (C) A representative Western blot used to obtain the data shown in panel (B).

Figure 5. Correlation of cat recombinant TPMT allozyme activity and immunoreactive protein. The figure shows the correlation of levels of enzyme activity and immunoreactive protein for 9 cat TPMT allozymes after transient expression in COS-1 cells. Data for the wild type allozyme have been highlighted.

Experimental Group	Primer Name	Primer Location	Primer Sequence
cDNA Cloning	F(-43)	Exon 2	GCATTTAAGGCATCTGTTTGTAGGCA
	R765	Exon 9	GATATTACTTGTTTTTACTGGTCT
5’-RACE	R65	Exon 2	CGGTTTTTCTGTACCTCGGTATCGGGGTACTCTTTAACAT
	Universal Primer	Anchor	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT
	Nested Universal	Anchor	AAGCAGTGGTATCAACGCAGAGT
Intron Length Determination	F(-76)	Exon 1	GCCGCGGGGTCCCTGTTGCGAGCT
	R65	Exon 2	CGGTTTTTCTGTACCTCGGTATCGGGGTACTCTTTAACAT
	F39	Exon 2	CCCGATACCGAGGTACAGAA
	R180	Exon 3	CTCGCCTTTAAGGAAAGTATCC
	F153	Exon 3	GCATTTGGATACTTTCCTTAAAGGCGAGAATGT
	R350	Exon 4	CCAGGAATTTCCATGATGGGCTCTTCCGAGTA
	F260	Exon 4	TGGGTGTGGAAATCAGTGAGCTTGGGATTC
	R417	Exon 5	GGGAAGATCAAAAAGGTTGCAACAGTACAG
	F376	Exon 5	GGGAACATTTCACTGTACTG
	R470	Exon 6	GCAACTAATGCTCCTCTATCCCAGATCC
	F428	Exon 6	TTGGCAAATTTGACAGGATCTGGGATAGAG
	R540	Exon 7	GTAGCGAAACCCTTTCCTCGTTAGGGACAGCA
	F509	Exon 7	TGCTGTCCCTAACGAGGAAAGGGTTTCGCTAC
	R608	Exon 8	TCAGCATCTGGAACATAAAACGGTGGGCCT
	F580	Exon 8	GGCCCACCGTTTTATGTTCCAGAT
	R692	Exon 9	CCCCAACTTTTATGTCGTTCTTCAAAAACATC
Site Directed Mutagenesis	F11MUT	Exon 2	CAAGCACTT*C*AATTGATGTTAAAG
	R34MUT	Exon 2	CTTTAACATCAATT*G*AAGTGCTTG
	F14MUT	Exon 2	GCACTTTAA*C*TGATGTTAAAGAGTACC
	R40MUT	Exon 2	GGTACTCTTTAACATCA*G*TTAAAGTGC
	F34MUT	Exon 2	GAGTACCCC*A*ATACCGAGGTACAG
	R57MUT	Exon 2	CTGTACCTCGGTAT*T*GGGGTACTC
	F324MUT	Exon 4	GGAAGAGCCCATC*C*TGGAAATTCCTG
	R349MUT	Exon 4	CAGGAATTTCCA*G*GATGGGCTCTTCC
	F686MUT	Exon 9	GTTGGGGAATTG*T*CTACATTG
	R706MUT	Exon 9	CAATGTAG*A*CAATTCCCCAAC

RFLP	I3F(-127)M13	Intron 3	CTGTGAACGTGAGTTTATGCTAATCCCT
	R294M13	Exon 4	TTCCCGAATCCCAAGCTCACTGAT
	I3F(-23)M13	Intron 4	GTGAATAACATGATTTCGTGCAGGT
	I4R167M13	Intron 4	AGCTATCACCTTTCAAGCCCTAGTTCT
Gene Resequencing	F(-43)M13	Exon 2	GCATTTAAGGCATCTGTTTGTAGGCA
	I2R162M13	Intron 2	CACGCCAGAGATGCGCTGTATTTCA
	I2F(-109)M13	Intron 2	GGGGATATTAAGTGAGATAGTGCA
	I3R283M13	Intron 3	CTTGAGACCCCACCACTGGT
	I3F(-127)M13	Intron 3	CTGTGAACGTGAGTTTATGCTAATCCCT
	I4R167M13	Intron 4	AGCTATCACCTTTCAAGCCCTAGTTCT
	I4F(-118)M13	Intron 4	TTCTTGTTTAACTACATATTCTCTCCCT
	I5R136M13	Intron 5	GGAGCCTGGAGTGTGTGCTTCAGA
	I5F(-165)M13	Intron 5	CCCATACCCGCATCAACACGTC
	I6R223M13	Intron 6	TCAGAAGGGACGGTGCCACAAG
	I6F(-41)M13	Intron 6	CAAGAGAAATGTAACACCTTACT
	I7R102M13	Intron 7	TGTGATCTAAATCAGGAGCAT
	I7F(-155)M13	Intron 7	GATGAGCACTGGGTGTTAATATGTAAGGGAAAT
	I8R263M13	Intron 8	GGCTCAGTGGGTGAAGCGTCTGACTCT
	I8F(-152)M13	Intron 8	CAGAGTCCAGAGTTTGCTCTTA
	R765M13	Exon 9	GATATTACTTGTTTTTACTGGTCT

Table 1. **Cat TPMT primer sequences**. Primers have been grouped based on the type of experiment in which they were used. "F" is forward; "R", reverse; "I", intron; and "MUT" indicates primers used to create a restriction site or an alteration in sequence during site-directed mutagenesis. Underlined italicized nucleotides are those altered by the "MUT" primers used to perform site-directed mutagenesis. With the exception of those that hybridized within introns, primers were numbered from the "A" in the cDNA ATG translation initiation codon, which was designated (+1). Negative numbers were located 5′ and positive numbers 3′ to that position. Intron-based primers were numbered from the initial nucleotide at the 5′-terminus of the intron (positive numbers) or the initial nucleotide at the 3′-terminus (negative numbers). All primers used for resequencing the gene and for the RFLP assays included M13 tags at their 5′-ends. The tag sequences were 5′-TGTAAAACGACGGCCAGT-3′ for forward primers and 5′-CAGGAAACAGCT ATGACC-3′ for reverse primers. RACE is rapid amplification of cDNA ends.

Sex	Status	N	RBC TPMT Activity (mean ± SD)
Female	Intact	7	4.7 ± 1.7
	Spayed	46	4.2 ± 1.7
Male	Intact	1	4.5
	Neutered	41	4.5 ± 2.0

Table 2. Cat RBC TPMT activity. Average RBC TPMT activity stratified by sex in intact, spayed or neutered animals is shown for those cats for which this information was available. None of these groups differed significantly on the basis of ANOVA ($p = 0.93$).

Location In Gene	SNP Position	WT Nucleotide	Variant Nucleotide	Amino Acid Alteration	Frequency (N=24)	Frequency (N=89)	TPMT Activity, % Variance Associated (N=24)	TPMT Activity, % Variance Associated (N=89)	Nucleotide Associated with High Activity
Exon 2	20	T	C	Leu7Ser	2.1	5.1	2.9	1.4	T
Exon 2	23	T	C	Ile8Thr	39.6	49.4	5	0.1	C
Exon 2	43	G	A	Asp15Asn	10.4	7.3	0.8	0.1	G
Intron 2	I2 (96)	A	G	---	15.2	---	5.8	---	---
Intron 3	I3 (-30)	G	T	---	16.7	---	1.9	1.1	G
Exon 4	337	A	C	Met113Leu	47.9	49.4	0.8	1.6	C
Intron 4	I4 (16)	G	T	---	4.4	---	6.7	---	---
Intron 4	I4 (107)	T	C	---	10.4	---	0.8	---	---
Intron 4	I4 (124)	A	G	---	10.4	---	0.8	---	---
Intron 5	I5 (13)	G	A	---	29.2	---	0.9	---	---
Intron 5	I5 (29)	T	C	---	41.7	---	17	4.2	C
Intron 5	I5 (51)	C	T	---	39.6	---	6.7	---	---
Intron 5	I5 (-47)	A	C	---	6.3	---	0.7	---	---
Intron 5	I5 (-26)	A	G	---	12.5	---	6.9	---	---
Exon 6	470	C	T	Silent	31.3	33.2	10	3.4	T
Exon 6	503	T	C	Silent	18.8	17.4	9.2	1.8	C
Intron 6	I6 (47)	C	T	---	14.6	---	15	---	---
Intron 6	I6 (62)	C	T	---	20.8	---	5.3	---	---
Intron 6	I6 (103)	A	G	---	4.2	---	0	---	---
Intron 6	I6 (126)	C	T	---	6.3	4.5	4.5	9.2	C
Intron 6	I6 (143)	C	T	---	18.8	17.9	9.2	2.7	T

Intron 6	I6 (153)	C	G	---	6.3	---	1.1	---	---
Intron 7	I7 (-71)	G	A	---	12.5	---	2.1	---	---
Exon 8	588	G	A	Silent	6.3	---	0.7	---	---
Intron 8	I8 (53)	T	C	---	12.5	---	2.1	---	---
Intron 8	I8 (75)	A	T	---	10.4	4.5	36	18	A
Intron 8	I8 (135)	C	G	---	31.3	---	1.7	---	---
Intron 8	I8 (172)	G	A	---	6.3	---	2.2	---	---
Intron 8	I8 (-75)	C	T	---	33.3	---	6.3	---	---
Intron 8	I8 (-46)	C	G	---	10.4	---	4	---	---
Exon 9	698	A	T	Asp233Val	10.4	4.5	36	18	A

Table 3. Cat *TPMT* polymorphisms. The locations, nucleotide polymorphisms, alterations in encoded amino acids as a result of those SNPs and frequencies for all polymorphisms observed are listed. The N = 24 group refers to DNA samples from animals selected for resequencing as a result of high or low RBC TPMT phenotypes, while the N = 89 group refers to the entire population sample of cats for which DNA was available. The portion of variance in level of RBC TPMT activity associated with each polymorphism for the N = 24 and the N = 89 groups, as well as nucleotides associated with high RBC TPMT activity for each polymorphism are also listed. Percentage values for the association of polymorphisms with activity variance are not additive because data for some of the SNPs (e.g., those in intron 6) are redundant as a result of linkage.
ND = not determined.

Polymorphism Pair		D′ Value
E2(20)	I3(-30)	1
E2(23)	E2(43)	-1
E2(23)	E4(337)	-0.78
E2(23)	I5(29)	-0.71
E2(23)	E6(503)	1
E2(23)	I6(143)	1
E2(43)	I3(-30)	1
E2(43)	E4(337)	1
I3(-30)	E6(470)	-1
E4(337)	I5(29)	0.70
E4(337)	E6(503)	-1
E4(337)	I6(143)	-1
I5(29)	E6(503)	-1
I5(29)	I6(143)	-1
E6(470)	E6(503)	1
E6(470)	I6(143)	1
E6(503)	I6(143)	0.92
I8(75)	E9(698)	1

Table 4. Linkage disequilibrium between pairs of cat *TPMT* SNPs. Pairwise linkage disequilibrium analyses were performed for the 12 cat TPMT SNPs that were assayed in all 89 animals studied. E is "exon" and "I" is intron. Only comparisons that resulted in absolute D′ values ≥ 0.7 or ≤ (-0.7) with p values < 0.001 have been included in the table.

Allozymes	Frequency %	Codon 7	Codon 8	Codon 15	Codon 113	Codon 233
*1	**38.2**	Leu	Ile	Asp	Met	Asp
*2	**38.2**	Leu	**Thr**	Asp	**Leu**	Asp
*3	**6.2**	Leu	**Thr**	**Asn**	**Leu**	Asp
*4	**5.1**	Leu	Ile	Asp	**Leu**	Asp
*5	**5.1**	**Ser**	Ile	Asp	Met	Asp
*6	3.4	Leu	**Thr**	Asp	Met	**Val**
*7	**1.7**	Leu	**Thr**	Asp	Met	Asp
*8	1.1	Leu	**Thr**	Asn	Met	Asp
*9	1.1	Leu	Ile	Asp	Met	**Val**

Table 5. Cat TPMT allozymes. The EM-algorithm was used to analyze data for the 5 nonsynonymous cSNPs observed. The 9 resultant allozymes, both unambiguous and inferred, are listed. Variant amino acids are shown as white against a black background. Allozymes with "bold" frequency values were unambiguous.

Allele	Frequency %	20	23	43	I3(-30)	337	I5(29)	470	503	I6(126)	I6(143)	I8(75)	698
1A	**16.2**	T	T	G	G	A	T	C	T	C	C	A	A
1B	**14.6**	T	T	G	G	A	T	**T**	**C**	C	**T**	A	A
1C	3.4	T	T	G	G	A	**C**	C	T	C	C	A	A
1D	1.7	T	T	G	G	A	T	**T**	**C**	**T**	**T**	A	A
1E	1.7	T	T	G	G	A	T	**T**	**C**	C	C	A	A
1F	1.1	T	T	G	G	A	T	**T**	T	C	**T**	A	A
2A	**23.3**	T	**C**	G	G	**C**	**C**	C	T	C	C	A	A
2B	**10.1**	T	**C**	G	G	**C**	**C**	**T**	T	C	C	A	A
2C	1.1	T	**C**	G	G	**C**	T	**T**	T	C	C	A	A
2D	0.6	T	**C**	G	G	**C**	T	C	T	C	C	A	A
2E	0.6	T	**C**	G	G	**C**	T	C	T	**T**	C	A	A
2F	0.6	T	**C**	G	G	**C**	**C**	**T**	T	**T**	C	A	A

Table 6. **Cat *TPMT* haplotype analyses.** Haplotype analysis was conducted for the ***1*** and ***2*** allozymes, allozymes present in 76% of the population sample studied. Variant nucleotides with respect to the reference sequence (***1A***) are shown as white against a black background. Initial haplotype designations (***1*** or ***2***) were made on the basis of the amino acids at codons 8 and 113, with the WT sequence at nucleotide 23 and 337 (Ile8 and Met113) assigned the ***1*** designation and those with the variant sequences encoding Thr8 and Leu113 designated ***2***. Letter designation were assigned on the basis of descending frequencies. Haplotypes with "bold" frequency values were unambiguous.

Allozyme	Enzyme Activity, % (N = 12)	Immunoreactive Protein, % (N = 5)	6-MP K_m, mM (N = 3)	AdoMet K_m, μM (N = 3)
1 (WT)	100	100	0.70 ± 0.067	1.8 ± 0.4
2 (Thr8/Leu113)	130 ± 3.0**	130 ± 8**	0.79 ± 0.16	2.6 ± 0.62
3 (Thr8/Asn15/Leu113)	15 ± 0.45*	18 ± 2.3*	0.97 ± 0.082	2.9 ± 0.16
4 (Leu113)	43 ± 1.1*	51 ± 8.4*	0.82 ± 0.013	3.6 ± 0.21
5 (Ser7)	127 ± 2.7**	130 ± 7.3**	1.0 ± 0.044	3.1 ± 0.16
6 (Thr8/Val223)	110 ± 3.9**	130 ± 6.9**	2.3 ± 0.20*	8.4 ± 2.0**
7 (Thr8)	30.0 ± 1.5*	37 ± 6.7*	0.89 ± 0.030	2.4 ± 0.86
8 (Thr8/Asn15)	7.4 ± 0.26*	0.9 ± 0.5*	2.7 ± 0.31*	3.8 ± 0.74
9 (Val223)	41 ± 1.6*	30 ± 6.7*	3.2 ± 0.27*	3.8 ± 0.6

Table 7. Cat TPMT allozyme expression and substrate kinetic data. The table lists basal levels of enzyme activity and immunoreactive protein with respect to that seen for the WT allozyme (***1***). Apparent K_m values for the 2 cosubstrates for the reaction, 6MP and AdoMet, are also listed. All values are mean ± SEM. ** = $p < 0.05$ and * = $p < 0.001$ as compared with the WT allozyme.

Figure 1

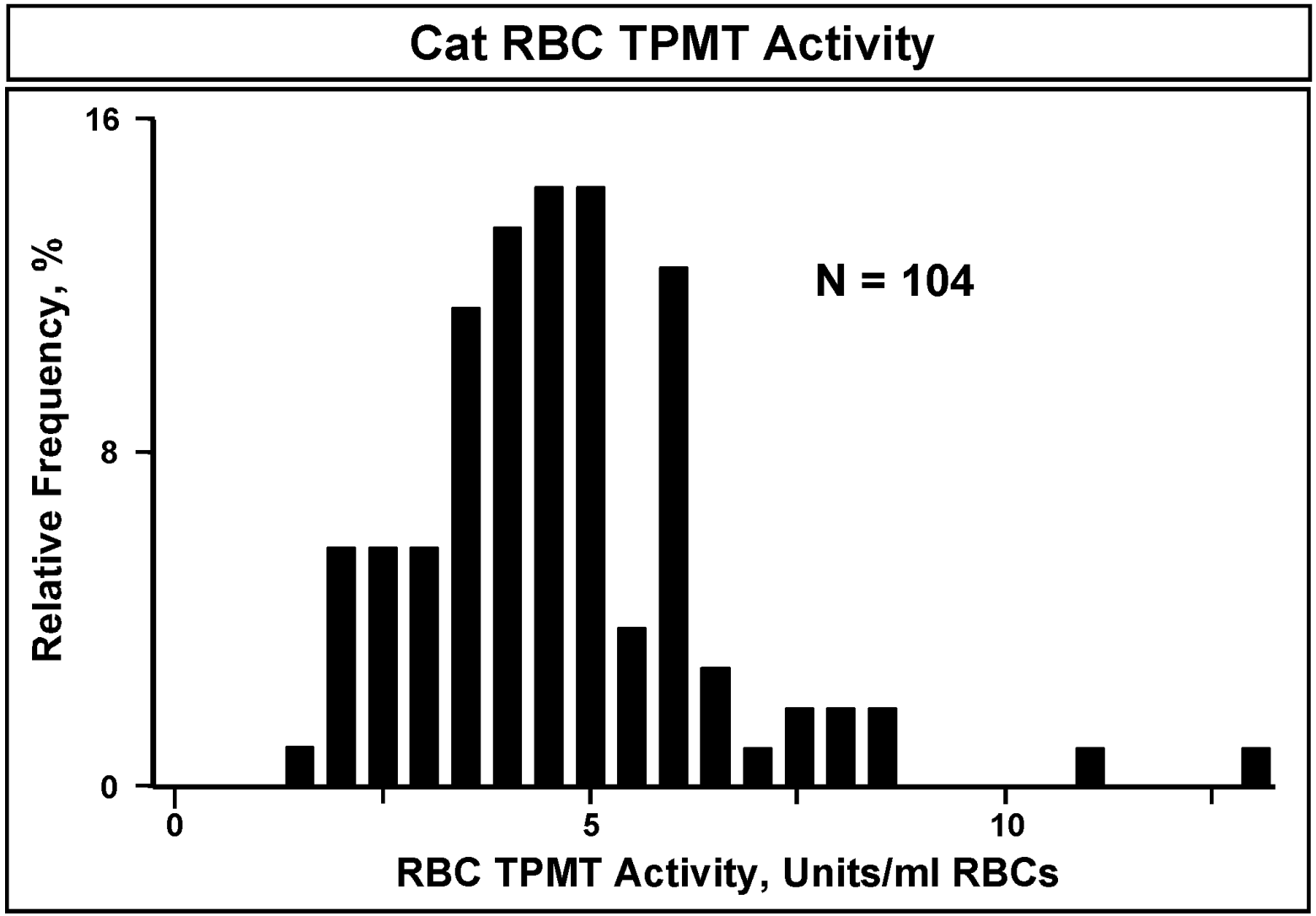

Figure 2

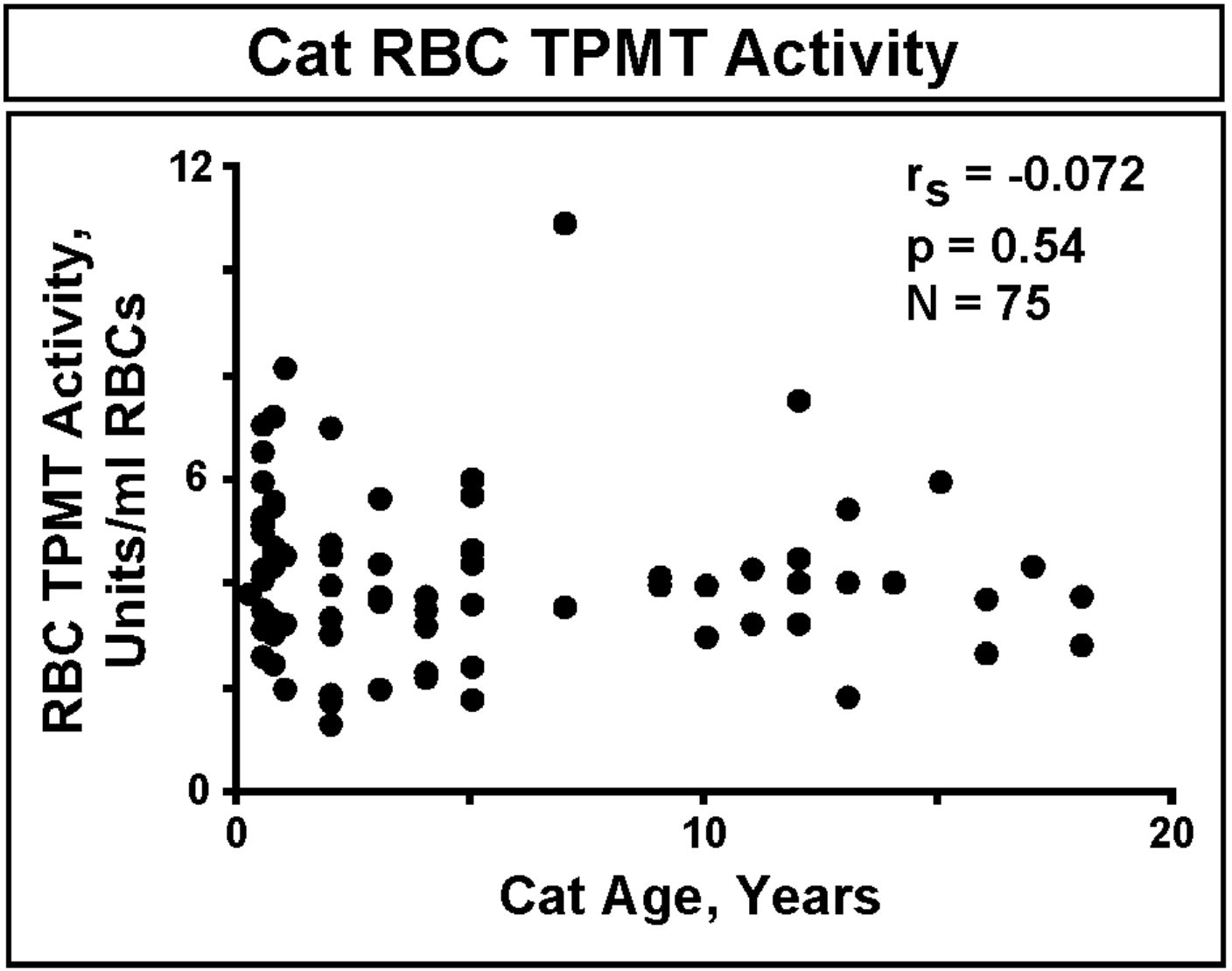

Figure 3

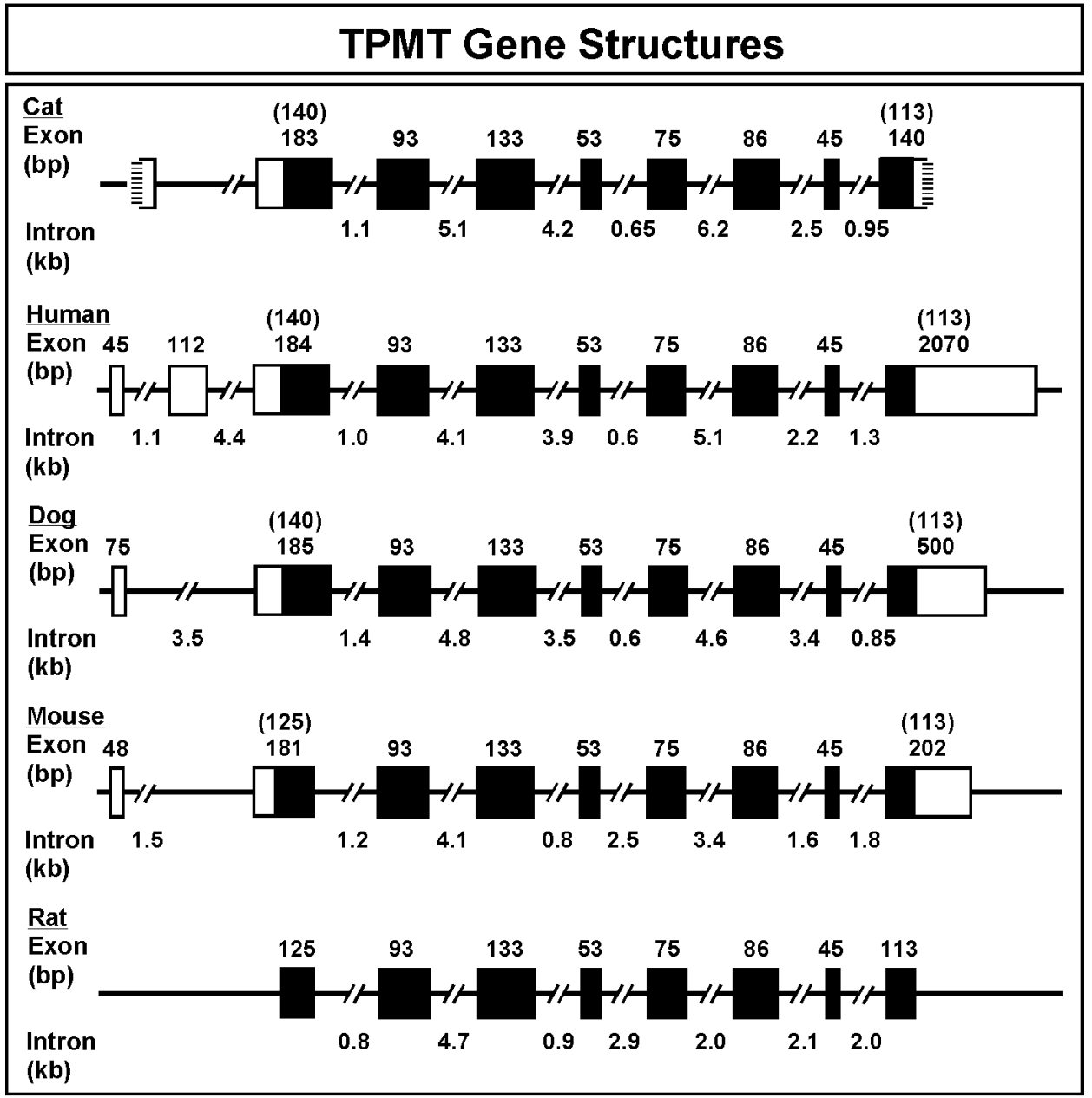

Figure 4

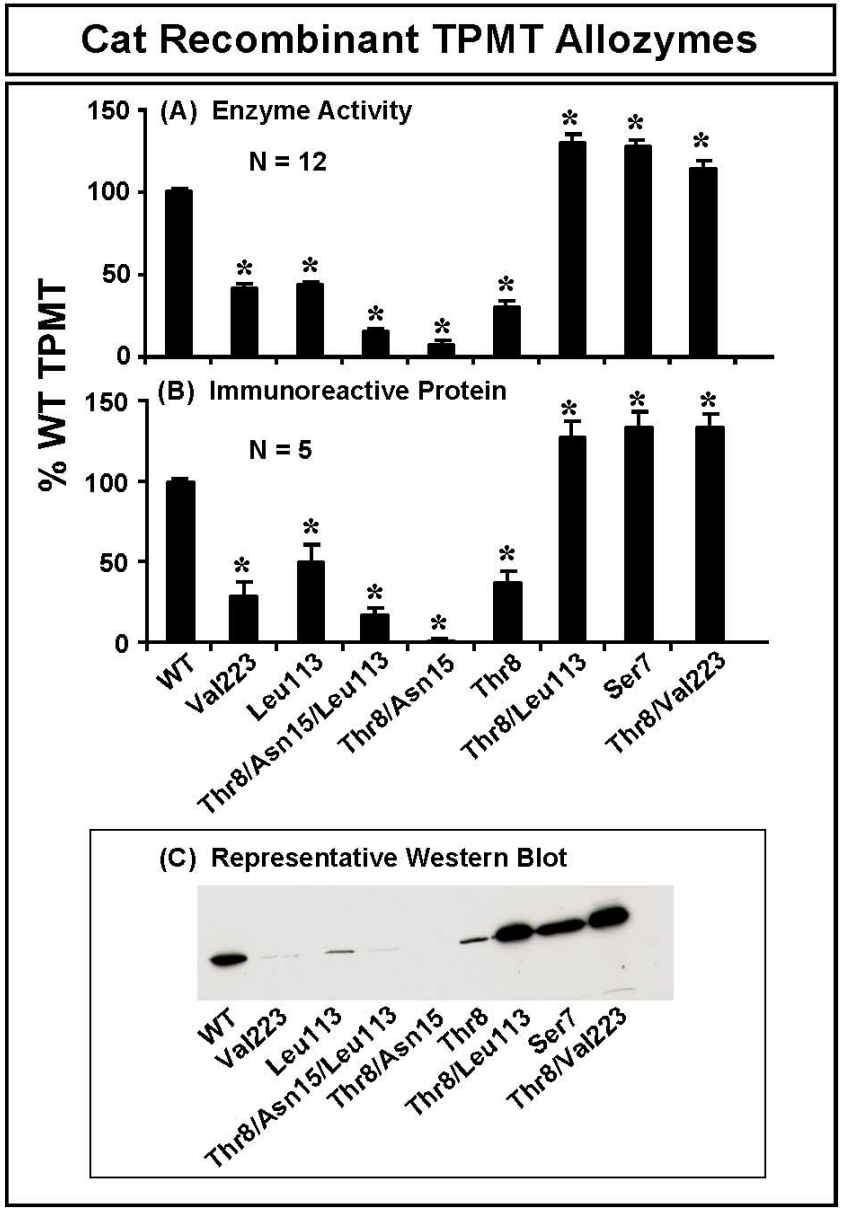

Figure 5