



*Short Communication*

## DNA sequence variants in the carbonyl reductase 1 (*cbr1*) gene in seven breeds of *Canis lupus familiaris*

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**ABSTRACT.** The anticancer anthracyclines doxorubicin and daunorubicin are used to treat a variety of cancers in dogs. The therapeutic utility of anthracyclines is limited by cardiotoxicity in some cases. Synthesis of anthracycline alcohol metabolites by carbonyl reductase 1 (CBR1) is crucial for the pathogenesis of cardiotoxicity. We hypothesize that genetic polymorphisms in canine *cbr1* contribute to the variable pharmacodynamics of anthracyclines in dogs. DNA sequence variants in canine *cbr1* were investigated in DNA samples from dogs of seven breeds. Thirteen SNPs were detected in canine *cbr1*. A 10-bp deletion in the 5'-untranslated region (5'-UTR) was found in specimens from the Labrador Retriever, Beagle, Siberian Husky, and Boxer breeds. The 5'-UTR also included a polymorphic “hot spot” region immediately downstream of the 10-bp

deletion. DNA sequence variants in the “hot spot region” ranged from 1 to 21 bp in length. Bioinformatics searches identified a cluster of three to six potential binding sites for the transcription factor Sp1 in the DNA segment containing both the “hot spot” region and the 10-bp deletion. This information provides a foundation to allow us to investigate whether DNA sequence variants in the 5'-UTR of canine *cbr1* impact the pharmacodynamics of anticancer anthracyclines in dogs.

**Key words:** Anthracycline-related cardiotoxicity; Anthracyclines; Domestic dog (*Canis lupus familiaris*); Carbonyl reductase 1 (*cbr1*); Cancer; Doxorubicin

## INTRODUCTION

The anticancer anthracyclines doxorubicin and daunorubicin are used to treat a variety of cancers in dogs including lymphomas, hemangiosarcoma, osteosarcoma, mammary tumors, and squamous cell carcinomas (Simon et al., 2006). The therapeutic utility of anthracyclines is limited by the development of cardiotoxicity in some animals. The average incidence of anthracycline-related cardiotoxicity in dogs is  $\approx 18\%$ , and cardiotoxicity has been documented in up to 69% of animals (Gillings et al., 2009). Anthracycline-related cardiotoxicity may induce sudden death by arrhythmias, or it may culminate in congestive heart failure (Herman et al., 1983; Herman and Ferrans, 1998; Morrison, 2002; Astra et al., 2003). To the best of our knowledge, body weight is the only documented potential risk factor for anthracycline-related cardiotoxicity in dogs (Gillings et al., 2009). Additional risk factors for anthracycline-related cardiotoxicity in dogs remain to be defined.

The pathogenesis of anthracycline-related cardiotoxicity is mediated by a combination of oxidative stress and metabolic perturbations induced by C-13 anthracycline alcohol metabolites (e.g., doxorubicinol and daunorubicinol) (Minotti et al., 2004). Carbonyl reductase 1 (CBR1) catalyzes the synthesis of cardiotoxic C-13 alcohol metabolites. The role of CBR1 activity during the pathogenesis of anthracycline-related cardiotoxicity has been firmly established through biochemical and genetic studies in mice, humans and rabbits (Forrest et al., 2000; Mordente et al., 2001; Olson et al., 2003). Interestingly, single nucleotide polymorphisms (SNPs) in the human *CBRs* impact the pharmacodynamics of anthracyclines and the risk of anthracycline-related cardiotoxicity (Lal et al., 2008; Blanco et al., 2008, 2011). We hypothesize that SNPs in canine *cbr1* may contribute to the variable pharmacodynamics of anthracyclines in dogs. Here, we documented DNA sequence variants in canine *cbr1* by analyzing DNA samples from dogs of 7 breeds.

## DNA samples

DNA samples from unrelated Labrador Retrievers (N = 14), German Shepherds (N = 15), Beagles (N = 13), Boxers (N = 14), Poodles (N = 12), Siberian Huskies (N = 15), and Golden Retrievers (N = 14) were acquired from the CHIC repository (Orthopedic

Foundation for Animals, OFFA). DNA was isolated from peripheral blood using standard methodology.

### Detection of *cbr1* polymorphisms

Canine *cbr1* exons 1, 2, 3 and corresponding flanking sequences (*Canis lupus familiaris*, chromosome 31, GenBank reference sequence NC\_006613.2) were amplified by polymerase chain reaction (Appendix 1). Amplification products were sequenced with forward and reverse primers in an Applied Biosystems 3130xl Genetic Analyzer. SNP analysis was performed with a web-based tool (<http://lpgws.nci.nih.gov/perl/snp/snp.cgi.pl>). The presence of SNPs, nucleotide insertions and nucleotide deletions was verified by visual inspection of the electropherograms.

## RESULTS AND DISCUSSION

The 3 exons of canine *cbr1* and up to 359 bp from the 5'-untranslated region (5'-UTR) were amplified to analyze the presence of DNA sequence variants in the coding and potential promoter regions, respectively. Thirteen SNPs were detected in canine *cbr1*, including 2 synonymous SNPs, 7 SNPs in the 5'-UTR and 4 intronic SNPs (Tables 1 and 2). A 10-bp deletion in the 5'-UTR was present in specimens from the Labrador Retriever, Beagle, Siberian Husky, and Boxer breeds. The 5'-UTR included a polymorphic “hot spot” region immediately downstream of the 10-bp deletion. The “hot spot” included sequence variants ranging from 1 to 21 bp in length. None of the 61 DNA samples (including 11 samples from Boxer dogs) showed the corresponding 10-bp fragment (5'-CACGAGACCC-3') reported in the GenBank reference sequence from a Boxer dog (Table 1). These findings highlight the polymorphic nature of the dog's genome.

Bioinformatics searches focused on the proximal 5'-UTR of canine *cbr1* (400 bp screened) pinpointed a cluster of 3 to 6 conserved motifs for the transcription factor Sp1 (5'-GCCACGCC-3') (McDowell et al., 2005). The number of Sp1 motifs, 10-bp deletion status (yes/no), and corresponding DNA sequences for the “hot spot” region are listed in Table 3. Of note, the Sp1 cluster is located in the DNA segment containing both the “hot spot” region and the polymorphic 10-bp deletion (length: 44 to 64 bp). In this segment, the GenBank reference sequence has a total of 4 Sp1 motifs (Table 3). Interestingly, one Sp1 motif is abrogated by the 10-bp deletion. In contrast, the presence of “hot spot” variants 3, 5, 7, and 9 resulted in an additional Sp1 motif. Similarly, 2 additional Sp1 motifs were created when the relatively long “hot spot” variant 8 (length: 21 bp) was present (Tables 1 and 3). Our previous studies have shown that the aryl hydrocarbon receptor pathway regulates the expression of human *CBRI* through conserved xenobiotic response elements (*XREs*) in the gene promoter region (Lakhman et al., 2007). Here, we pinpointed a conserved *XRE* (5'-CACGCCA-3') in the vicinity of the Sp1 cluster in the 5'-UTR of canine *cbr1* (306 bp upstream of the translation initiation codon, A<sub>+1</sub>TG). Future studies are warranted to investigate whether the *XRE*, in concert with the variable number of Sp1 motifs, impacts the transcription of canine *cbr1* and, consequently, the pharmacodynamics of anticancer anthracyclines in dogs (Wang et al., 1998).

**Table 1.** DNA variants in canine *cbr1* (5'-UTR and exon 1) - *Canis lupus familiaris* chromosome 31, whole genome shotgun sequence (NCBI reference sequence: NC\_006613.2)\*.

	33434924 Reference: G Variant: C		33434941 Reference: A Variant: C		33434956 Reference: G Variant: C		33434985 Reference: A Variant: C		33435007 Reference: C Variant: G		33435047~33435056 Reference sequence: AGGCCACGCC (10-bp deletion)	
	<i>p</i>	<i>q</i>	<i>p</i>	<i>q</i>	<i>p</i>	<i>q</i>	<i>p</i>	<i>q</i>	<i>p</i>	<i>q</i>	<i>p</i> Deletion	<i>q</i> No deletion
Labrador (N = 14)	1.00	0.00	0.50	0.50	1.00	0.00	1.00	0.00	0.82	0.18	0.07	0.93
German Shepherd (N = 14)	1.00	0.00	0.89	0.11	1.00	0.00	1.00	0.00	0.11	0.89	0.00	1.00
Beagle (N = 5)	0.80	0.20	0.80	0.20	0.50	0.50	1.00	0.00	0.70	0.30	0.40	0.60
Boxer (N = 11)	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	0.73	0.27	1.00	0.00
Poodle (N = 6)	0.75	0.25	0.75	0.25	1.00	0.00	1.00	0.00	0.25	0.75	0.00	1.00
Siberian Husky (N = 5)	0.30	0.70	0.30	0.70	0.80	0.20	0.20	0.80	0.90	0.10	0.20	0.80
Golden Retriever (N = 6)	0.92	0.08	0.58	0.42	1.00	0.00	1.00	0.00	0.50	0.50	0.00	1.00
Interbreed (N = 61)	0.90	0.10	0.72	0.28	0.94	0.06	0.93	0.07	0.55	0.45	0.26	0.74
Amino acid change	None 5'-UTR		None 5'-UTR		None 5'-UTR		None 5'-UTR		None 5'-UTR		None 5'-UTR	
			33435057~33435066 Reference sequence: CACGAGACCC (10 bp)**		33435132 Reference: A Variant: C		33435156 Reference: C Variant: A		33435291 Reference: C Variant: A		33435507 Reference: G Variant: A	
					<i>p</i>	<i>q</i>	<i>p</i>	<i>q</i>	<i>p</i>	<i>q</i>	<i>p</i>	<i>q</i>
Labrador (N = 14)			0.07 (variant 3)	0.07 (variant 4)	0.96	0.04	0.68	0.32	0.00	1.00	N/A	N/A
German Shepherd (N = 14)			0.65 (variant 5)	0.21 (variant 6)								
Beagle (N = 5)			1.00 (variant 6)		0.93	0.07	0.93	0.07	0.00	1.00	0.06	0.94
Boxer (N = 11)			(N = 10) <sup>§</sup>								(N = 8) <sup>§</sup>	
Poodle (N = 6)			0.20 (variant 2)	0.20 (variant 4)	1.00	0.00	0.80	0.20	0.57	0.43	0.80	0.20
Siberian Husky (N = 5)			0.60 (variant 7)									
Golden Retriever (N = 6)			1.00 (variant 7)		1.00	0.00	1.00	0.00	0.36	0.64	0.86	0.14
Interbreed (N = 61)			0.50 (variant 5)	0.50 (variant 6)	1.00	0.00	0.75	0.25	0.00	1.00	0.25	0.75
Amino acid change			0.80 (variant 6)	0.20 (variant 7)	1.00	0.00	0.30	0.70	0.20	0.80	0.90	0.10
			0.25 (variant 1)	0.50 (variant 8)	0.70	0.30	0.90	0.10	0.00	1.00	0.50	0.50
			0.25 (variant 9)		(N = 5) <sup>§</sup>		(N = 5) <sup>§</sup>		(N = 5) <sup>§</sup>		(N = 5) <sup>§</sup>	
			(N = 4) <sup>§</sup>									
			0.02 (variant 1)	0.02 (variant 2)	0.97	0.03	0.80	0.20	0.15	0.85	0.14	0.86
			0.02 (variant 3)	0.04 (variant 4)	(N = 60) <sup>§</sup>		(N = 60) <sup>§</sup>		(N = 60) <sup>§</sup>		(N = 40) <sup>§</sup>	
			0.22 (variant 5)	0.36 (variant 6)								
			0.26 (variant 7)	0.04 (variant 8)								
			0.02 (variant 9)									
			(N = 55) <sup>§</sup>									
			None 5'-UTR		None 5'-UTR		None 5'-UTR		Gly/Gly (Open reading frame)		None Intron 1	

\*GenBank reference sequence from a Boxer dog (<http://www.ncbi.nlm.nih.gov/genome/guide/dog/>). The translation initiation codon (A<sub>1</sub>TG) is located at positions: 33435163-33435165. \*\*Variant 1: 5'-C-3' (1 bp); variant 2: 5'-CAGGCCACG-3' (9 bp); variant 3: 5'-AGGCCACGCC-3' (10 bp); variant 4: 5'-CAGACCACGC-3' (10 bp); variant 5: 5'-CAGGCCACGCC-3' (11 bp); variant 6: 5'-CAGACCACGCC-3' (11 bp); variant 7: 5'-CTGGCCACGCC-3' (11 bp); variant 8: 5'-CAGGCCACGCCTAGCCACGCC-3' (21 bp); variant 9: 5'-CAGACCACGCCTAGCCACGCC-3' (21 bp). N/A = not analyzed. <sup>§</sup>In some cases, the number of samples analyzed was different from the totals shown in the first column. *p* and *q* = allele frequencies.

**Table 2.** DNA variants in canine *cbr1* (exons 2 and 3) - *Canis lupus familiaris* chromosome 31, whole genome shotgun sequence (NCBI reference sequence: NC\_006613.2)\*.

	Exon 2				Exon 3			
	33436150		33436191		33436196		33439456	
	<i>p</i>	<i>q</i>	<i>p</i>	<i>q</i>	<i>p</i>	<i>q</i>	<i>p</i>	<i>q</i>
Labrador (N = 14)	0.27	0.73	1.00	0.00	0.79	0.21	0.86	0.14
German Shepherd (N = 15)	0.83	0.17	0.53	0.47	0.00	1.00	1.00	0.00
Beagle (N = 13)	0.75	0.25	1.00	0.00	0.75	0.25	1.00	0.00
Boxer (N = 14)	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00
Poodle (N = 12)	0.43	0.57	1.00	0.00	1.00	0.00	1.00	0.00
Siberian Husky (N = 15)	0.28	0.72	1.00	0.00	0.40	0.60	0.93	0.07
Golden Retriever (N = 14)	0.06	0.94	0.96	0.04	0.06	0.94	1.00	0.00
Interbreed	0.56	0.44	0.91	0.09	0.58	0.42	0.97	0.03
Amino acid variation	(N = 77) <sup>§</sup>	(N = 82) <sup>§</sup>	None	None	(N = 71) <sup>§</sup>	(N = 96) <sup>§</sup>	Glutamic acid/glutamic acid Open reading frame	
	Intron 2	Intron 2	Intron 2	Intron 2	Intron 2	Intron 2		

\*GenBank reference sequence from a Boxer dog (<http://www.ncbi.nlm.nih.gov/genome/guide/dog/>). The translation initiation codon (A<sub>-1</sub>TG) is located at positions: 33435163-33435165. <sup>§</sup>In some cases, the number of samples analyzed was different from the totals shown in the first column. *p* and *q* = allele frequencies.

**Table 3.** Number of SpI motifs, 10-bp deletion status (yes/no), and corresponding DNA sequences for the “hot spot” region in the 5'-UTR of canine *cbr1*.

Canine breed	33435047-33435056 Reference sequence: AGGCCACGCC (10-bp deletion)	33435057-33435066 Reference sequence: CACGAGACCC (10 bp) “hot spot” region DNA sequence (length)	Condition (number of samples)	Number of SpI motifs
Boxer (GenBank NC_006613.2) Labrador (N = 14)	No	5-CACGAGACCC-3' (10 bp)	Not applicable	4
	Yes	5-AGGCCACGCC-3' (10 bp)	Homozygous (N = 1)	4
	No	5-CAGACCACGC-3' (10 bp)	Homozygous (N = 1)	4
		5-CAGGCCACGCC-3' (11 bp)	Homozygous (N = 8)	5
German Shepherd (N = 14)		5-CAGACCACGCC-3' (11 bp)	Homozygous (N = 2)	4
	No	5-CAG(G/A)CCACGCC-3' (11 bp)	Heterozygous (N = 2)	5 or 4
		5-CAGACCACGCC-3' (11 bp)	Homozygous (N = 10)	4
		Sequence not determined	Not determined (N = 4)	4±
Beagle (N = 5)	No	5-CAGGCCACGC-3' (9 bp)	Homozygous (N = 1)	4
		5-CAGACCACGC-3' (10 bp)	Homozygous (N = 1)	4
Boxer (N = 11)	Yes	5-CTGGCCACGCC-3' (11 bp)	Homozygous (N = 3)	4
	Yes	5-CTGGCCACGCC-3' (11 bp)	Homozygous (N = 11)	4
Poodle (N = 6)	No	5-CAGGCCACGCC-3' (11 bp)	Homozygous (N = 3)	5
		5-CAGACCACGCC-3' (11 bp)	Homozygous (N = 3)	4
Siberian Husky (N = 5)	No	5-CAGACCACGCC-3' (11 bp)	Homozygous (N = 4)	4
	Yes	5-CTGGCCACGCC-3' (11 bp)	Homozygous (N = 1)	4
Golden Retriever (N = 6)	No	5-C-3' (1 bp)	Homozygous (N = 1)	4
		5-CAGGCCACGCCTAGCCACGCC-3' (21 bp)	Homozygous (N = 1)	6
		5-CAG(G/A)CCACGCCTAGCCACGCC-3' (21 bp)	Heterozygous (N = 2)	6 or 5
	Sequence not determined	Not determined (N = 2)	4±	

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## APPENDIX

Appendix 1. Reagents and conditions for the amplification of canine *cbr1* (exons 1, 2, 3 and flanking sequences).

Exon	Forward primer	Reverse primer	Reaction mixture	Reaction conditions	Product length
1	5'-ACACCCGGAAAGCAAAACC-3'	5'-GGTTGGAAAGCACTGCAACCCGC-3'	Reaction volume: 50 µL per sample 50 ng dog genomic DNA (template) 200 nM forward and reverse primers (Invitrogen, Carlsbad, CA, USA) 200 µM dNTPs* 2 mM MgCl <sub>2</sub> * 0.5 M GC-rich resolution solution* 1X GC-rich PCR buffer with DMSO* 0.04 U/µL GC-rich PCR enzyme Mix*	Activation: 95°C, 3 min Cycling (N = 35) Denaturation: 95°C, 30 s Annealing: 54°C, 30 s Extension: 72°C, 45 s Final extension: 72°C, 7 min	842 bp
2	5'-CGCCAACCTGCCGTCTGT-3'	5'-GTGGGGCAGAGCAACAGAGTTC-3'	Reaction volume: 50 µL per sample 50 ng dog genomic DNA (template)	Activation: 94°C, 5 min Cycling (N = 35)	450 bp
3 (Set 1)	5'-GTAGACATCAGATACC-3'	5'-TCAGCATCTGAGGGCAAC-3'	200 nM forward and reverse primers (Invitrogen) 200 µM dNTPs‡ 2 mM MgCl <sub>2</sub> §	Denaturing: 94°C, 30 s Annealing: 68.9°C, 45 s Extension: 72°C, 1 min	586 bp
3 (Set 2)	5'-GACACAAAAGAGCGTGAC-3'	5'-AGGTGTAGCAGATTTCTC-3'	1X PCR buffer‡ 0.025 U/mL HotStart-IT Taq Polymerase‡	Final extension: 72°C, 3 min	639 bp

\*GC-rich PCR System and dNTPack supplied by Roche Applied Science (Indianapolis, IN, USA). ‡Reagents from Affymetrix Inc. (Santa Clara, CA, USA).