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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 V_{max} 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.

Glutathione S-transferases (GSTs) are a family of cytosolic enzymes that conjugate glutathione to a variety of reactive molecules. These GSTs are responsible for detoxifying endogenous lipid peroxides, as well as a number of xenobiotics including clinically used drugs, environmental pollutants, pesticides, and herbicides.[1–3] The major human GSTs with clinical relevance to date are GST-mu (GSTM1), GST-theta (GSTT1) and GST-pi (GSTP1).

Low activity variants in human GSTs are common. Gene deletions ("null" genotypes) in *GSTM1* and *GSTT1* are found in approximately 50% and 20% of Americans, respectively,[4] and a low activity genotype in *GSTP1* (Ile105Val) is found in 28–75% of individuals depending on ethnicity.[5] Low activity GST variants affect clinical responses to antineoplastic drugs.[6–9] Low activity GST variants also increase the risk of various cancers in humans, including Hodgkin and non-Hodgkin lymphomas, leukemias, and cancers of the lung, breast, skin, and bladder.[10–19] These risk associations have been attributed to impaired detoxification of environmental carcinogens.

Abbreviations:

DCNB	1,2-dichloro-4-nitrobenzene
GST	glutathione-S-transferase

Understanding of GST functional variation in dogs is limited. This is an important knowledge gap given that dogs are commonly used in preclinical drug toxicity studies as predictors of human response. A previous study in purpose-bred beagles found 45-fold variability in individual liver GST-theta activity toward a prototypical GST substrate, DCNB (1,2-dichloro-4-nitrobenzene).[20,21] In fact, 12% of beagle dogs were found to have very low conjugation activity for DCNB, and virtually undetectable expression of a GST-theta ortholog in their livers.[20] However, the molecular mechanism for this GST-theta defect was not characterized.

We recently resequenced the canine ortholog of human *GSTT1* in dogs of various breeds, and found several polymorphisms with predicted functional relevance. Furthermore, we found a single nucleotide polymorphism in canine *GSTT1* that was significantly associated with lymphoma risk in dogs.[22] However, the predicted amino acid sequence of reference canine GSTT1 was different from that of the GST-theta protein that was absent in 12% of beagle dogs.[20] We hypothesized that a second GST-theta enzyme was expressed in canine liver, and that this isozyme was responsible for the low activity GST-theta variation previously documented in research colony dogs. Therefore, the objective of our study was to determine the molecular basis for this low activity GST-theta phenotype and its allele frequency in dogs of other breeds.

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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.[20,21] 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.[a] Activities for conjugation of DCNB to glutathione were measured by a standard spectrophometric assay with 1 mM DCNB, 5 mM glutathione and 250 μg cytosolic protein/mL reaction,[23] 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, MPPELFLDLYSPPPRAIYIFALKNGIP, of the GST-theta enzyme that was poorly expressed in liver cytosol of dogs with low DCNB conjugation[24] was used to screen for a homologous DNA sequence in the canine genome (canFam3.1; genome.ucsc.edu/). 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 as LOC477558, located at chr26:28565303-28567518. An alignment of XM_851878.5 was performed against the human genomic and transcript databases from the National Center for Biotechnology Information (NCBI), by megaBLAST (Basic Alignment Research Tool). A multiple sequence alignment tool,[b,25] was used to align the canine sequence with all known or predicted canine GST-theta transcripts and with human *GSTT1* and *GSTT2,* and found <63% homology with other human and canine GST-theta sequences (Table 1). Finally, another web-based tool[c] was used to align the entire 729 bp coding region for canine XM_851878 sequence with multiple mammalian genomes. The highest homology scores (>550) were observed for other members of the Carnivora order such as the cat, ferret, and giant panda. In contrast, low homology scores (<300) were seen for mammals such as human, cow, and pig. These results together were strong evidence that a novel *GSTT* gene locus was present in dogs and other closely related species. Therefore, we propose that the gene locus presently annotated as LOC477558 be named *GSTT5*.

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 kits[d,e] with random oligo primers. Gene-specific primers (F: 5′ GTAGCTCAGAGGAAGTAGGT, R: 5′ CACAC AGCACGTTTATTGC) then were used to amplify the predicted *GSTT5* coding region from liver cDNA, by commercial PCR reagents[f] (thermocycler conditions available on request). Dye-terminator sequencing of *GSTT5* amplicons was performed[g] by internal sequencing primers (F: 5′ TGAGCCGCAAGTACCAGACG, R: 5′ CACAGGTAGACATTGGTAGC). Sequences were aligned and screened for polymorphisms by commercial software.[h] 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′ CTAGGAGGGAGGGCTGGT, R: 5′ GGCAAGCAGGCAAGAGAG).

Immunoblotting of GSTT5 Protein in Canine Livers

The peptide sequences of canine GSTT1 and GSTT5 were aligned[b,25] to identify unique amino acid sequences that could be used to generate isoform-specific antibodies. The peptide AVQLPATNVYLCKSLL was unique to canine GSTT5 and was used to generate rabbit anticanine GSTT5 polyclonal sera by a commercial vendor.[i] 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[j] 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),[k] was used to detect dog beta-actin, followed by densitometry.[l,26]

Quantitative PCR (qPCR) of GSTT5 Transcripts in Canine Livers

Quality of total RNA samples was assessed by a commercial nano chip;[m] RNA samples with an RNA integrity number (RIN) ≥7 were considered of adequate quality for qPCR.[27] Complementary DNA (cDNA) was generated by a commercial master mix,[n] and qPCR was performed by a real-time cycler and commercial reagents according to the manufacturer's instructions.[o] *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 *NcoI* restriction sites (F: 5′ TAGGGATCCATGCCGCCGGAGCTGTTTC, R: 5′ ATTCCATGGGCGCCGAGCCTCTCCAG). The resulting 746-bp fragment was cloned into the plasmid pCR2.1 by TOPO cloning.[p] The correct orientation of the resulting construct was confirmed by

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 byClustal Omega.[25]

Gene	mRNA	*cfGSTT5*	*cfGSTT1*	*cfGSTT2*	*cfGSTT3*	*cfGSTT4*	*hsGSTT1*	*hSGSTT2*
cfGSTT5	XM_851878	100.00	62.79	61.16	60.03	58.52	60.72	61.85
cfGSTT1	XM_534751	62.79	100.00	66.67	82.16	62.10	89.63	64.58
cfGSTT2	XM_003433436	61.16	66.67	100.00	64.72	56.73	64.17	81.50
cfGSTT3	XM_003639928	60.03	82.16	64.72	100.00	61.40	81.88	63.61
cfGSTT4	XM_543530	59.52	62.10	56.73	61.40	100.00	61.54	59.08
hsGSTT1	NM_000853	60.72	89.63	64.17	81.88	61.54	100.00	63.47
hsGSTT2	NM_001080843	61.85	64.58	81.50	63.61	59.08	63.47	100.00

sequencing, using standard plasmid-specific primers, and the *BamHI-NcoI* fragment was cloned into a bacterial expression vector.[q] A stop codon was introduced by site directed mutagenesis.[r] and specific primers (F: 5′ CAAGCTTCGAATTCCATGTTAGC CGAGCCTCTCCAGCAG, R: 5′ CTGCTGGAGAGGCTCGGC-TAACATGGAATTCGAAGCTTG).

The 6-bp deletion variant was also generated by site-directed mutagenesis[r] by a second set of primers (F: 5′ CTCGGGT CAGCCTGTGGAGCGGC, R: 5′ GCCGCTCCACAGGCTGAC CCGAG). Both constructs were used to transform *E. coli*,[s] and protein expression was induced with isopropyl β-D-1-thiogalacto-pyranoside (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.[t]

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.[u] 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 ($P < 0.0001$).

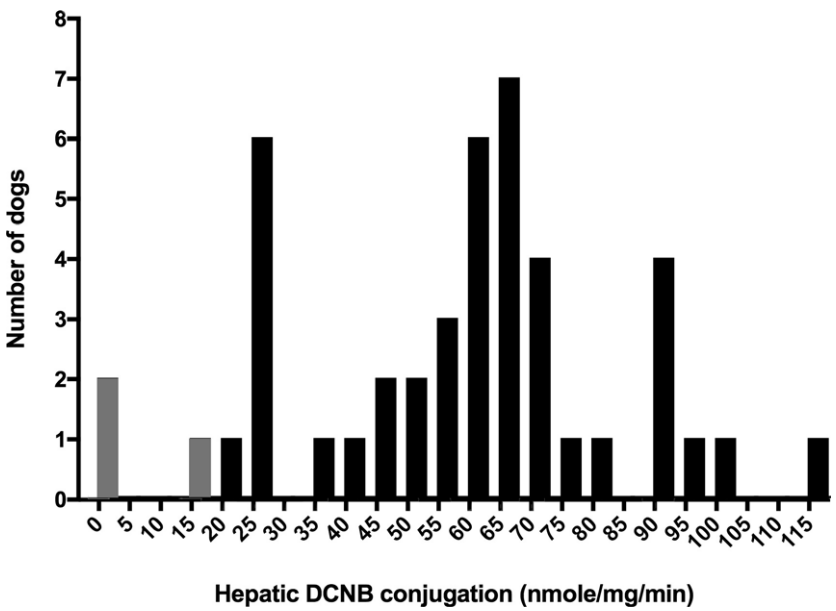

Fig. 1. Histogram of GST-theta activities toward the prototypic substrate DCNB in livers from 45 dogs of various breeds. Enzyme activities in individual dogs are grouped together in bins spanning 5 nmol/mg/min. The gray bars represent three dogs homozygous for a 6 bp coding deletion in *GSTT5*.

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

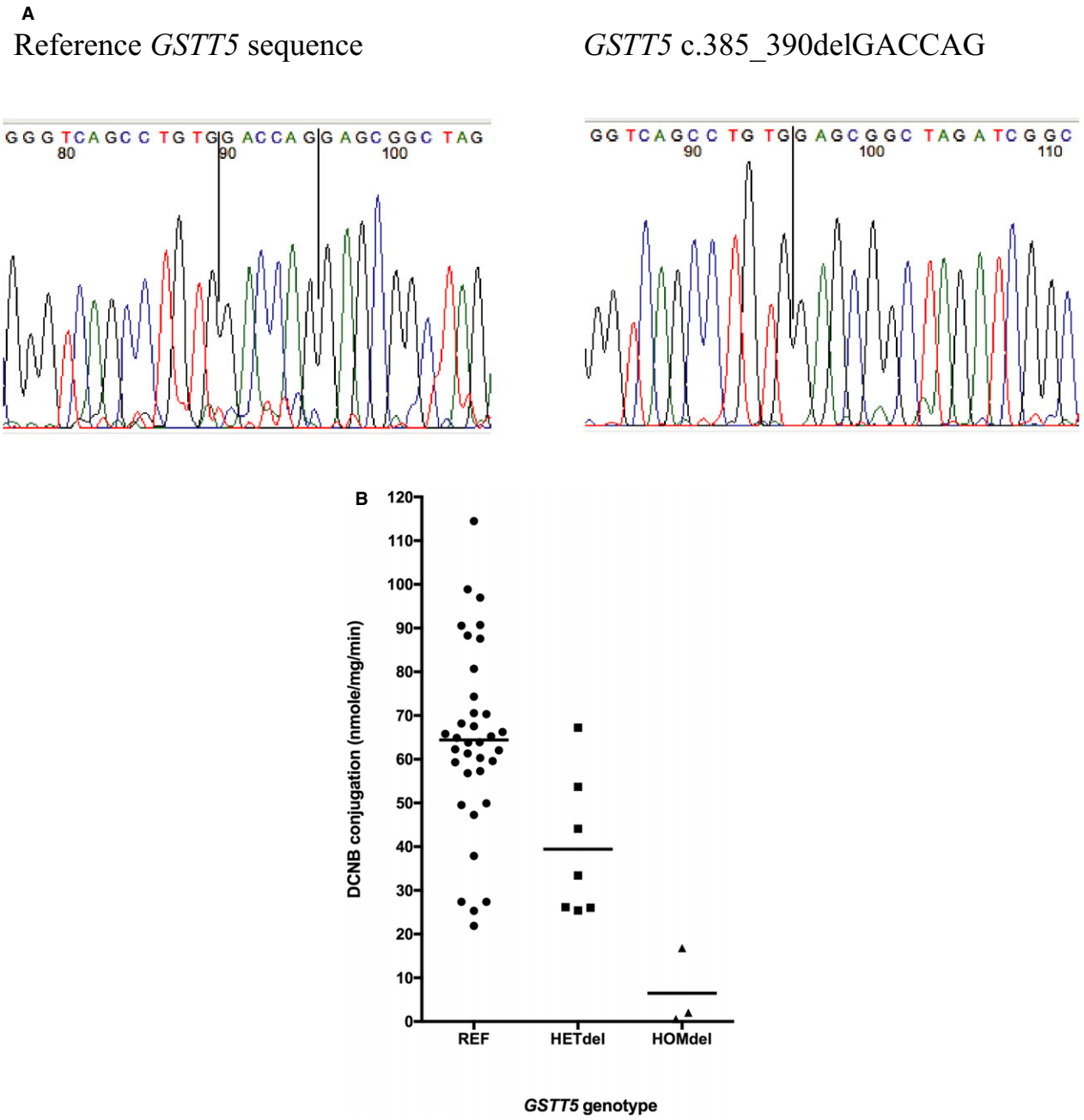

Fig. 2. **(A)** Representative DNA chromatograms for a dog with the reference *GSTT5* genotype (left) and a dog with a homozygous *GSTT5* 6-bp deletion (right; c.385_390delGACCAG, p.Asp129_Gln130del). **(B)** DCNB activities by *GSTT5* genotype. The horizontal bar in each scatterplot represents the group 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]

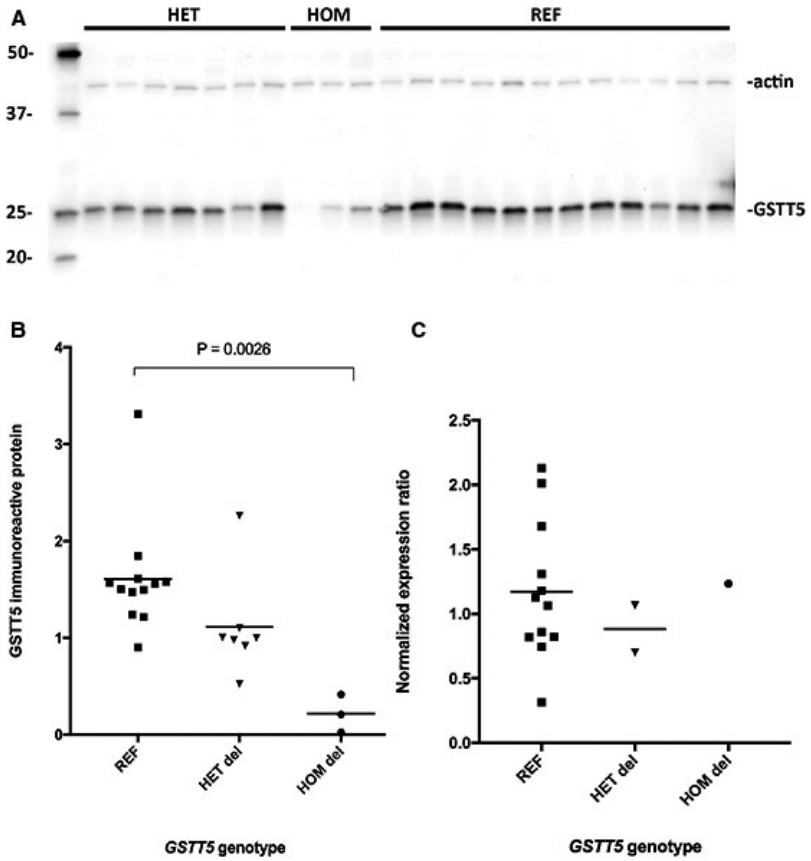

Fig. 3. GSTT5 protein and mRNA expression in canine livers. **(A)** Immunoblot image, and **(B)** densitometry values for immunoreactive GSTT5 protein in 22 canine livers by *GSTT5* genotype. Densitometries were normalized for β-actin expression; 2.5 μg cytosolic protein per lane. $P = 0.0026$ between reference (REF) and homozygous variant (HOM del) livers. **(C)** *GSTT5* transcript levels by qPCR in 15 genotyped canine livers. Transcript levels were normalized to *GAPDH* and *B2M* expression.

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_{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, MPPELFLDLYSPPPRAIYIFALKNGIP,[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

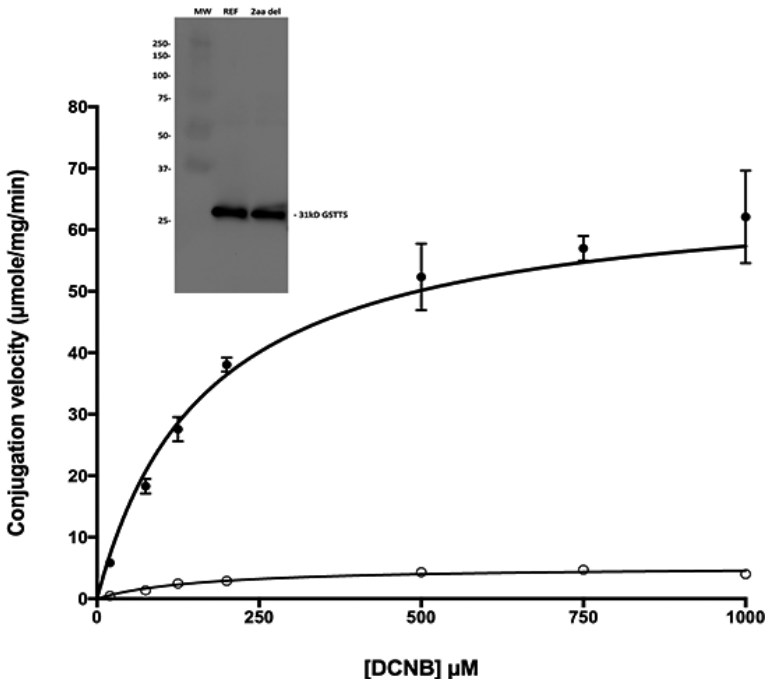

Fig. 4. Enzyme activities for DCNB conjugation by the recombinant GSTT5 enzyme (filled circles) and by the GSTT5 variant Asp129_Gln130del (open circles). Interpolated lines represent the Michaelis-Menten model fit for each enzyme; error bars indicate the SD for 3 separate experiments. Insert: Immunoblot of recombinant reference (REF) and variant (2 aa del) GSTT5 proteins; 10 ng protein loaded in each lane.

reported to be a specific substrate for GSTT5 (i.e. the canine GST-theta isoform formerly called GST-D or isozyme Yd_fYd_f), compared to canine homologs of GST-mu and GST-pi.[24] 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 proteasomal degradation of the defective protein in vivo, which would not be detected in the in vitro bacterial system.[28]

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.[20,21] 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.

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Footnotes

[a] Bio-Rad Laboratories, Hercules, CA
[b] Clustal Omega (http://www.ebi.ac.uk/)
[c] BLAT, (genome.ucsc.edu/)
[d] RNeasy Midi Kit (Qiagen, Valencia, CA)
[e] High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA)
[f] AmpliTaq Gold 360 MasterMix (Life Technologies, Grand Island, NY)
[g] Big Dye reagents (Applied Biosystems, Foster City, CA)
[h] Staden Package software (http://staden.sourceforge.net/)
[i] Panigen Inc, Blanchardville, WI
[j] Promega, Madison WI
[k] Abcam, Cambridge MA
[l] ImageJ (https://imagej.nih.gov/ij/)
[m] Total RNA Nano Chip (Agilent Technologies, Santa Clara, CA)
[n] Superscript VILO Mastermix cDNA Synthesis Kit (Invitrogen, Carlsbad, CA)
[o] Roche Lightcycler 96 with the Fast Start Essential DNA Probes Master Kit (Roche Diagnostics, Indianapolis, IN)
[p] TOPO TA cloning kit (Invitrogen, Carlsbad CA)
[q] pRSETA vector (Invitrogen, Carlsbad CA)
[r] Quickchange II Site Directed Mutagenesis Kit (Agilent, Santa Clara, CA)
[s] BL21 (DE3) pLysS (EMD-Millipore, Merck KGaA, Darmstadt, Germany)
[t] Prism (GraphPad Software, Inc. La Jolla, CA)
[u] Chief & NIH Distinguished Investigator, Cancer Genetics and Comparative Genomics Branch, National Human Genome research Institute.

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

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

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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