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Genotype frequency of *ATP7A* and *ATP7B* mutation-related copper-associated hepatitis in a Japanese guide dog Labrador retriever population

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ABSTRACT. The incidence of copper-associated hepatitis in Labrador retriever in Japan has not been examined. This study examined the genotype frequencies of *ATP7B*:c.4358G>A, a mutation responsible for copper-associated hepatitis, and *ATP7A*:c.980C>T, a modifier of this disease, in Labrador retrievers of guide dog associations in Japan. Genetic material was collected by buccal swabs from 253 Labrador retrievers and genotyping was performed for the *ATP7B* and *ATP7A* mutations. The gene frequency was 0.107 for *ATP7B*:c.4358A. For *ATP7A*:c.980C, the gene frequencies were 0.703 in females and 0.368 in males. In this study, we established genotyping methods for the *ATP7B*:c.4358G>A and *ATP7A*:c.980C>T mutations. Based on the genotyping results, the risk of copper-associated hepatitis in the study population was 0.80% in males and 1.05% in females.

KEY WORDS: *ATP7B*, copper-associated hepatitis, genotype frequency, Labrador retriever

Copper accumulation in hepatocytes results in chronic hepatitis, known as Wilson disease in humans. This disease has been described in several species, including humans [2], dogs [5], cats [1], and rats [16], and copper accumulation in the hepatocytes is its common manifestation. A null mutation in *COMMD1,* which codes the channel protein for copper transport on the hepatocytes, has been reported to cause copper toxicosis in Bedlington terriers [6]. Recently, *ATP7B*:c.4358G>A mutation on a gene that plays a role in copper transportation was found in copper-associated hepatitis in Labrador retrievers [5]. Besides the *ATP7B* mutation, it was found that *ATP7A*:c.980C>T mutation was negatively associated with copper-associated hepatitis in Labrador retrievers [5]. *ATP7A* is a copper transporting ATPase like *ATP7B* [5]. A mutation in *ATP7A* causes an X-linked copper deficiency disorder, known in humans as Menkes disease [7]. Dogs with *ATP7B*:c.4358G>A show copper accumulation in the hepatocytes because copper transport to the Golgi apparatus is inhibited [5]. The *ATP7A*:c.980C>T mutation causes downregulation of copper absorption by the small intestinal epithelium and, indirectly, decreases copper accumulation in the hepatocytes. Thus, the c.980C allele is related to the development of copper-associated hepatitis [5]. Therefore, both genes affect copper-associated hepatitis in Labrador retrievers. Since the mutation in *ATP7B* is also responsible for Wilson disease in humans [2], Labrador retrievers are an important model for the human disease.

Labrador retrievers work as guide dogs and are companion animals in Japan. Some guide dog associations in Japan primarily own Labrador retrievers. Dogs that present a low risk for health problems are preferred as guide dogs. In copper-associated hepatitis, copper gradually accumulates in the hepatocytes before the animal shows clinical symptoms [12]. Once hepatitis develops, it is difficult to cure it. However, it is possible to control the health of dogs at risk of developing copper-associated hepatitis by feeding them with a low-copper diet before they progress to the severe hepatitis stage [3, 4]. Genotyping of *ATP7B* might be a useful approach to identify dogs susceptible to the disease before the onset of hepatitis [12]. Moreover, breeding control based on genotyping data could decrease the incidence of copper-associated hepatitis in dogs as was done with progressive retinal atrophy [14].

The incidence of copper-associated hepatitis in Labrador retrievers in Japan has not been examined. It is important to reveal this information and identify affected dogs so their health can be monitored and maintained. Although Labrador retrievers are a major breed in Japan known to have chronic hepatitis, the rate of dogs with copper deposition is low [9]. That report was based

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on clinical examination, not genotyping of mutations. This study aimed to examine the frequencies of the *ATP7B* and *ATP7A* mutations in relation to copper-associated hepatitis in Labrador retrievers.

Buccal swabs were collected, regardless of consanguinity, from 253 healthy Labrador retriever dogs (95 males and 158 females, aged 1–7 years) owned by guide dog associations in Tochigi, Kanagawa, Aichi, Kyoto, Hyogo, and Fukuoka prefectures in Japan. Since approximately 2,000 dogs are owned by guide dog associations in Japan, totally 12.8% dog were examined for mutations. Swabs were stored on DNA storage cards (Flinders Technology Associates Elute cards, QIAGEN Tokyo, Tokyo, Japan). After washing the cards three times with distilled water, genomic DNA was eluted in nuclease-free water (ThermoFisher Scientific Japan Group, Tokyo, Japan) by incubation for 20 min at 95°C. Eluates were stored at −20°C until use.

An internal probe (Eprobe, K.K. DANAFORM, Yokohama, Japan) was used to detect the *ATP7B*:c.4358G>A mutation. The Eprobe primers were as follows: Forward, 5′- GGAGTCCCACCGCCGGTAGAGGTACGAAGCCCAAGCGCAGAGCC-3′ and Reverse, 5′-GGGAGGACAGAGACACCTTGCTGAC-3′. The underlined letters indicate artificial mismatches. The primer's 3′-ends were designed to specifically match *ATP7B* because the sequence around *ATP7B*:c.4358G>A shows high homology with *MCC* (mutated in colorectal cancers). The artificial mismatches were introduced to increase specificity to *ATP7B* (Supplementary Fig. 1). The Eprobe was designed to match the wild-type G allele against the anti-sense chain: 5′- CGTGGCCCGCG-3′. The underlined C shows the mutation site. A turn-back sequence was introduced at the 5′-end of the forward primer to prevent the PCR product from forming secondary structures [8, 10]. PCR was carried out in a reaction mixture containing 1x E-Taq (K.K. DANAFORM), 4 µM of the forward primer, 0.1 µM of the reverse primer, 0.4 µM of Eprobe, and 10 ng of genomic DNA. Thermal cycling conditions were as follows: initial denaturation at 98°C for 30 sec, followed by 50 cycles of denaturation at 98°C for 10 sec, annealing at 60°C for 10 sec, and extension at 72°C for 20 sec. Genotyping was performed using a melting curve analysis from 40 to 95°C with a real-time PCR instrument (MyGo Mini; IT-IS Life Science Ltd., Dublin, Ireland) after completing the PCR reaction. Data obtained from the Eprobe was confirmed by sequence analysis for 5 GG, 10 GA, and 5 AA of each genotype as previously [13].

High-resolution melting (HRM) analysis was used to detect the *ATP7A*:c.980C>T mutation. The assay primers, which amplified a 78 bp fragment, were as follows: Forward, 5′-TGCCATAGTAAAGTACAATGCAAGC-3′ and Reverse, 5′-TGTCCTGGTGATATGGCCTCTA-3′. The PCR was carried out in a reaction mixture containing 1x Precision Melt Supermix (Bio-Rad, Hercules, CA, USA), 0.2 µM of each primer, and 10 ng of genomic DNA. Thermal cycling conditions were as follows: initial denaturation at 95°C for 120 sec, followed by 50 cycles of denaturation at 95°C for 10 sec, and annealing/extension at 60°C for 15 sec. Genotyping was performed by a melting curve analysis from 50 to 95°C with MyGo Mini. Three PCR products selected from each genotype were sequenced to confirm the genotype, as previously described [13].

The obtained genotype frequencies were analyzed by χ^2 test for Hardy-Weinberg equilibrium. Deviations between the measured and expected values were regarded as statistically significant at $P<0.05$.

The Eprobe genotyping clearly distinguished between the three *ATP7B*:c.4358G>A genotypes (Fig. 1A). Furthermore, the HRM analysis could discriminate between the *ATP7A*:c.980C>T genotypes (Fig. 1B). The *ATP7A* homozygous group contained hemizygous genotypes (CY and TY) because the gene is on the X chromosome. The representative sample sequencing results were consistent with the respective genotypes (data not shown). The *ATP7B*:c.4358G>A genotype frequency in the 253 Labrador retrievers is shown in Table 1. The gene frequencies of the G and A alleles were calculated to be 0.893 and 0.107, respectively. Thus, the expected genotype frequencies of the GG, GA, and AA genotypes were 0.798, 0.191, and 0.011, respectively (χ^2=1.923,

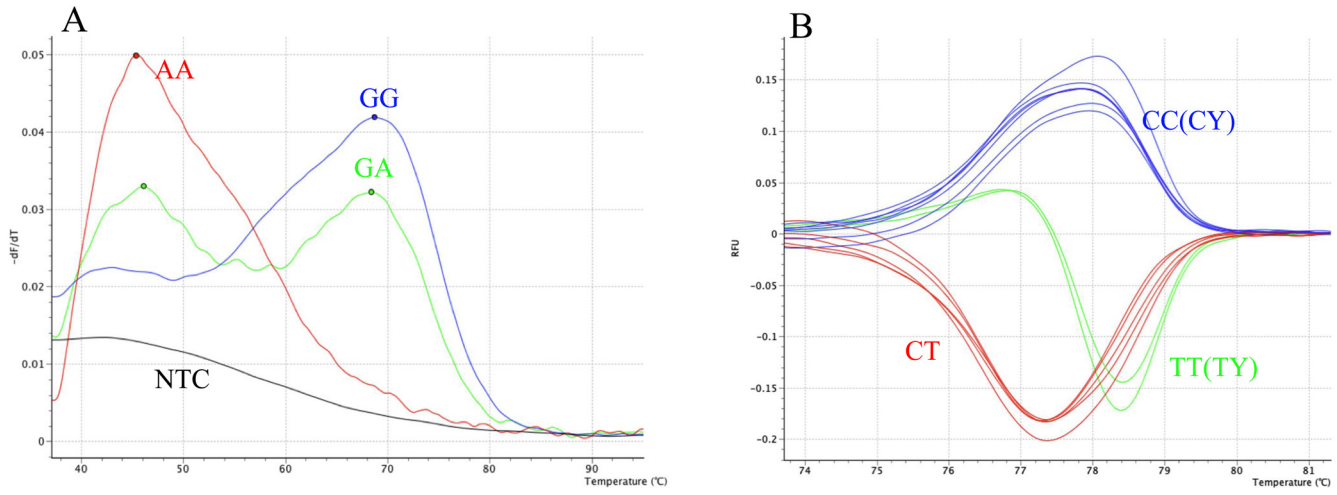

Fig. 1. Genotyping of the *ATP7A* and *ATP7B* mutations in Labrador retrievers. **A**. Genotyping of the *ATP7B*:c.4358G>A mutation by the Eprobe. The G and A alleles were distinguished by melting analysis of the polymerase chain reaction (PCR) product. The G allele had a higher melting temperature than the A allele. GA had two peaks corresponding to the two alleles. NTC, no-template control. **B**. Genotyping data of the *ATP7A*:c.980C>T mutation based on high-resolution melting (HRM) analysis. The genotypes could be clearly distinguished. Note that the CC and TT genotypes include CY and TY, the hemizygotes of the C or T allele in males. NTC, no template control.

Table 1. Genotype frequencies (%) of *ATP7B* and *ATP7A* mutations in Japanese guide dog Labrador Retirivers

ATP7B (c.4358G>A)	N	GG	GA	AA	A allele frequency
	253	80.6	17.4	2.0	0.107
ATP7A (c.980C>T)	N	CC	CT	TT	C allele frequency
Female	158	51.9	36.7	11.4	0.703
		CY*	TY*		
Male	95	73.7	26.3		0.368

N, the number of dogs examined. *CY, TY: the C allele is on X chromosome, thus Y chromosome is shown as the allele.

P=0.377, φ=2), indicating that the genotypes were in Hardy-Weinberg equilibrium.

The *ATP7A*:c.980C>T genotype frequency in the male and female Labrador retrievers is shown in Table 1. The gene frequency of the C allele was calculated at 0.703 for females and 0.368 for males. Therefore, the expected genotype frequencies of the CC, CT, and TT in females were 0.494, 0.418, and 0.088, respectively (χ^2=3.203, *P*=0.202, φ=2), and the expected genotype frequencies of CY and TY in males were 0.703 and 0.297, respectively (χ^2=0.533, *P*=0.466, φ=1). These results indicated that the male and female genotypes were in Hardy-Weinberg equilibrium. The study population frequency of the homozygous *ATP7B*:c.4358G>A carrying the C allele of *ATP7A*, a genetic combination related to the risk of developing copper-associated hepatitis, was 0.80% in males and 1.05% in females.

This study established real-time PCR genotyping methods for *ATP7B* and *ATP7A* mutations related to canine copper-associated hepatitis. The design of the *ATP7B* mutation primers was particularly limited because of the high homology between *ATP7B* and *MCC*. However, the specificity of the *ATP7B* primers was ensured by designing them with *ATP7B*-specific ends. Furthermore, the binding of the Eprobe was inhibited by the secondary structure with the GC-rich sequence of the PCR product. The turn-back sequence inserted at the 5′-end of the forward primer improved the Eprobe binding specificity by preventing secondary structure formation in the PCR product. The specific primers for *ATP7B* and the turn-back primer facilitated accurate genotyping of the *ATP7B*:c.4358G>A mutation.

Copper-associated hepatitis in Labrador retrievers is a progressive disease in which copper gradually accumulates in the hepatocytes. Copper-associated hepatitis could be controlled by a low-copper diet during the early stage of the disease [4]. Although liver biopsy for pathological examination and determination of the copper level is recommended for copper-associated hepatitis diagnosis, the procedure is highly invasive. However, it is possible to prevent hepatitis onset in dogs at risk of developing the disease because they carry a homozygous *ATP7B*:c.4358G>A mutation [11, 12]. The genotyping approach established in this study could detect such dogs before they develop hepatitis so that preventive measures could be taken. Most Labrador retrievers owned by the guide dog associations in Japan work as guides. Such working dogs need to remain as healthy as possible. Therefore, pre-symptomatic diagnosis of copper-associated hepatitis by genotyping for the *ATP7B* and *ATP7A* mutations in dogs with increased levels of liver enzymes could benefit the dogs and their owners.

The estimated risk of copper-associated hepatitis calculated in this study based on the *ATP7B* and *ATP7A* mutations was 1.05% in female and 0.80% in male Labrador retrievers of guide dog associations in Japan. These genotype results support the low rate of copper-associated hepatitis in Labrador retrievers reported based on pathological examination [9]. Most chronic hepatitis in Labrador retrievers might be caused by factors other than copper accumulation. Indeed, the heritability of both copper-associated hepatitis mutations was calculated to be 12.5% [5]. Breeding control to prevent homozygous *ATP7B*:c.4358G>A mutation, the key to copper-associated hepatitis development, might be possible by identifying dogs without the mutation through genotyping. Indeed, the guide dog associations in Japan have successfully decreased the incidence of progressive retinal atrophy by selective breeding [14]. It might be possible to use the same measure for copper-associated hepatitis. However, it might be difficult to control chronic hepatitis only by purging the *ATP7B* and *ATP7A* mutations.

A limitation of this study was that the mutation of *RETN*, another negative modifier of copper-associated hepatitis, was not examined because of low frequency obtained from the study performed at Netherland [15]. A mutation in *RETN* (c.19C>T) decreases copper accumulation in the hepatocytes, like the *ATP7A* mutation [15]. The detailed mechanism through which *RETN* exerts its inhibitory effect on copper accumulation has not been elucidated yet. The frequency of the T allele in the Netherlands was 0.089 [15], indicating that the genotype frequency of the homozygous T allele was 0.79%. Although *RETN* allelic frequency has not been examined in Japan, some dogs with the T allele might be present. Therefore, the incidence of copper-associated hepatitis in the Labrador retrievers of the guide dog associations in Japan might be slightly lower than the rate shown in this study. Another limitation was that client-owed Labrador retrievers were not included in this study. This group might have a different genetic status based on population genetics. Therefore, the present data is restricted to the guide dog population in Japan.

We have identified the genotype frequencies of the *ATP7B* and *ATP7A* mutations related to copper-associated hepatitis in Labrador retrievers belonging to guide dog associations in Japan. Genotyping could detect dogs with copper-associated hepatitis at the pre-symptomatic stage of the disease. Selective breeding based on these genotypes could decrease the incidence of copper-associated hepatitis in these working dogs.

CONFLICTS OF INTEREST. None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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