A 6-bp Deletion Variant in a Novel Canine Glutathione-S-Transferase Gene (GSTT5) Leads to Loss of Enzyme Function

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Objectives: Glutathione-S-transferases (GSTs) detoxify reactive xenobiotics, and defective GST gene polymorphisms increase cancer risk in humans. A low activity GST-theta variant was previously found in research beagles. The purpose of our study was to determine the molecular basis for this phenotype and its allele frequency in pet dogs.

Methods: Banked livers from 45 dogs of various breeds were screened for low GST-theta activity by the substrate 1,2-dichloro-4-nitrobenzene (DCNB), and were genotyped for variants in a novel canine GST gene, GSTT5. Whole-genome sequences from 266 dogs were genotyped at one discovered variant GSTT5 locus.

Results: Canine livers ranged 190-fold in GST-theta activities, and a GSTT5 exon coding variant 385_390delGACCAG (Asp129_Gln130del) was significantly associated with low activity (P < 0.0001) and a marked decrease in hepatic protein expression (P = 0.0026). Recombinant expression of variant GSTT5 led to a 92% decrease in Vmax for DCNB (P = 0.0095). The minor allele frequency (MAF) for 385_390delGACCAG was 0.144 in 45 dog livers, but was significantly higher in beagles (0.444) versus nonbeagles (0.007; P = 0.0004). The homozygous genotype was significantly over-represented in Pembroke Welsh corgis (P < 0.0001) based on available whole-genome sequence data.

Conclusions: An Asp129_Gln130del variant in canine GSTT5 is responsible for marked loss of GST-theta enzyme activity. This variant is significantly over-represented in purpose-bred laboratory beagles and in Pembroke Welsh corgis. Additional work will determine the prevalence of this variant among other purebred dogs, and will establish the substrate range of this polymorphic canine enzyme with respect to common environmental carcinogens.

Key words: Carcinogen; Detoxification; Dog; Glutathione conjugation; Pharmacogenetics.
Materials and Methods

DCNB Activity Screen in Canine Livers

Banked canine livers from 45 adult dogs euthanized after other research studies were screened for outlier low DCNB conjugation activities. The dogs included 23 from our laboratory (17 purpose-bred hounds and 6 beagles), and 22 provided by Dr Michael Court at Washington State University (9 mixed-breed dogs, 5 greyhounds, 4 Chihuahuas, 3 beagles, and 1 Labrador retriever). Liver cytosol was prepared by standard differential centrifugation, and cytosolic protein was quantitated by the Bradford protein assay. Activities for conjugation of DCNB to glutathione were measured by a standard spectrophotometric assay with 1 mM DCNB, 5 mM glutathione and 250 μg cytosolic protein/mL reaction, and were expressed in nmol DCNB conjugated/mg cytosolic protein/min.

In Silico Analysis of Canine GST-theta with Reported Low Activity

The N-terminal sequence, MPPEFLDLVSPPPRAIY1-FALKNGGP, of the GST-theta enzyme that was poorly expressed in liver cytosol of dogs with low DCNB conjugation was used to screen for a homologous DNA sequence in the canine genome. A sequence was found on chromosome 26 with 96.6% homology, which corresponded to the spliced dog EST cDNA GR900508 (liver) and a canine glutathione S-transferase theta-1-like mRNA (accession XM_851878.5). The transcript aligned to the exonic regions of the gene locus identified on chromosome 26 with 96.6% homology, which corresponded to the canine sequence with all known or predicted canine GST-theta isoforms.

Resequencing of GSTT5 in Liver Samples

Total liver cDNAs were prepared from the 23 liver samples that were collected in our laboratory and screened for DCNB activities. Total RNA was isolated and was converted to cDNA by commercially available kit with random oligo primers. Gene-specific primers (F: 5′ GTAGCTCGAGGAATGTAGGT, R: 5′ CACACAGCAGTTTATGTCG) were then used to amplify the predicted GSTT5 coding region from liver cDNA, by direct PCR reagents (thermocycler conditions available on request). Direct-terminator sequencing of GSTT5 amplicons was performed by internal sequencing primers (F: 5′ TGAGCGGCAATCGAGCGACG, R: 5′ CACAGTACATGGTACG). Sequences were aligned and screened for polymorphisms by commercial software. Genomic DNA samples from the remaining 22 livers from Dr Michael Court’s laboratory were genotyped at a single variant GSTT5 locus by different primers (F: 5′ CTAGGAGGGAGGCTGGT, R: 5′ GGCAAGCAGGCAAGAGAG). The peptide sequences of canine GSTT1 and GSTT5 were aligned to identify unique amino acid sequences that could be used to generate isoform-specific antibodies. The peptide AVQL-PATNYLCKSL was unique to canine GSTT5 and was used to generate rabbit anticanine GSTT5 polyclonal sera by a commercial vendor. Liver cytosol was subject to immunoblotting with rabbit anti-canine GSTT5 polyclonal sera as the primary antibody (1:25,000) and affinity-purified goat anti-rabbit HRP conjugate as the secondary antibody (1:10,000). For relative quantitation of GSTT5, a monoclonal mouse-anti-human beta-actin linked to horseradish peroxidase (1:2500) was used to detect dog beta-actin, followed by densitometry.

Quantitative PCR (qPCR) of GSTT5 Transcripts in Canine Livers

Quality of total RNA samples was assessed by a commercial nano chip. RNA samples with an RNA integrity number (RIN) ≥7 were considered of adequate quality for qPCR. Complementary DNA (cDNA) was generated by a commercial master mix, and qPCR was performed by a real-time cycler and commercial reagents according to the manufacturer’s instructions. GAPDH and B2M were amplified concurrently as housekeeping genes. Primers for qPCR are listed in Table S1.

Synthesis and Characterization of Recombinant GSTT5 Proteins

The reference cDNA for canine GSTT5 was amplified by PCR with primers containing BamHI and Ncol restriction sites (F: 5′ TAGGGATCCATGCGCGGAGCTGTTC, R: 5′ ATTCATGGCGCGAGCTTC). The resulting 746-bp fragment was cloned into the plasmid pCR2.1 by TOPO cloning. The correct orientation of the resulting construct was confirmed by sequencing.

Table 1. Percent Identity Matrix for the polymorphic GST-theta transcript of interest (ultimately named GSTT5), other known or predicted canine (cf) GSTT transcripts, and human (hs) GSTT1 and GSTT2. Alignment data were generated by Clustal Omega.

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sequencing, using standard plasmid-specific primers, and the BamHI-NcoI fragment was cloned into a bacterial expression vector. A stop codon was introduced by site-directed mutagenesis and specific primers (F: 5’ CAAGCTTCGAATTCCATGTTAGCCGAGCTCTCCAGCAG, R: 5’ CTGCTGGAGAGGCTGGCTGTCGAAGCTTG). Both constructs were used to transform E. coli, and protein expression was induced with isopropyl-β-D-thiogalactopyranoside (IPTG). His-tagged GSTT5 proteins were purified by nickel affinity chromatography and elution with 300 mM imidazole. Protein purity was confirmed by silver staining of gels after polyacrylamide gel electrophoresis. Immunoreactive protein expression and activities toward DCNB were determined as described for individual canine livers.

**Statistical Analyses**

Data for DCNB activities and GSTT5 immunoreactive protein in individual canine livers were compared based on GSTT5 genotype by analysis of variance (ANOVA) followed by Tukey’s multiple comparison tests, and for recombinant proteins with reference or variant sequences by an unpaired t-test, with *P* < 0.05 considered significant. Minor allele frequencies were compared among breeds by 2-tailed Fisher’s exact test. Kinetic parameters were estimated using commercial software.

**Results**

**DCNB Activity and GSTT5 Resequencing in Canine Livers**

There was a 190-fold range in DCNB conjugation activities in the 45 livers screened (mean activity, 56.7 nmol/mg/min; range, 0.6–114.5 nmol/mg/min; Fig 1). GSTT5 cDNA was amplified from the 23 livers from our laboratory and genomic DNA was isolated from the remaining 22 livers. A homozygous 6-bp deletion in exon 4, c.385_390delGACCAG (p.Asp129_Gln130del; Fig 2A), was found in the 3 livers with the lowest DCNB activities (median, 2.0 nmol/mg/min; range, 0.6–16.8 nmol/mg/min). This variant also was found in heterozygous form in 7 other livers with intermediate DCNB activities (mean, 40.4 nmol/mg/min; range, 25.4–67.2 nmol/mg/min), whereas 35 livers with the reference GSTT5 sequence had mean DCNB activities of 64.1 nmol/mg/min (range, 21.9–114.5 nmol/mg/min). The DCNB activities were significantly lower in both the heterozygous and homozygous variant livers compared to the reference genotype livers (*P* < 0.0001; Fig 2B). The overall minor allele frequency (MAF) of GSTT5 c.385_390delGACCAG was 0.144 in the 45 livers, with 6.7% of dogs having the homozygous variant genotype. In the small sample of beagles, the MAF for the 6-bp deletion variant was 0.444, with 2 of 9 beagles (22%) having the homozygous variant genotype. This allele frequency in beagles was significantly higher than in nonbeagles (MAF 0.007, 1 of 36 homozygous; *P* = 0.0004).

In addition to allele frequencies in 45 livers, we determined the MAF for the GSTT5 6-bp deletion variant in whole-genome sequencing data from 281 dogs provided by the laboratory of Dr Elaine Ostrander. Sequences could be genotyped at this locus in 266 domestic dogs of 117 different breeds. The overall MAF for c.385_390delGACCAG was 0.045, with 6 homozygotes observed (3 of 3 Pembroke Welsh corgis, 1 of 5 Border collies, 1 of 13 golden retrievers, 1 of 4 soft-coated Wheaten terriers, and 0 of 4 beagles). In the Ostrander population, the 6-bp deletion variant was significantly over-represented in the Pembroke Welsh corgi (*P* < 0.0001).

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**Fig. 1.** Histogram of GST-theta activities toward the prototypic substrate DCNB in livers from 45 dogs of various breeds. Enzyme activities in individual dogs are grouped together in bins spanning 5 nmol/mg/min. The gray bars represent three dogs homozygous for a 6 bp coding deletion in GSTT5.
Liver GSTT5 Protein and Transcript Expression by Genotype

GSTT5 protein expression was analyzed in 22 genotyped canine livers (3 homozygous for the 6-bp deletion variant, 7 heterozygotes, and 12 with the reference GSTT5 genotype). GSTT5 protein expression was significantly lower in livers homozygous for Asp129_Gln130del compared to the reference genotype ($P = 0.0026$; Fig 3A–B). mRNA expression data were available for 15 livers with high RNA quality, including one homozygote and 2 heterozygotes, and did not appear to differ by GSTT5 genotype (Fig 3C).

Recombinant Protein Expression

To confirm a genotype-phenotype relationship between the GSTT5 6-bp deletion variant and impaired activity, recombinant reference and variant GSTT5

Fig. 2. (A) Representative DNA chromatograms for a dog with the reference GSTT5 genotype (left) and a dog with a homozygous GSTT5 6-bp deletion (right; c.385_390delGACCAG, p.Asp129_Gln130del). (B) DCNB activities by GSTT5 genotype. The horizontal bar in each scatterplot represents the group mean. Activities were significantly lower in livers heterozygous (HETdel) or homozygous (HOMdel) for the GSTT5 c.385_390delGACCAG allele compared to the reference (REF) genotype ($P < 0.0001$). [Color figure can be viewed at wileyonlinelibrary.com]
proteins were expressed and purified, and immunoreactive protein and DCNB activities were measured. Immunoreactive protein expression in vitro was comparable for both reference and variant GSTT5. Purified recombinant GSTT5 showed nearly 970-fold enrichment in mean activity at 1 mM DCNB (62.1 μmol/mg/min) compared to liver cytosol (64.1 nmol/mg/min). When kinetics were compared between reference and variant recombinant proteins, the 6-bp variant showed a 92% decrease in \( V_{\text{max}} \) for DCNB conjugation on a per mg protein basis (5.3 ± 0.2 μmol/mg/min versus 67.1 ± 7.9 μmol/mg/min; \( P = 0.0095 \); Fig 4), with similar \( K_m \) values (164 ± 22 μM and 168 ± 27 μM, respectively; \( P = 0.76 \)).

**Discussion**

Polymorphisms in genes that encode GST enzymes are important in the epidemiology of cancer risk in humans, but little is known about GST variants in dogs. Previous studies had found polymorphic activity for the GST-theta substrate, DCNB, in beagle dog livers, with some dogs having virtually no activity.\(^{20,21}\) These latter dogs were reportedly missing a GST-theta protein with an N-terminal amino acid sequence, MPPEFLDLYSPPRAYIYFLKNGIP,\(^{24}\) which we matched to a novel canine GST gene on chromosome 26. This gene was not highly homologous to any known human GSTT gene, and was therefore given the unique name of GSTT5.

We next looked at the relationship between individual variability in canine hepatic DCNB activities and genetic variants in canine GSTT5 cDNA. We found a 190-fold range in activities, which is similar to the range (0.0–189.9 nmol/mg/min) previously reported for DCNB in liver cytosol samples from 280 research beagle dogs.\(^{21}\) In our study, the 3 livers with the lowest activities had a homozygous 6 bp coding deletion, c.385_390delGACCAG, in exon 4 of the GSTT5 gene. Activities for livers with this polymorphism in the homozygous or heterozygous states were significantly lower than reference livers, with homozygous livers showing a 90% decrease in mean activities. However, there was some overlap between heterozygote and homozygote activities, and the livers with the next lowest activities after the homozygotes (activities of 21.9–27.4 nmol/mg/min; \( n = 7 \)) were a mixture of 3 heterozygotes and 4 reference GSTT5 genotypes. Other canine GST enzymes possibly could contribute to variability in DCNB activity phenotypes. However, DCNB is
reported to be a specific substrate for GSTT5 (i.e. the canine GST-theta isoform formerly called GST-D or isozyme Yd1Yd), compared to canine homologs of GST-mu and GST-pi. It is also possible that variants in other GSTT5 loci may modulate enzyme activity.

In addition to decreased DCNB activities, mean immunoreactive GSTT5 protein in homozygous variant livers also was decreased by 90% compared to reference livers, with no apparent change in transcript levels in a limited number of samples. These findings suggest that the Asp129_Gln130 deletion leads to decreased GSTT5 expression in vivo, either because of impaired protein translation or enzyme stability. Expression of the variant enzyme in a nonmammalian system (E. coli) generated protein that was of comparable yield to the reference enzyme, with a 92% decrease in catalytic activity on a per mg basis. This finding suggests that the missing amino acids Asp129 and Gln130 are important, directly or indirectly, for catalytic activity. Based on an NCBI blast search for putative domains in GSTT5, amino acids 129–130 are located adjacent to the enzyme’s substrate binding pocket, but the $K_m$ was not different between expressed variants. These findings, together with the endogenous liver expression data, suggest that GSTT5 385_390delGACCAG leads to an unstable protein with loss of catalytic activity. Low expression in vivo compared to relatively unchanged expression in vitro may be a consequence of mammalian protemosomal degradation of the defective protein in vivo, which would not be detected in the in vitro bacterial system.

The overall MAF for GSTT5 385_390delGACCAG was 0.144 in our sample of 45 livers, and was significantly higher (0.444) in a small subpopulation of purpose-bred laboratory beagles, with 2 of 9 (22%) beagles being homozygous. This observation is consistent with the previous finding of low DCNB activities in 12% of research colony beagles. Our study was limited by a relatively low number of individual dog livers available, many of which were from purpose-bred or mixed-breed dogs. Therefore, we also determined allele frequencies for this GSTT5 variant from whole-genome sequence data in 266 dogs, and found an overall MAF of 0.045, with Pembroke Welsh corgis significantly over-represented (3 of 3 dogs homozygous). Ongoing work will screen for the frequency of this dysfunctional allele in additional pet beagles and other purebred dogs.

In summary, we discovered a 6-bp deletion variant in the coding region of canine GSTT5 that is responsible for marked loss of enzyme activity with respect to the prototypical GST-theta substrate, DCNB. Ongoing work will determine the substrate range of this polymorphic canine enzyme, including therapeutic drugs and carcinogens, which will help to establish the clinical and toxicological relevance of this variant in both companion dogs and dogs used in preclinical research.

Source of Funding

SC was supported by the UW-SVM Summer Scholars Program (NIH T35 OD01078) and the 12-Month Mentored Research Program (NIH T32 OD010999). KL was funded by the 12-Month Mentored Research Program (NIH T32 OD010999) and under NIH awards UL1TR000427 and TL1TR000429.
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Footnotes

1. Bio-Rad Laboratories, Hercules, CA
2. Clustal Omega (http://www.ebi.ac.uk/)
3. BLAT, (genome.ucsc.edu/)
4. RNeasy Midi Kit (Qiagen, Valencia, CA)
5. High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA)
6. Amplitaq Gold 360 MasterMix (Life Technologies, Grand Island, NY)
7. Big Dye reagents (Applied Biosystems, Foster City, CA)
8. Staden Package software (http://staden.sourceforge.net/)
9. Panigen Inc, Blanchardville, WI
10. Abcam, Cambridge MA
12. Total RNA Nano Chip (Agilent Technologies, Santa Clara, CA)
13. Superscript VILO Mastermix cDNA Synthesis Kit (Invitrogen, Carlsbad CA)
14. Roche Lightcycler 96 with the Fast Start Essential DNA Probes Master Kit (Roche Diagnostics, Indianapolis, IN)
15. TOPO TA cloning kit (Invitrogen, Carlsbad CA)
16. pRSETA vector (Invitrogen, Carlsbad CA)
17. Quickchange II Site Directed Mutagenesis Kit (Agilent, Santa Clara, CA)
18. BL21 (DE3) pLysS (EMD-Millipore, Merck KGaA, Darmstadt, Germany)
19. Prism (GraphPad Software, Inc. La Jolla, CA)
20. Chief & NIH Distinguished Investigator, Cancer Genetics and Comparative Genomics Branch, National Human Genome research Institute.

Acknowledgments

The authors thank Dr Michael Court at Washington State University for sharing banked liver samples, and Drs Elaine Ostrander and Brian Davis at the Cancer Genetics and Comparative Genomics Branch, National Human Genome Research Institute, National Institutes of Health, for generously sharing whole-genome sequencing data on 281 domestic dogs. We also thank Bradley Mayer for assistance with genotyping, Martha Johnson and Mark Fulton for early work with the DCNB assay, and Tiffany Nguyen from the Integrated Biological Sciences Summer Research Program for initial work on GSTT5 expression.

Conflict of Interest Declaration: Authors declare no conflict of interest.

Off-label Antimicrobial Declaration: Authors declare no off-label use of antimicrobials.

References


Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. Primers used for qPCR of GSTT5 transcripts in normal canine livers