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SHORT COMMUNICATION Clinical Case Report

Case Report: Congenital methemoglobinemia in a cat with the reduced NADH-cytochrome b5 reductase 3 activity and missense mutations in *CYB5R3*

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Abstract

A one-year-old castrated male mixed-breed cat was referred for detailed examination of long-term pale mucous membrane without any clinical episodes that may cause cyanosis. While no causative abnormalities were detected in thoracic radiography and echocardiography, and arterial partial pressure of oxygen was within normal value, methemoglobin concentration of the cat was increased to 30% and cytochrome b5 reductase activity, which converts methemoglobin to hemoglobin, was reduced to 2.0 IU/gHb (13.4 ± 1.7 IU/gHb in control cats, n = 3), indicating congenital methemoglobinemia. Sequence analysis of *CYB5R3*, which codes cytochrome b5 reductase, showed two missense mutations found in the patient, one of which was predicted to affect protein function.

Key Words: Cyanosis; Cytochrome b5 reductase; Methemoglobinemia

Methemoglobin results from oxidation of the iron moiety in hemoglobin from the Fe^{2+} to the Fe^{3+} state. Red blood cells are continuously exposed to oxygen-free radicals with carrying oxygen in a high concentration, resulting in persistent formation of methemoglobin which is not capable of reversibly binding oxygen and is impaired in oxygen delivery. Endogenous enzymatic reactions catalyzed primarily by NADH-cytochrome b5 reductase 3 (cytochrome b5 reductase), and negligibly by NADPH methemoglobin reductase reduce methemoglobin back to hemoglobin to maintain methemoglobin concentration at less than 1% of total hemoglobin[2,7,17)].

Methemoglobinemia is characterized by high concentration of methemoglobin in blood, and is classified into congenital or acquired, depending on the cause of increased methemoglobin. While acquired methemoglobinemia results from increased oxidation of hemoglobin overwhelming the protective mechanisms in red blood cells by toxins and drugs such as lidocaine or procaine,

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congenital methemoglobinemia is caused by decreased enzymatic reduction of methemoglobin mostly due to genetic abnormalities in genes involved in the reaction[7]. In humans, over 40 different mutations of *CYB5R3* which codes cytochrome b5 reductase have been described to damage enzymatic activity in patients with congenital methemoglobinemia[17]. Although many cases of persistent methemoglobinemia associated with red cell cytochrome b5 reductase deficiency has been recognized in dogs and cats, no causative mutations of *CYB5R3* have been identified in these animal species[9,11,15].

Here we report a cat with congenital methemoglobinemia, where genetic analysis for *CYB5R3* was performed to identify possibly responsible mutations that lead to decreased cytochrome b5 reductase activity.

A one-year-old castrated male mixed-breed cat, weighing 5.1 kg, was referred to Hokkaido University Veterinary Teaching Hospital for detailed examination of long-term pale mucous membrane without any clinical episodes that may cause cyanosis such as coughing or respiratory distress. At presentation, the cat was bright, alert, and responsive. On physical examination, the oral and the nasal plane of the cat were distinctly cyanotic. No cardiac or pulmonary abnormalities were detected on auscultation. Although mildly enlarged right heart and mild enlargement of the right atrium and trivial tricuspid regurgitation were pointed out on thoracic radiographs and echocardiograms, these were not regarded as the cause of the cyanosis. Moreover, no clinical signs of poisoning were observed.

Venous blood sample showed brown color on white filter paper after exposure to room air, indicating the presence of methemomglobinemia[11] in which methemoglobin concentration was determined to be 30% by the previous method[18] (1% in control mixed-bread cats, n = 2). The cat was not anemic, exhibiting hematocrit, red blood cell count, and hemoglobin values of 43%, 9.2×10^6/μl, and 14.4 g/dl, respectively, and arterial partial pressure of oxygen was normal (89 mmHg).

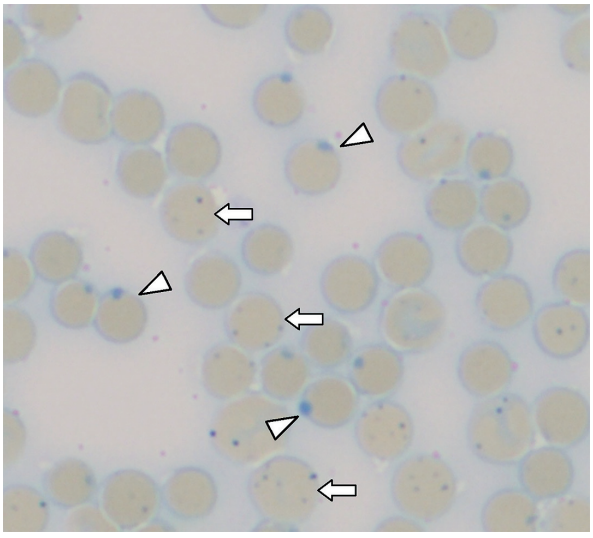

Fig. 1. Increased Heinz body formation in the patient's red blood cells. Red blood cells from the patient cat were attained with new methylene blue. Several typical red blood cells containing a single large Heinz body (arrowheads) or multiple small Heinz bodies (arrows) are indicated. ×100 objective.

In addition, in the patient cat, approximately 30% of red blood cells contained Heinz bodies (Fig. 1), while Heinz body-containing cells were up to 5% in normal cats[5]. Heinz body formation occurs through an abundant formation and accumulation of methemoglobin and subsequent conversion of methemoglobin to hemichrome with resultant oxidative denaturation and precipitation of hemoglobin[19,20]. In general, Heinz body formation is not critical in methemoglobinemia in humans and dogs. However, the presence of Heinz bodies in the patient cat is not surprising, because cats are well recognized as the species whose hemoglobin is most susceptible to oxidation and denaturation and large numbers of circulating Heinz bodies may develop without concurrent anemia[5,6,12]. The molecular basis for the ease of Heinz body formation in cats has been attributed to the presence of 8 freely reactive sulfhydryl groups in the feline hemoglobin (only two are reactive in the human hemoglobin)[14], the ease of dissociation of feline hemoglobin from tetramers to dimers[10], and the unique nonsinusoidal vascular structure of the feline spleen that allows

unimpeded passage of red cells containing Heinz bodies[3].

Since the current case showed methemoglobinemia with no clinical signs of toxicosis and inability to identify a source of oxidant that could cause methemoglobinemia, a congenital disorder was suggested. We therefore analyzed cytochrome b5 reductase activity of red blood cells of the patient by using spectrophotometric assays[4]. The cytochrome b5 reductase activity in the patient cat was 2.0 IU/gHb and was remarkably lower than the activity in control mixed-breed cats (13.4 ± 1.7 IU/gHb, mean $\pm$ S.D., $n = 3$), demonstrating a significant methemoglobinemia associated with cytochrome b5 reductase deficiency. Congenital methemoglobinemia in cats is known to be extremely rare with the fact that there have been only five cases reported[11]. It has also been reported that affected animals with congenital methemoglobinemia have cyanotic mucous membranes with exercise intolerant or lethargic at times, but they frequently have no clinical signs of the disease[11] as observed in our case.

To study the molecular basis of cytochrome b5 deficiency in the cat, sequence analysis to confirm genetic abnormalities of *CYB5R3* was performed comparing with human soluble cytochrome b5 reductase[21]. Total DNA was extracted from white blood cells separated from venous blood samples of the patient cat and a healthy mixed-breed cat with QIAamp DNA Mini Kit (QIAGEN, CA, USA). As no reference sequences existed for cat cytochrome b5 reductase mRNA in GenBank, comparative genomic technique that utilized human *CYB5R3* (NM_001171661) was applied to predict the sequence of the homologous gene in the feline genome (ICGSC Felis_catus_8.0). The fact that the amino acid sequence of feline cytochrome b5 reductase predicted from the open reading frame of feline *CYB5R3*, found in the chromosome B4 was shown to have 92.3% sequence homology with human indicates structural conservation of the enzyme (Fig. 2). The gene-specific primers were designed from the sequence of intron in predicted cat's *CYB5R3* gene to perform a series of PCR experiments to determine the full length DNA sequence of all 8 exons (Table 1). The PCR products of each exon (3 clones for each) were sequenced by Sanger sequence method using a 3500 genetic analyzer (Applied Biosystems, CA, USA). Three independent clones for each PCR product were totally coincided in their nucleotide sequences in the patient and the control cat and the nucleotide sequence obtained for the control cat was identical to that appeared in the feline genomic sequence (ICGSC Felis_catus_8.0). Sequence alignment analysis between the patient and normal cat revealed the presence of nucleotide changes at two positions leading to missense mutations in exon 2 (F36L) and exon 6 (Y179H) in the patient (The sequence of normal cat has been deposited in the DDBJ with accession number LC259008). In the present study, all three clones for the PCR products of exon 2 and exon 6 from the patient cat contained F36L and Y179H mutations, respectively, suggesting that the cat was homozygous for these mutations. This is compatible with that cytochrome b5 reductase activity in the cat was only 15% of that in the control cat, and is of suggestive that the mutant allele could be propagated to a certain extent. However, we could not analyze genetic background of the mutations because the current case was a stray and mixed-breed cat.

Finally, to address functional relevance of the two mutations found in this cat, we projected prediction models as to whether the amino acid substitution might affect protein function based on the structure and function of a protein with PolyPhen or the degree of conservation of amino acid residues of a protein with SIFT. PolyPhen scores of F36L and Y179H were 0.997 and 0.000, respectively, where scores ranging 0.85 to 1.0 are predicted to be damaging[1]. SIFT scores were 0.02 for F36L and 0.53 for Y179H, respectively, where scores ranging 0.0 to 0.1 are predicted to be deleterious[16]. From these results, F36L mutation could be considered to be involved in the reduced

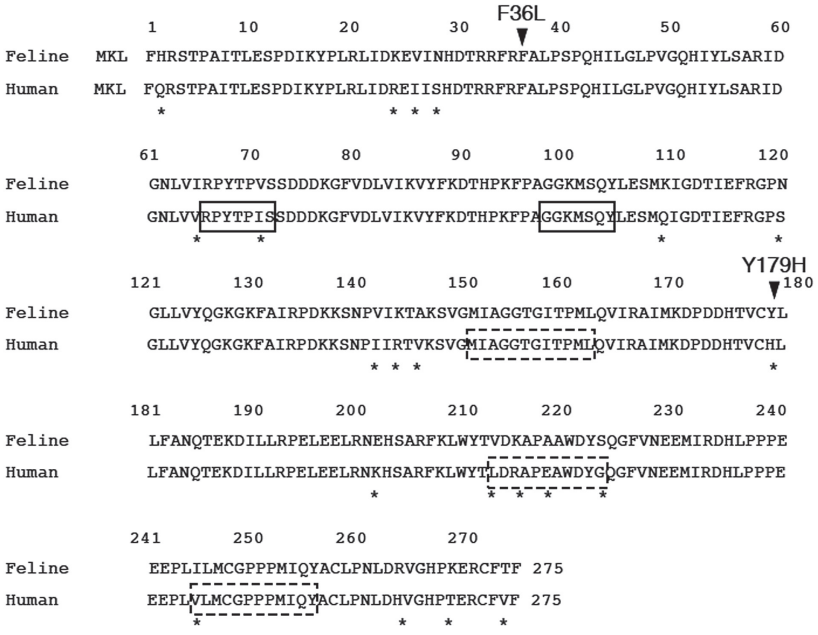

Fig. 2. The predicted amino acid sequence of feline cytochrome b5 reductase and the mutations found in the patient cat. The amino acid sequence of feline cytochrome b5 reductase predicted from the putative feline *CYB5R3* gene is shown in comparison with that of human cytochrome b5 reductase in red blood cells, and the amino acid residues (one letter indication) are numbered for mature proteins (for details, see reference 15 and GenBank accession number NM_001171661). Amino acid residues which are different between feline and human and the substitutions of amino acid residues found in the patient cat (F36L and Y179H) are indicated by asterisks and arrowheads, respectively. The sequences composing FAD-binding domain and NADH-binding domain in human cytochrome b5 reductase are indicated by solid and dotted rectangles[17].

Table 1. PCR primers used in this study

Exon		Sequence	Location (felCat8)	Annealing temperature (amplified fragment size)
1	Sense	5'-CGTGGGTCTGTCACTGTCAT-3'	chrB4:135900726-135900707	60°C (240 bp)
	Reverse	5'-GCCAACAAACACTGTGATGC-3'	chrB4:135900506-135900487	
2	Sense	5'-TCAGCATCGTTTCTCACACTG-3'	chrB4:135897332-135897312	60°C (246 bp)
	Reverse	5'-CTCCCTAGTTCAAGGGCAAA-3'	chrB4:135897106-135897087	
3	Sense	5'-CTCCAGAACTCCCGGAATC-3'	chrB4:135896819-135896801	60°C (239 bp)
	Reverse	5'-TGCCAATCATTCAGAAACCA-3'	chrB4:135896600-135896581	
4	Sense	5'-ACTGTGGGAATGTCTTGCAG-3'	chrB4:135894660-135894641	59°C (240 bp)
	Reverse	5'-AGAGTCACCCACCCAGACAG-3'	chrB4:135894440-135894421	
5	Sense	5'-CCCCGTCTCCTTCTACCTTC-3'	chrB4:135894049-135894030	60°C (175 bp)
	Reverse	5'-ACCCTGACCCGTGACATCT-3'	chrB4:135893894-135893875	
6	Sense	5'-GTGAGCTCCCCAAGGTCAG-3'	chrB4:135893852-135893834	60°C (250 bp)
	Reverse	5'-TGTGTCGGGAACACTCACTG-3'	chrB4:135893622-135893603	
7	Sense	5'-TGGGAGTTTCTGGGAGTCTG-3'	chrB4:135889027-135889008	60°C (242 bp)
	Reverse	5'-GCTTCCTCCCCTCACCTTC-3'	chrB4:135888804-135888786	
8	Sense	5'-CTGGGATTCACCTTGTCTCC-3'	chrB4:135881430-135881411	60°C (300 bp)
	Reverse	5'-GCGGGAAATGACGTTAAAGA-3'	chrB4:135881150-135881131	

The nucleotide sequence and its origin in the feline genome, annealing temperature, and amplified fragment size of the PCR primers for sequencing analysis are shown. Locations of the nucleotide sequences are derived from the putative *CYB5R3* gene in feline genome (Felis_catus_8.0/felCat8) as described in the text.

activity of the enzyme in the patient. Genetic mutations of *CYB5R3* has been known to lead to two different clinical phenotypes in human patients[17]. Cyanosis is the only clinical symptom in type I methemoglobinemia. On the other hand, additional severe mental retardation and neurologic impairment are recognized in type II methemoglobinemia. It has been reported that whereas genetic mutations identified in type I methemoglobinemia were found to have amino acid substitutions throughout the structure of the protein and not directly involved in FAD or NADH binding, type II methemoglobinemia mutations were found to lead to a truncated protein or amino acid substitutions directly involved in FAD or NADH binding[8]. Given the fact that the amino acid sequences for cytochrome b5 reductase between human and cat were highly conserved by the alignment analysis, we could speculate that both of the two amino acid substitutions identified in the cat may not be directly involved in FAD or NADH binding (Fig. 2). In fact, it has been reported that R32Q mutation close to F36L could lead to Type I methemoglobinemia in human[13]. Collectively, type I methemoglobinemia could occur also in cats by the similar mechanism in human patients.

In conclusion, we found genetic mutations that lead to amino acid substitutions in a cat with possible type I methemoglobinemia. Functionally decreased enzyme activity for the cytochrome b5 reductase protein with the mutations found in the cat should be confirmed to determine the causative mutations in the *CYB5R3* gene in congenital methemoglobinemia in cats.

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