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Glutathione S-transferase theta genotypes and environmental exposures in the risk of canine transitional cell carcinoma

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Abstract

Introduction: Transitional cell carcinoma (TCC) in humans is associated with environmental exposures and variants in glutathione S-transferase (GST) genes. Scottish Terriers have a high breed risk for TCC, but the relationship between genetic and environmental risk in dogs is not fully understood.

Hypotheses: Scottish Terriers have a higher frequency of GST-theta variants compared to lower risk breeds. Dogs with TCC of any breed have a higher frequency of GST-theta variants along with higher environmental exposures, compared to controls.

Animals: One hundred and five Scottish Terriers and 68 controls from lower risk breeds; 69 dogs of various breeds with TCC, and 72 breed- and sex-matched unaffected geriatric dogs.

Methods: In this prospective case-control study, dogs were genotyped for 3 canine GST-theta variants: *GSTT1* I2+28 G>A, a *GSTT1* 3′UTR haplotype, and *GSTT5* Asp129_Gln130del. Owners of dogs with TCC and unaffected geriatric controls completed a household environmental questionnaire.

Results: The *GSTT1* 3′UTR haplotype and *GSTT5* Asp129_Gln130del variants were significantly *underrepresented* in Scottish Terriers (minor allele frequency [MAF] = 0.000 for both), compared to dogs from lower risk breeds (MAF = 0.108 and 0.100; $P \leq .0002$). Dogs with TCC did not differ from unaffected geriatric controls across the 3 investigated loci. Transitional cell carcinoma was associated with household insecticide use (odds ratio [OR] = 4.28, 95% confidence interval [CI] = 1.44-12.33, $P = .02$), and was negatively associated with proximity to a farm (OR = 0.49, 95% CI = 0.25-0.99, $P = .04$).

Conclusions and Clinical Importance: Low-activity GST-theta loci are unlikely contributors to TCC risk in dogs. Increased risk is associated with household insecticide use, and possibly with less rural households.

KEYWORDS
bladder cancer, canine, chemical exposure, pharmacogenetics, pharmacogenomics

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; CI, confidence interval; GST, glutathione S-transferase; GSTT1, glutathione S-transferase theta 1; GSTT5, glutathione S-transferase theta 5; MAF, minor allele frequency; OR, odds ratio; TCC, transitional cell carcinoma.

1 | INTRODUCTION

Transitional cell carcinoma (TCC) of the bladder in dogs is a muscle-invasive cancer that can lead to bleeding, pain, and urinary obstruction. Treatment is often met with relapse and has a high case of fatality rate.[1,2] The Scottish Terrier breed has a high risk for TCC, and this is also influenced by environmental exposures[2-4]; however, the mechanisms for this breed-environment interaction are not understood.

In humans, roughly half of TCC risk is attributed to cigarette smoking,[5] about 20% to occupational exposures,[6] and a small fraction to medical exposures[7] or isolated familial risk.[8] Low-grade chronic exposures from chemicals in the household environment might contribute to the remaining risk. Human TCC is associated with residence in areas with higher industrial activity,[9] and with environmental tobacco smoke exposure among non-smokers.[10,11] Both air pollution and tobacco smoke contain 4-aminobiphenyl, arsenic, and acrolein, which are known bladder carcinogens.[12-14] Exposure to high levels of phenoxyherbicides,[15] arsenic contamination in well water,[13] and chlorination by-products in tap water[16] are also linked to human TCC.

Individual susceptibility to TCC in humans is further modified by genetic variability in biotransformation pathways, such as glutathione S-transferases (GSTs), which are important in the detoxification of foreign chemicals. Low activity variants in the genes encoding GST-theta, GST-mu, and GST-pi have been associated with human TCC risk in various studies.[17-21] In particular, a null variant in *GSTT1*, with complete absence of enzyme expression, was significantly associated with human TCC in a meta-analysis of 50 studies.[22]

Naturally occurring TCC in dogs shares many biologic similarities with muscle-invasive TCC in humans, suggesting shared tumor etiologies.[1,23] As in humans, TCC in dogs tracks with areas of higher industrial activity[9] and exposure to phenoxyherbicides and pesticides.[3] In addition, Scottish Terrier dogs exhibit an 18-fold higher breed risk for TCC compared to mixed breed dogs.[1] However, even this high breed risk is modified by diet[24] and phenoxyherbicide exposures.[3] This suggests that TCC involves interactions between genetic and environmental risk factors in dogs as well as in humans.

We have characterized polymorphisms in 2 canine GST genes to date, *GSTT1* and *GSTT5*.[25-27] In canine *GSTT1*, an intronic variant (I2+28 G>A) is predicted to perturb splicing of *GSTT1* mRNA,[25] and a 3′UTR haplotype containing 101_102insT leads to a 50% decrease in gene expression.[26] In *GSTT5*, a 6 base pair coding deletion in exon 4 (385_390delGACCAG; Asp129_Gln130del) virtually eliminates enzyme activity.[27]

We hypothesized that low-functioning GSTT variants, along with specific environmental exposures, would be associated with TCC risk in dogs. The primary aim of this study was to compare GSTT genotype frequencies in 2 populations: Scottish Terriers versus lower risk dog breeds, and dogs with TCC of any breed compared to unaffected geriatric control dogs. A secondary aim was to evaluate the association between bladder TCC in dogs and potential household or environmental exposures to chemical bladder carcinogens.

2 | METHODS

2.1 | Glutathione S-transferase genotyping in Scottish Terriers and lower risk breeds

To investigate the frequency of GST-theta variants in the breed at highest risk for TCC, we first recruited Scottish Terriers along with control dogs from lower risk breeds (this excluded Shetland Sheepdogs, West Highland White Terriers, Beagles, Wire Haired Fox Terriers, Eskimo dogs, Keeshonds, and Samoyeds, which are also at higher than average risk for TCC).[1,2] Scottish Terriers were primarily recruited through ongoing clinical studies at the Purdue University College of Veterinary Medicine. Scottish Terriers of any health status were allowed for this breed-focused phase of the study.

Control dogs were recruited from client- and staff-owned pets at the teaching hospitals at the University of Wisconsin and Purdue University, and from banked DNA from previous studies.[25,26] Control dogs were matched for sex, neuter status, and age (within 1 year) to the Scottish Terrier dogs, and were clinically healthy at the time of recruitment. DNA samples were obtained from all dogs using buccal swabs (MasterAmp, Epicentre, Madison, Wisconsin), and genomic DNA was isolated using a commercial kit (DNeasy Blood and Tissue Kit, Qiagen, Hilden, Germany) as previously described.[26] All study procedures were approved by the Institutional Animal Care and Use Committee at each institution, and all dog owners provided written informed consent before enrollment.

2.2 | Glutathione S-transferase genotyping and questionnaires in TCC cases and controls

Dogs with TCC were recruited from the University of Wisconsin-Madison and from Purdue University, and through collaborations with Colorado State University, the University of Georgia, Texas A&M University, and several private veterinary oncology and primary care practices. The diagnosis of TCC was based on visualization of a bladder or urethral mass on abdominal ultrasound or cystoscopy, combined with positive urine cytology, bladder histopathology, or the BRAF (Raf family serine/threonine-protein kinase B gene) mutation detection in voided urothelial cells.[28,29] Confirmed TCC cases could be of any breed, sex, or age. Clinically unaffected control dogs were recruited through the University of Wisconsin-Madison, through outreach at dog events, and by advertisements through breed associations, kennel clubs, and the National Canine Cancer Foundation. Control dogs were matched by breed, sex, and neuter status to TCC dogs, and must have reached the median age of onset of canine TCC (≥8 years for Scottish Terriers and ≥11 years for other breeds),[1] with no history of systemic cancer and no unresolved lower urinary tract signs within the year before recruitment. Because of budget constraints of the study, healthy dogs were not screened for occult bladder tumors with abdominal ultrasound or BRAF assays. When a precise breed- and sex-matched control could not be identified for a purebred case, a mixed breed dog with partial breed match to the case was allowed. For mixed breed cases, we estimated breed composition based on

veterinary medical records and the dog's physical appearance and body weight. We then matched the dog to the most relevant size category (toy, small, medium, large, and giant) as defined previously.[30,31] Control dogs for these mixed-breed cases were utilized from the same size categories (eg, toy, small, medium, large, or giant) as the TCC cases.

Buccal brush samples for genomic DNA isolation were obtained from all TCC cases and controls, and owners completed a questionnaire about their dog's household environment, including residential locale (proximity to factories, farms, or vehicle traffic), household smoking status, lawn treatments, dog's primary water source (well, municipal, bottled, other), and the use of flea and tick products (see questionnaire in Supporting Information). Proximity of each participating household to possible industrial sources of chemical carcinogens was confirmed using the resident address and the "nearby" function on Google Maps, with the search terms "chemical plant," "municipal dump," "landfill," "manufacturer" (eg, rubber, textile, leather), "coal plant," "incineration plant," and "crematorium." Google Earth satellite images are reported to be current within 1-3 years of the time of online access. Google Maps also provides the active status of factories and other potential sources of pollution, which we confirmed using the sources' websites whenever available. Owner-reported household proximity to a farm was confirmed via similar methodology, using "farm" as a search term, and through the "Google Earth" view on Google Maps to confirm physical presence of farmland within 1 mile from the household.

2.3 | Glutathione S-transferase theta variant resequencing

Three polymorphic loci in canine GST-theta genes were directly re-sequenced: *GSTT1* I2+28 G>A; the *GSTT1* 3′UTR haplotype containing 101_102 insT, and the 6 base pair coding deletion in exon 4 of *GSTT5* (385_390delGACCAG; Asp129_Gln130del). Primers were designed from the canine genome assembly (CanFam3.1) as previously described.[26,27] Sequence alignment and polymorphism screening were carried out using SerialCloner v2.6 (SerialBasics) and FinchTV chromatogram reader software (Geospiza Inc).

2.4 | Statistical analyses

Minor allele frequencies (MAFs) and genotype frequencies at each GSTT locus were calculated for all dogs, and were compared between Scottish Terriers and lower risk breeds, and between TCC cases and controls, using Fisher's exact tests. Categorical data for reported household and environmental exposures were also compared between cases and controls using Fisher's exact test, with generation of odds ratios (ORs) and 95% confidence intervals (CIs). Google Maps data were used for statistical analyses of proximity data to potential industrial sources of pollution. Owner-reported proximity data were evaluated for accuracy compared to Google Maps data using Fisher's exact tests. All analyses were conducted using Prism Graphpad Statistical Software (Version 7.0d), with an unadjusted, exploratory significance threshold of $P < .05$.

3 | RESULTS

3.1 | Glutathione S-transferase genotyping in Scottish Terriers

A total of 105 Scottish Terriers were recruited, 68 of which were matched by sex and age to control dogs from lower risk breeds. Baseline breed genotypes for the 3 GST-theta variant loci results were determined for all 105 Scottish Terriers, and were statistically compared between the 68 Scottish Terriers and their matched controls. The matched Scottish Terriers and lower risk breed group each contained 40 females (40 spayed) and 28 males (26 neutered). The top 5 represented breeds in the lower risk group were mixed breed (16), Golden Retriever (12), Labrador Retriever (10), Alaskan Malamute (6), and Siberian Husky (4). The remaining breeds in the lower risk group include Australian Cattle Dog, Newfoundland, Greyhound, German Shorthaired Pointer, Welsh Corgi, Brittany Spaniel, Bernese Mountain Dog, Cocker Spaniel, Doberman Pinscher, Toy Poodle, English Pointer, Weimaraner, Chihuahua, German Shepherd, and Shih Tzu.

Allele and genotype frequencies were compared between breed groups (Table 1). Contrary to our hypothesis, the low functioning *GSTT1* 3′UTR haplotype containing 101_102insT was not overrepresented in Scottish Terriers (MAF = 0.000, even across all 105 dogs), and was significantly more prevalent in the lower risk population (MAF = 0.110, $P < .0001$). Similarly, the *GSTT5* Asp129_Gln130del

TABLE 1 Genotype and minor allele frequencies (MAFs) of variant loci in canine *GSTT1* and *GSTT5* among Scottish Terriers (a breed at high risk for transitional cell carcinoma [TCC] and sex- and age-matched dogs from breeds at lower risk for TCC)

GST locus	Scottish Terriers	Other breeds not at high risk for TCC	*P* value
***GSTT1* I2+28**	66 dogs	63 dogs	
Genotype (n)			
GG (REF)	62	60	1.00
GA	3	1	
AA	1	2	
MAF	0.038	0.040	1.00
***GSTT1* 3′UTR haplotype**	65 dogs	60 dogs	
Genotype (n)			
REF	65	49	.0002
HET insT	0	9	
HOM insT	0	2	
MAF	0.000	0.108	<.0001
***GSTT5* exon 4 6 bp del**	60 dogs	55 dogs	
Genotype (n)			
REF	60	45	.0004
HET DEL	0	9	
HOM DEL	0	1	
MAF	0.000	0.100	.0002

allele was also absent in Scottish Terriers (MAF = 0.000, across all 105 dogs), but was found in 10% of the lower risk population (MAF = 0.100, P = .0002). Allele frequencies at the *GSTT1* I2+28 locus did not differ between breed groups (Table 1).

3.2 | Glutathione S-transferase genotyping in TCC cases and controls

A total of 100 dogs with TCC were recruited, of which 70 were successfully matched with at least 1 clinically healthy older control dog. Of these, 69 cases had both questionnaire data and buccal swab samples available (Table 2). Two Shetland Sheepdog cases did not have a perfect match, so Shetland Sheepdog-mixes were used. In total, 69 dogs with TCC and 72 unaffected control dogs were genotyped for *GSTT1* I2+28 G>A, the *GSTT1* 3′UTR variant haplotype, and *GSTT5* Asp129_Gln130del.

However, dogs with TCC did not differ from controls in genotype or MAFs at any of the 3 investigated loci (Table 3). All of the Scottish Terriers sequenced in this part of the study had the reference alleles in the *GSTT1* 3′UTR and in *GSTT5*, as found in the initial Scottish Terrier population. Of note, 7 out of the 11 Scottish Terriers in this part of the study (6 cases and 1 geriatric control dog) were re-recruited from the initial 105 Scottish Terriers in the breed-genotyping population.

3.3 | Environmental exposures in TCC cases and controls

Owners completed an environmental questionnaire about their dog's exposure to environmental chemicals in the home and yard over the previous year, as well as household proximity to potential industrial sources of pollution. Proximity data were confirmed using the owner's home address and Google Maps, and the Google Maps data were used for statistical comparisons. Three dog owners did not specify a household address, so self-reported proximity data to nearby farms and industrial sources were used for statistical analyses in these cases.

Dogs with TCC were significantly more likely to live in a household that reported using insecticide treatment in the previous year, compared to unaffected controls (OR = 4.28, 95% CI = 1.44-12.63, P = .02; Table 4). Dogs with TCC were also significantly *less* likely to live near (within a mile of) a farm (OR = 0.49, 95% CI = 0.25-0.99, P = .04; Table 4). There was no association between TCC risk and household smoking (OR = 1.30, 95% CI = 0.35-3.98, P = .76), well water for drinking (OR = 0.56, 95% CI = 0.25-1.24, P = .17) or use of 1 or more flea or tick control products (OR = 0.70, 95% CI = 0.32-1.57, P = .42).

To assess the accuracy of owner responses regarding household proximity (within 1 mile) to potential sources of environmental pollution, we compared owner responses with objective data obtained from the owner's address and Google Maps. Owner-reported household proximities to a farm, as well as self-classified household locale as "rural" or "farm," were both in significant agreement with objective data from Google maps (P = .0001 for both). Owner-reported proximity to a chemical plant also significantly agreed with Google Maps data (P = .004), but owner-reported data for other industrial sites did not correlate. Overall, proximity to industrial sites (chemical and manufacturing plants, dumps, landfills, coal plants, or incinerators) was low across the case-control population, with no more than 7 households located within a mile of each of these sites, either reportedly or objectively.

TABLE 2 Demographic data from dogs with transitional cell carcinoma (TCC) of the bladder and healthy breed- and sex-matched controls over the median age of onset of TCC in dogs (≥8 years in Scottish Terriers and ≥11 years in other breeds)

Signalment	TCC (n = 69)	Controls (n = 72)
Median age (range)	11 (4-16) y	12 (8-16) y
Sex		
FS	44	48
FI	2	1
MN	21	21
MI	2	2
Common breeds (n > 3)		
Mixed	20 (29.0%)	22 (29.2%)
Beagle	7	7
Shetland Sheepdog	6	4
West Highland Terrier	6	6
Dachshund	6	6
Scottish Terrier	5	6
Border Collie	3	3
Min Schnauzer	3	3
Method of TCC diagnosis		
Histopathology	51	N/A
Cytology	18	
Imaging		
Ultrasound only	17	N/A
Cystoscopy only	7	
Multimodal imaging[a]	45	

Abbreviations: FI, female intact; FS, female spayed; MI, male intact; MN, male neutered.
[a]Some combination of abdominal ultrasound, radiographs, cystoscopy, abdominal CT, or surgical visualization.

4 | DISCUSSION

We first investigated the prevalence of low-activity GST-theta variants in Scottish Terriers, a breed at high risk for TCC,[1] compared to healthy sex- and age-matched dogs from lower risk breeds. Contrary to our hypothesis, variants in *GSTT1* and *GSTT5* were not overrepresented in Scottish Terriers, and in fact, the low expression *GSTT1* 3′UTR haplotype and the inactive *GSTT5* variant were absent in Scottish Terriers in this study population. This observation is most likely a breed phenomenon that is unrelated to TCC risk. However, it was also possible that the *active* reference alleles were related in some way to TCC risk in this breed. Although GSTs are predominantly detoxifying

TABLE 3 Glutathione S-transferase (GST)-theta genotype and minor allele frequencies (MAFs) in dogs with transitional cell carcinoma (TCC) and in older breed- and sex-matched control dogs. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated relative to the reference genotype or allele

GST locus	TCC	Controls	OR	95% CI	*P* value
***GSTT1* I2+28**	66 dogs	68 dogs			
Genotype (n)					
GG (REF)	56	56	0.83	0.32-2.02	.82
GA	8	11			
AA	2	1			
MAF	0.091	0.096	0.95	0.40-2.15	1.00
***GSTT1* 3′UTR haplotype**	69 dogs	68 dogs			
Genotype (n)					
REF	53	50	0.84	0.39-1.85	.70
HET insT	12	14			
HOM insT	4	4			
MAF	0.145	0.162	0.74	0.46-1.66	.74
***GSTT5* exon 4 6 bp del**	68 dogs	67 dogs			
Genotype (n)					
REF	61	64	2.45	0.63-8.97	.33
HET Del	7	3			
HOM Del	0	0			
MAF	0.051	0.022	2.37	0.64-8.55	.33

enzymes, GST-theta enzymes can, less commonly, bioactivate some chemical substrates to more reactive carcinogenic compounds.[32,33]

To test the hypothesis that either active or low activity variants at these GSTT gene loci contribute to TCC risk in dogs, we performed a case-control study comparing GST-theta genotypes in dogs with TCC to unaffected older breed- and sex-matched controls. However, we found similar allele and genotype frequencies between groups across the 3 variant GST-theta loci, which did not support a clinically relevant association between GSTT and bladder cancer in dogs.

In humans, the null *GSTT1* variant has been associated with increased bladder cancer risk in several studies.[17,21,22,34] However, other studies have associated the fully active *GSTT1* genotype with TCC.[35-38] These discordant findings could be explained by uncharacterized differences in environmental chemical exposures among populations.[39] There is still a poor understanding of which chemical carcinogens are substrates for specific GST enzymes, and whether the glutathione-conjugated products are more or less mutagenic, in either humans or dogs. Further work is needed to characterize the roles of specific canine GST enzymes in bladder carcinogen biotransformation. In addition, low-functioning variants in the genes encoding GST-mu (*GSTM1*) and GST-pi (*GSTP1*) also influence TCC risk in humans, and can interact with *GSTT1* genotype.[36,40-44] Follow-up studies are underway to evaluate whether genetic variants in canine *GSTM1* and *GSTP1* contribute to TCC risk in dogs.

In addition to genotyping, we collected environmental questionnaire data from case and control households to survey for possible environmental exposures associated with bladder cancer risk in dogs. We found that dogs with TCC were 4 times more likely to come from a home that reported the use of household insecticides in the previous year. Environmental insecticide exposure has been epidemiologically and mechanistically associated with several cancers in humans, including bladder cancer, leukemia, and lymphoma.[45,46] Active compounds in several classes of common household pesticides, such as organophosphates, synthetic pyrethroids, and neonicotinoids, have been shown to induce genotoxic effects such as aneuploidy and chromosomal breaks in mammalian lymphocytes at low concentrations mimicking realistic exposure conditions.[47] Our questionnaire combined all environmental insecticide types (described as those for "ants, wasps, and termites") into 1 category of exposure, and we did not ask owners to identify specific chemicals. Future studies will need to collect more refined information about specific insecticide exposures.

Bladder cancer in both humans and dogs has been associated with urban and industrial environments.[9,48,49] In our study, dogs with TCC were significantly less likely to live within 1 mile from a farm. This observation could be a surrogate for a more urban home environment, with more vehicle emissions and industrial activity. However, we were not able to capture an association between TCC risk and households self-categorized as "urban" (Table 3), nor did we find risk associations with vehicle emissions, or proximity to manufacturing sites using objective Google Maps data. Although these objective data can overcome uncertainty in owner reports, they are still subject to bias in the accuracy of search terms and in the timing of Google Maps data collection relative to the duration of residence in an area. An interesting approach used by another research group was to measure the number of employees per household in manufacturing jobs as a surrogate for proximity to industrial activity.[9,48] Follow-up studies should include a

TABLE 4 Selected household environmental data from owners of dogs with transitional cell carcinoma (TCC) of the bladder, and older sex- and breed-matched unaffected control dogs

Owner reported data		TCC n (%)	Controls n (%)	OR	95% CI	*P* value
Neighborhood	Respondents	69	72			
	Suburban	42 (60.9%)	42 (58.3%)	-	-	-
	Non-suburban	27 (39.1%)	27 (37.5%)	1.00	0.51-1.96	1.00
	Urban	13	11	1.18	0.50-2.94	.81
	Rural	13	13	1.00	0.44-2.29	1.00
	Farm	1	3			
	Unknown	0	3			
Proximity to industrial sources[a]	Respondents	69	72			
	None	62 (89.9%)	61 (84.7%)	-	-	
	One or more	7 (10.1%)	11 (15.3%)	0.63	0.24-1.77	.45
Drive-by traffic[b]	Respondents	68	68			
	Minimal	25 (36.8%)	26 (38.2%)	-	-	
	Moderate or heavy	43 (63.2%)	42 (61.8%)	1.06	0.53-2.14	1.00
Proximity to a farm	Respondents	69	71			
	>1 mile	45 (65.2%)	34 (47.9%)	-	-	
	Within 1 mile	24 (34.8%)	37 (52.1%)	0.49	0.25-0.99	.04
Household tobacco use[c]	Respondents	68	72			
	Non-smoking	62 (91.2%)	67 (93.1%)	-	-	
	Smoking	6 (8.8%)	5 (6.9%)	1.30	0.35-3.98	.76
Herbicide[d] or insecticide treatment	Respondents	68	70			
	Neither	8 (11.8%)	19 (27.1%)	-	-	
	Herbicides[c] only	14 (20.16%)	14 (20.0%)	2.38	0.81-6.58	.17
	Insecticides only	18 (26.5%)	10 (14.3%)	4.28	1.44-12.6	.02
	Both	28 (41.2%)	27 (38.6%)	2.46	0.97-6.73	.10
Water source	Respondents	66	69			
	Not well water	53 (80.3%)	48 (69.6%)	-	-	
	Well water	13 (19.7%)	21 (30.4%)	0.56	0.25-1.24	.17
Flea/tick product use	Respondents	69	71			
	None used	18 (26.1%)	14 (19.7%)	-	-	
	At least 1	51 (73.9%)	57 (80.3%)	0.70	0.32-1.57	.42
	Spot-on only	26 (37.7%)	30 (42.3%)	0.67	0.30-1.63	.51
	Shampoo only	1 (1.4%)	0 (0%)	NA[e]	NA	NA
	Collar only	2 (2.9%)	3 (4.2%)	0.52	0.08-2.87	.64
	Pill only	13 (18.8%)	11 (15.5%)	0.92	0.33-2.63	1.00
	Other only	5 (7.2%)	2 (2.8%)	1.94	0.31-10.74	.68
	More than 1	4 (5.8%)	11 (15.5%)	0.28	0.09-0.99	.07

[a]Household proximity to industrial sources was defined as within a mile of a chemical plant, municipal dump, landfill, rubber/leather/textile manufacturing plant, coal plant, or incinerator/crematorium.
[b]Traffic categories were defined as minimal (dead end or 1-way rural road), moderate (neighborhood roadways), or heavy (near a highway).
[c]Any tobacco (cigarette, cigar, or pipe) use by household members or visitors within the past year.
[d]Lawn or household treatment within the previous year.
[e]NA, cannot be calculated.

variety of surrogate markers to capture households in urban or industrial environments, or ideally include direct measurements of chemical exposures in affected dogs.

In humans, about half of all TCC cases are found in cigarette smokers,[5] and additional cases are linked to second-hand tobacco smoke.[10] In our study, as in a previous report,[50] we did not find an

association between household smoking status and TCC risk in dogs. We found notably few dog owners (n = 11 overall) who identified their homes as "smoking" environments, suggesting either a low prevalence in this study population or bias in self-reporting. Follow-up studies should include quantitative measures of second-hand smoke exposure in dogs, such as urinary cotinine.[51]

We did not find an association between herbicide ("weed killer") use and TCC, which is contrary to a previous study that linked phenoxyherbicide use with TCC in Scottish Terriers.[3] We did not ask about specific brands names or specific herbicide chemicals, which could have improved the accuracy of this question. 2,4-Dichlorophenoxyacetic acid (2,4-D) is the most common phenoxyherbicide in household use, and has been found in canine urine after lawn exposures.[4] Although 2,4-D does not appear to be directly mutagenic, its soil metabolites are reactive and mutagenic.[52,53] Follow-up studies are needed to measure concentrations of specific herbicides and insecticides in the urine of dogs with TCC, compared to controls.

The use of topical flea and tick products was previously associated with canine TCC in a dose-dependent manner, especially among obese dogs.[50] In our population, the use of flea and tick control products was not found to be a risk factor. This difference is likely because of the changes in the active ingredients and solvent composition of modern flea products, and a decline in the use of flea and tick dips containing reactive organic solvents.[54] We also did not observe an association between drinking water source and TCC in our case-control population. Although human TCC has been associated with both arsenic contamination in well water[13] and high levels of chlorinated byproducts (trihalomethanes) in municipal water,[16] 1 study in dogs did not find a relationship between calculated trihalomethane exposure in municipal water and TCC.[55] Direct measurements of urinary arsenic and trihalomethanes in affected and control dogs could better address this question.

Our study has several limitations. First, we only genotyped 3 GST-theta loci, and variants in other GST isoforms can modify bladder cancer risk in humans.[17,21,40,56] We also did not include covariates such as diet and body condition score. We used questionnaire data as a surrogate for many household exposures, which can be influenced by recall bias, differences in question interpretation, and varying levels of awareness of the household environment. In fact, we found poor correlation between owner reports and Google Maps data for household proximity to most industrial sites. This might have been due, in part, to a low prevalence of nearby industrial activity for most households queried. Our TCC case population was drawn primarily from veterinary teaching hospitals, which are not typically situated in large urban areas, and this population might not be representative of dogs in other regions across the country. Finally, because of budget limitations, geriatric control dogs were not screened with a urinary ultrasound in addition to clinical history. Requiring a urinary ultrasound could possibly have detected a few control dogs with early disease, but would not have been likely to affect our overall results.

This is the first molecular epidemiologic investigation of cancer risk in dogs that considers pharmacogenetic variability in a carcinogen biotransformation pathway. Our findings indicate that previously identified low activity variants in canine *GSTT1* and *GSTT5* are not likely to play a major role in canine TCC risk, at least among dogs referred to veterinary specialists. Our results also support an epidemiologic association between insecticide use and bladder cancer in dogs of various breeds. Furthermore, a rural environment, as reflected by proximity to a farm, might decrease TCC risk in dogs.

Work is currently underway to explore the relationship between polymorphisms in canine *GSTM1* and *GSTP1* and TCC risk in dogs, and to identify the major canine GSTs involved in the biotransformation of candidate environmental bladder carcinogens. We are also working to measure urinary concentrations of environmental chemicals in dogs with and without TCC. These data will allow us to make mechanism-based predictions for future studies of gene-environment interactions in TCC risk. The overall goal is to better understand the causes of TCC in dogs, and to support evidence-based cancer prevention strategies.

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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.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

All study procedures were approved by the IACUCs at the University of Wisconsin-Madison and Purdue University, and all dog owners provided written informed consent prior to enrollment.

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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