Study of DNA Polymorphisms of the *CMAH* gene in Dogs and Cats

Summary of Doctoral Thesis

Yumiko Uno

Graduate School of Veterinary Medical and Life Science

Nippon Veterinary and Life Science University

1. Introduction

N-glycolylneuraminic acid (Neu5Gc) and N-acetylneuraminic acid (Neu5Ac) are sialic acids that are commonly found in mammalian cells. Neu5Gc is synthesized from its precursor, Neu5Ac, by cytidine-5′-monophospho-N acetylneuraminic acid hydroxylase (CMAH), which is encoded by the *CMAH* gene. Most mammals have both Neu5Gc and Neu5Ac, but humans and ferrets have only Neu5Ac due to loss-of-function mutations. In cats, the Neu5Gc and Neu5Ac on erythrocyte membranes are the blood type A and type B antigens, respectively, and each type results from mutations in the *CMAH* gene that affect the production of Neu5Gc and Neu5Ac. The AB blood group system (A, B, and AB blood types) of cats is of major importance in feline transfusion medicine. A DNA screening scheme involving *CMAH* variants that can accurately determine blood types within the feline AB blood group system would be of great use and is highly desirable to complement phenotypic tests. Even though the *CMAH* gene has been well characterized in cats, not much is known about the *CMAH* gene in dogs. In general, all European dogs have Neu5Ac, but not Neu5Gc, while Asian dogs have either of these two sialic acids. Furthermore, it was recently reported that canine and feline parvoviruses preferentially recognize Neu5Gc. Therefore, in this study, we characterized for the first time the dog *CMAH* gene. Furthermore, we also studied the association between blood types and the cat *CMAH* gene, and compared the results to those of the dog *CMAH* gene.

2. cDNA cloning and expression of the dog *CMAH* gene

A 1737-bp open reading frame (ORF) of the dog *CMAH* gene was amplified by reverse transcription polymerase chain reaction (RT-PCR) using cDNA prepared from dog bone marrow. The cDNA was predicted to encode a protein of 578 amino acid residues. The

ORF of *CMAH* was composed of 14 exons by comparison with the dog genome sequences (NC_006617.3). The amino acid sequence was found to be highly similar to the corresponding sequences in cat (93%), pig, chimpanzee, mouse, and rat. To determine the expression of the dog *CMAH* gene in different tissues, we also performed RT-PCR using total RNA extracted from 28 tissues. The expression of the dog *CMAH* gene was found in many tissues, but not all tissues; the cat *CMAH* gene has been reported to be expressed in almost all tissues. Therefore, similar to the *CMAH* gene in other animals, the dog *CMAH* gene was considered to be well-conserved, and to have a similar function as the cat *CMAH* gene. However, different mechanisms might exist between dogs and cats for the expression of this gene.

3. Discovery of DNA polymorphisms in the dog *CMAH gene*

Fourteen exons of *CMAH* containing coding regions were amplified by PCR from genomic DNA samples prepared from 11 dogs of 11 breeds, and their sequences were determined. DNA polymorphisms were identified by comparing each sequence with the reference sequence (NC_006617.3). We identified 15 SNPs (4 exonic and 11 intronic) and an indel in the 11 dogs. Three of the four exonic SNPs were synonymous: c.15 T>C (p.lle5lle) in exon 2, and c.1701G>A (p.Pro567Pro) and c.1713G>A (p.Arg571Arg) in exon 14. However, c.554A>G (p.Lys185Arg) in exon 5 was a non-synonymous SNP, where only 1 Shiba dog had the A allele while the other breeds had the G allele. Since most European dogs express Neu5Ac, but not Neu5Gc, we assumed that the G allele at c.554 A>G was associated with Neu5Ac.

4. Distribution of the c.554 A>G SNP of *CMAH* in seven dog breeds and correlation of the *CMAH* gene at the c.554A>G locus with Neu5Ac expression

To characterize the missense SNP (c.554A>G SNP), we investigated its distribution by analyzing the sequences of the *CMAH* exons in seven different breeds using 229 genomic DNA samples. We found that the G allele was widely distributed among six of the seven tested breeds. However, the genotyping results for the Shiba dogs were different from those of the six other breeds at position c.554, making the Shiba dog the most polymorphic breed at c.554 A>G.

To investigate c.554A>G in dogs with the presence or absence of Neu5Ac expression, we examined 56 Shiba dogs and 29 Labrador Retrievers for the binding of lectin to Neu5Ac. Thereafter, we genotyped the dogs at c.554A>G. Results showed that the Shiba dogs could be divided into two groups of phenotypes, i.e., positive (44/56) or negative (12/56) for Neu5Ac. In contrast, all of the Labrador Retriever dogs were positive for the binding of lectin to Neu5Ac. In the Labrador Retriever dogs that showed positive binding of lectin to Neu5Ac, the G allele was more frequent than the A allele; however, in Shiba dogs that were negative for the binding, the G allele was also more frequent. Thus, these results did not clarify whether point mutations at c.554A>G influence the expression of Neu5Ac. In this study, the Shiba dogs did not carry the loss-of-function deletion of the *CMAH* gene that has been found in humans and ferrets. Recently, a promoter region responsible for the intestine-specific regulation of porcine *CMAH* has been found. Future studies will need to investigate the promoter region of the dog *CMAH* gene to determine the regulation of the expression of this gene.

5. Genotyping of the cat *CMAH* gene in type B and AB cats and comparison with dog one

We investigated the mutations and diplotypes of the cat *CMAH* gene by analyzing the nucleotide sequences of the four exons in the coding region of *CMAH* in 21 type B cats

and 6 type AB cats. We also compared the results to those of a previous report (Omi et al., 2016). In type B cats, the distribution of diplotypes was not so discordant to that described in the previous report, although some novel diplotypes were discovered. The genotype AA at c.268T>A was found in 43 of 55 of the type B cats in the previous and present studies. Considering that AA has not been found in the cats of the other types in our laboratory thus far, the genotype AA alone may determine type B cats. This new finding may be useful for predicting the phenotype of type B cats. Among the AB type cats, we identified c.364 C>T in 1 cat; this was the first time it had been found in Japan, although it has been recently reported by Gandolfi et al. (2016) in the United States. D 1 and D 13 were found in a type AB cat, although the previous study reported that these diplotypes corresponded to at least one intact *CMAH* allele (type A). Furthermore, a type AB cat had D 5, meaning that D 5 has been detected in all type A, B, and AB cats in our studies thus far (data not shown). In summary, further investigations on genotyping for predicting the phenotypes in type AB cats is needed. Therefore, we need to analyze other exons in which additional variants might be discovered. However, the present blood types were determined by the card agglutination (CARD) method, and some reports have cast doubt on its accuracy; as such, we cannot exclude the small possibility of phenotypic errors when determining the blood types.

Compared to the cat *CMAH* gene, the results on the dog *CMAH* gene suggest that variants with an amino acid substitution at the *CMAH* loci are not associated with the expression of Neu5Ac or Neu5Gc. Analysis of the regulation mechanism of mRNA expression may enable the cause of this difference to be identified. Therefore, future studies, such as investigations of the promoter region of *CMAH,* are needed. We also

need to improve the accuracy of phenotyping by Western-blotting or with alloantibodies in cat serum in order to clarify the association between genotypes and phenotypes.