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CASE REPORT

Genetic cause for congenital methemoglobinemia in an Australian Pomeranian dog

Shijia Zhou | Rick Tearle | Raziallah Jafari Jozani | Bethany Winra | Olaf Schaaf | Anthony Nicholson | Anne Peaston

School of Animal and Veterinary Sciences, The University of Adelaide, Roseworthy, South Australia, Australia

Correspondence
Anne Peaston, Companion Animal Health Centre, Building E40 Roseworthy Campus, University of Adelaide, Mudla Wirra Road, Roseworthy SA 5371, Australia.
Email: anne.peaston@adelaide.edu.au

Present address
Raziallah Jafari Jozani, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran.

Little is known about genetic causes of congenital methemoglobinemia in dogs. Here, we report a CYB_5R_3 mutation in a Pomeranian dog with congenital methemoglobinemia. A 6-year-old neutered female Pomeranian dog was investigated for cyanosis noticed during anesthesia for an orthopedic procedure. The history included lifelong mild exercise intolerance and bluish tongue. Methemoglobinemia was diagnosed using co-oximetry. The CYB_5R_3 gene was analyzed by comparing the patient's genomic DNA with the reference canine sequence. Mutation functional significance was investigated using snpEff and multispecies protein homology analyses. A homozygous missense single nucleotide CYB_5R_3 mutation (ATC → CTC at codon 194) caused a p.Ile194Leu substitution. The pIle194 residue is highly conserved in other mammals, supporting the likely pathogenicity of the substitution. The mutation described here is identical to that associated with familial methemoglobinemia in a family of Japanese Pomeranian dogs. This observation, together with the homozygous mutation found in our case, indicates that the mutant allele may be widespread within the Pomeranian breed internationally.

KEYWORDS

cyanosis, dog, methemoglobin, cytochrome b5 reductase, mutation

1 | CASE DESCRIPTION

Congenital methemoglobinemia (CM) is a rare disease in animals.[1] In humans, it is most commonly associated with a nicotinamide adenine dinucleotide (NADH)-cytochrome b5 reductase (CYB_5R_3) deficiency, but few veterinary studies have explored an underlying genetic cause.[2]

Three types of CM have been described. Type I CM is caused by CYB_5R_3 enzyme activity deficiency in erythrocytes. Type II CM is caused by CYB_5R_3 enzyme activity deficiency in all tissues, resulting from several different genetic changes that either affect the catalytic domains of the enzyme or truncate the enzyme. Type IV CM is rarely reported and is caused by a deficiency in cytochrome b5 (CYB_5).[3] Abnormal hemoglobins (Hbs) such as Hb-M and Hb-Hana also have been reported to cause CM.[4–7]

Before our investigation, another report documented type IV CM in a Pit Bull mixed breed dog, in which a 37 bp deletion in the promoter region and a missense mutation in 1 of the exons of the CYB_5 gene were suspected to be the genetic causes.[8] Another case of familial type II CM in Pomeranian dogs was reported in which the genetic abnormalities included a single nucleotide substitution in the promoter region and a missense mutation in the coding sequence of the CYB_5R_3 gene.[9]

In this report, we document the diagnostic investigation and genetic analysis of a Pomeranian dog referred to the Companion Animal Health Centre (CAHC), University of Adelaide, for marked cyanosis under general anesthesia and subsequently diagnosed with CM.

Abbreviations: CM, congenital methemoglobinemia; CYB_5, cytochrome b_5; CYB_5R_3, cytochrome b_5 reductase; Hb, hemoglobin; MetHb, methemoglobin; NADH, nicotinamide adenine dinucleotide; SNP, single nucleotide polymorphism.

2 | CASE SUMMARY

A 6-year-old spayed female Pomeranian dog was anesthetized for surgery to treat right medial patella luxation and suspected cranial cruciate ligament rupture. During anesthesia, marked cyanosis of the tongue

and sublingual vessels was noted; the surgery was abandoned; and after recovery from anesthesia, the dog was referred to the CAHC.

The owner reported that the dog had a long history of exercise intolerance and its tongue had always been blue. On presentation at the CAHC, the dog's vital signs were within reference ranges, but its tongue appeared dark blue, and SpO_2 was 97% when the dog was breathing room air. Orthopedic examination confirmed cranial cruciate ligament rupture and medial patella luxation. The dog was admitted to hospital with surgery tentatively planned for the next day. However, observation of marked darkening of the cyanotic tongue and gingiva when the dog was walked or handled, together with the history and physical examination findings, prompted further investigation.

Cyanosis can be classified clinically as either central or peripheral. Central cyanosis is a result of global deoxygenation of arterial blood as a consequence of cardiopulmonary diseases or decreased oxygen carrying capacity of circulating blood as is the case with methemoglobinemia. Peripheral cyanosis is caused by local reduction of oxygenated Hb. Central cyanosis is usually first observed in oral mucous membranes, especially the tongue, and peripheral cyanosis usually is limited to the affected region.[10] In our case, marked cyanosis of sublingual vessels was indicative of central cyanosis. However, no functional or structural cardiac abnormalities were detected during clinical examination or on echocardiography, and abdominal ultrasonography was unremarkable.[11] Airway and pulmonary diseases were unlikely given the dog's history, physical examination results, and unremarkable thoracic radiographs.[12] No abnormalities were observed on a serum biochemistry profile. Hematology abnormalities included moderate absolute erythrocytosis (red blood cells [RBC], 10.8×10^{12}/L; reference range, $4.9\text{-}8.2 \times 10^{12}$/L), marginal thrombocytopenia (platelet count, 183×10^9/L; reference range, $200\text{-}500 \times 10^9$/L), mild to moderate neutropenia (neutrophils, 1.5×10^9/L; reference range, $3.5\text{-}12 \times 10^9$/L), and lymphopenia (lymphocytes, 0.7×10^9/L; reference range, $0.9\text{-}3.5 \times 10^9$/L; Veterinary Diagnostic Laboratory, School of Animal and Veterinary Sciences, University of Adelaide, Roseworthy, South Australia). Absolute erythrocytosis in the presence of cyanosis suggested that the erythrocytosis was a physiologic response to tissue hypoxemia, most likely as a result of methemoglobinemia.[13]

Initial pulse oximetry and blood gas analysis using the epoc Blood Analysis System (Siemens Healthcare GMBH, Germany) indicated adequate oxygenation. However, because neither modality can detect methemoglobin (MetHb), venous and arterial blood were collected for MetHb assay using an ABL90 Flex blood gas machine (Radiometer) for co-oximetry. Published reference intervals for MetHb are ≤1.2%.[8] The dog's percentage MetHb was markedly increased, 26.3% in arterial blood and 26.0% in venous blood.

Repeat hematology analysis indicated persistent moderate erythrocytosis (RBC, 9.81×10^2/L; reference range, $5.4\text{-}8.5 \times 10^2$/L), adequate platelets (platelet count, 229×10^9/L, reference range, $200\text{-}520 \times 10^9$/L), persistent mild neutropenia (neutrophils, 2.1×10^9/L; reference range, $3.0\text{-}11.5 \times 10^9$/L), and lymphopenia (lymphocytes, 0.8×10^9/L; reference range, $1.0\text{-}4.8 \times 10^9$/L). In light of the history and clinical findings, we concluded that persistent modest neutropenia was most likely to be normal for this dog. Persistent slight lymphopenia was assessed as not being clinically relevant but may have reflected stress.[13]

3 | MATERIALS AND METHODS

To investigate the suspected underlying genetic cause of methemoglobinemia in this Pomeranian dog, DNA extracted from a whole-blood sample (QIAamp DNA Blood Mini Kit; QIAGEN Pty Ltd, Chadstone, Australia) was submitted to the Australian Genome Research Facility for Whole Genome Sequencing using the Illumina platform. A total of 426 853 809 paired end reads of 150 nt were generated. Reads were aligned to the reference genome *Canis familiaris* 3.1 using bwa.[14] Variants were called using GATK 4.0.[15] Protein altering variants of the CYB_5R_3 gene were identified, and the likely pathogenicity of each was assessed using snpEff and Release 105.[16]

4 | RESULTS

In CYB_5R_3 genomic DNA, 1 potentially pathogenic variant was identified at chr10: 22836951 (Figure 1). This position is covered by 45 reads, and although the reference genome reports an A, all 45 reads carried a C. This finding is a strong evidence that a homozygous variant occurred at this position, changing the codon from ATC→CTC, and consequently changing the amino acid encoded by this codon from isoleucine to leucine, p.Ile194Leu.

The NCBI *Canis lupus familiaris* Annotation Release 105 (Release 105), released to the public in September 2017, states that the CYB_5R_3 gene consists of 8 exons (https://www.ncbi.nlm.nih.gov/gene/474479, accessed December 18, 2018). According to Release 105, the mutation at chr10: 22836951 falls within exon 6 of the gene. However, in a similar study, using a different genome annotation scheme, the same mutation was assigned to exon 7.[9] Furthermore, the Ensembl Release 94, released to the public in October 2018, annotates 11 exons for the gene producing 2 splice variant transcripts differing in use of the first 3 exons, but having exons 4-11 in common, and placing the mutation in exon 9 (https://asia.ensembl.org/Canis_familiaris/Gene/Splice?db=core;g=ENSCAFG00000000953;r=10:22823473-22849757, accessed December 18, 2018).

To clarify the exact location of the mutation described both here and in another report,[9] we aligned our data with the stable, annotated transcript NCBI Reference Sequence for CYB5R3, NM_001048084.1.[17] The mutation site corresponds to the same c.580A→C in NM_001048084.1 as previously described.[9]

Our analysis also showed that the dog's genotype was heterozygous T/G at position 22 819 977 bp in the putative promoter region upstream of the CYB_5R_3 gene (Figure 2), whereas a previous report observed this site was homozygous for the G allele.[9] We found multiple other single nucleotide polymorphisms (SNPs) in the canine CYB_5R_3 genome sequence, but based on snpEff analysis, these were deemed unlikely to be of functional significance.

To investigate whether isoleucine is conserved at the same site in other species, we used Gapped BLAST to compare the sequence of CYB_5R_3 from 6 different mammalian species.[18] We found that pIle194 and the flanking sequence (particularly the upstream sequence) is highly conserved in 5 species, with 1 species encoding a conservative variant, valine, at position 194 (Table 1).

FIGURE 1 58-base sequence of CYB_5R_3 showing 1 missense variant in CYB_5R_3 gene. Each pink line represents an individual sequencing read, and characters written on each read (G, C, A, T) indicate a mismatched base in the read. At position 22 836 951, all 45 reads report an A→C variant. Figure generated from interactive genomic viewer

5 | DISCUSSION

Here we report a case of CM in a mature Pomeranian dog, discovered incidentally during investigation of abnormal mucous membrane color before surgical treatment for lameness. Systemic administration of methylene blue is appropriate for temporary relief of the clinical signs in situations such as advanced methemoglobinemia or when an animal undergoes surgery.[8] In our case, the canine lameness improved with medical management and surgery was not performed. Combined with the history of the canine ability to cope with its CM since birth, specific treatment for CM was deemed unnecessary. Genetic investigation identified a homozygous missense mutation in chr10: 22836951, within an exon common to the 2 splice variant CYB_5R_3 gene transcripts, resulting in p.Ile194Leu substitution, a strong candidate for causing CM in this dog.

In humans, several distinctly different membrane-associated or soluble forms of the CYB_5R_3 enzyme are encoded by the CYB_5R_3 gene, generated from splice variant transcripts that differ only in the combination of the first exons that are used.[19] Genome and protein annotations from NCBI and Ensembl support the existence in dogs of at least 2 enzyme isoforms generated in the same way. The missense variant at chr10: 22836951 (cDNA580A→C in NM_001048084.1) would be predicted to impact both forms of CYB_5R_3 enzymes, potentially producing a severe clinical phenotype.[19] In dogs and humans, the membrane-bound form of the CYB_5R_3 enzyme (Membrane-CYB_5R_3) is present in many tissue types and is involved in lipid metabolism among other functions.[19,20] As in humans, the soluble form of the CYB_5R_3 enzyme (Soluble-CYB_5R_3) is thought to be restricted to erythrocytes in dogs and is responsible for reducing MetHb.[21] This observation presents a puzzle because the clinical phenotype of our dog was not typical of Type II CM in humans who have severe clinical signs involving cyanosis, neurological dysfunction, and early death associated with deficiency of the membrane CYB5R3 isozyme. Instead, the phenotype of our dog was more typical of Type I CM associated with erythrocyte CYB_5R_3 enzyme deficiency. In another study,[9] it was speculated that the mutation may contribute to decreased protein half-life, decreased catalytic efficiency, or both. Therefore, a possible explanation for the mild phenotype could be related to different protein synthesis capacities of erythrocytes compared with other tissue cells. Mature erythrocytes rapidly lose the ability to synthesize proteins after loss of the nucleus

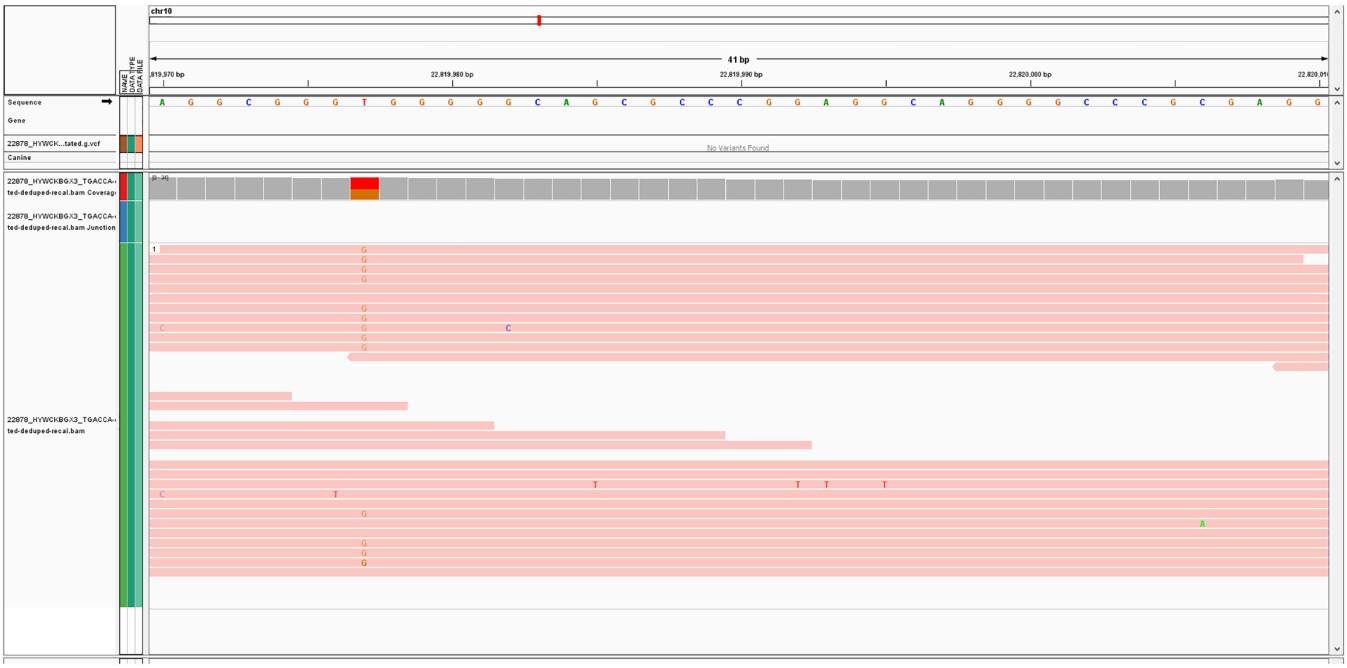

FIGURE 2 41-base sequence of the CYB_5R_3 gene promoter region showing heterozygous T and G single nucleotide polymorphism. Each pink line represents an individual sequencing read, and 13 out of 27 reads for this region reported G while the remainder reported reference genotype T and are therefore not illustrated. Figure generated from interactive genomic viewer

and translation of stored transcripts, and do not have the ability to increase protein production to compensate for decreased protein function or half-life.[22] In other tissues, cells can compensate by increasing Membrane-CYB_5R_3 expression.

A case report of CM in a Pomeranian puppy in the United States in 1999 noted that the dog showed cyanosis without any detectable cardiopulmonary abnormalities. Venous MetHb was increased and MetHb-reductase activity was decreased, confirming a diagnosis of CM, but genetic analysis was not performed.[1] During the course of the current clinical investigation, a Japanese research team also reported the p.Ile194Leu missense variant in several related Pomeranians with clinical phenotype Type I CM.[9] Their study confirmed decreased CYB_5R_3 enzyme activity in affected dogs, although computer-simulated analyses suggested that the p.Ile194Leu conservative substitution would have little effect on protein structure or function. The same group reported an additional SNP in the promoter region, c.1-235 T/G, with tested animals homozygous for the G allele.[9] In our dog, the genotype at this position in the promoter is heterozygous T/G (Figure 1), indicating that the promoter SNP is unlikely to be involved in the disease phenotype.

Isoleucine and leucine are branched chain amino acids with very similar structures differing only in the position of a methyl group in the side chain.[23] Their substitution for each other typically is viewed

TABLE 1 Multispecies amino acid comparison of the region flanking the pIle194Leu mutation in the dog in the current study[18]

Species:	Amino acid sequence surrounding the SNP:																		
Dog in the current study	P	M	L	Q	V	I	R	A	***L***	I	K	D	P	H	D	P	T	V	C	H
Canis lupus	P	M	L	Q	V	I	R	A	I	I	K	D	P	H	D	P	T	V	C	H
Homo sapiens	P	M	L	Q	V	I	R	A	I	M	K	D	P	D	D	H	T	V	C	H
Bos Taurus	P	M	L	Q	V	I	R	A	I	M	K	D	P	D	D	H	T	V	C	H
Ovis aries	P	M	L	Q	V	I	R	A	I	M	K	D	P	D	D	H	T	V	C	H
Felis catus	P	M	L	Q	V	I	R	A	I	M	K	D	P	D	D	H	T	V	C	Y
Mus musculus	P	M	L	Q	V	I	R	A	V	L	K	D	P	N	D	H	T	V	C	Y

pIle194 is shaded light brown; leucine and valine substitutions are shaded yellow.

as functionally conservative, although instances of marked functional alteration of proteins hosting this mutation have been reported.[23–25] When we compared the sequence of CYB_5R_3 from 6 different mammalian species, we found the amino acid isoleucine is conserved at p194 in 5 species, with 1 species having a different conservative substitution with valine (Table 1). Together, our data and that of published reports provide further support for the likely functional significance of the p.Ile194Leu missense mutation.

Our study has several limitations the most important of which is that it remains possible that the mutation reported here and in another report[9] has no pathological impact, and that other genomic alterations might affect, for example, expression or degradation of soluble-CYB_5R_3.[26] As previously reported,[9] we agree that to confirm the pathological significance of the p.Ile194Leu missense variant, further investigations should be considered, including expression analysis in erythrocytes and other tissues. In our case, it would be useful to directly measure this canine CYB_5R_3 enzymatic activity, in comparison with that of healthy dogs.[27] To probe enzyme properties, such as stability against heat and protease and affinity for NADH, in vitro protein expression systems could be devised to obtain purified reference and p.Ile194Leu mutated soluble and membrane CYB_5R_3 enzymes. We did not assess the clinical history or laboratory abnormalities of Pomeranian dogs related to this dog as this information was unknown. However, given that the canine genotype is homozygous, it is reasonable to suspect that close or more distant family members related to both dam and sire must be carrying the pIle194Leu mutation. Because the identical mutation was found in a family of Japanese Pomeranian dogs, it is possible that, barring the highly unlikely event that identical de novo mutations have arisen independently in different lines of Pomeranian dogs, the mutation may be present within the breed internationally. Genetic testing may help to detect carriers of the mutation so as to estimate its prevalence and enable it to be eliminated from the breed.

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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 work was conducted with the full agreement of and encouragement from the dog's owner.

HUMAN ETHICS APPROVAL DECLARATION

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

ORCID

Anne Peaston https://orcid.org/0000-0002-7501-2415

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