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ORIGINAL INVESTIGATION

Predicting copper toxicosis: relationship between the ATP7A and ATP7B gene mutations and hepatic copper quantification in dogs

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Abstract

This preliminary study summarizes the genotypes of 42 Labrador Retrievers and Labrador Retriever–Golden Retriever crosses and phenotypes a subset of ten of these dogs that are homozygous mutant, heterozygous, or homozygous normal for mutations in the *ATP7A* and *ATP7B* genes that have been associated with the development of copper toxicosis in Labrador Retrievers. The purpose of this study is to evaluate whether there is a correlation between *ATP7A* and *ATP7B* genotypes and clinical evidence of hepatic pathology in young, asymptomatic Labrador Retrievers. We evaluated serum ALT levels, hepatic copper concentrations, and hepatic histopathology from ten offspring where both parents had a least one copy of the *ATP7B* mutation. Five were homozygous mutant, four were heterozygous, and one was homozygous normal for comparison. None had increased serum ALT activity. All dogs homozygous for the *ATP7B* mutation had elevated hepatic copper concentrations compared to dogs heterozygous for the *ATP7B* mutation regardless of sex or presence of an *ATP7A* mutation with the mean hepatic copper concentration being 1464 ppm (reference range 100–330 ppm). Mean hepatic copper concentration in homozygous normal and heterozygous dogs was 328 ppm. In this preliminary analysis, we found that dogs that carry two copies of the *ATP7B* mutation have abnormally elevated hepatic copper levels despite having normal serum ALT activity. Our findings support the hypothesis that the *ATP7B* DNA test can predict defects in hepatic copper metabolism. Veterinarians can test for the *ATP7B* gene mutation to identify Labrador Retrievers at risk for copper toxicosis so that they can take steps to prevent development of copper-associated chronic hepatitis in their patients.

Introduction

Copper is an essential trace element, though in excess, accumulations lead to toxic manifestations due to free radical formation. For this reason, copper levels in the body are tightly regulated. The protein pathways involved in copper homeostasis have been studied extensively in humans and mice and have led to the discovery of several important gene mutations that disrupt the body's ability to maintain proper copper homeostasis. Specifically, in humans, mutations of the gene coding for copper transporter ATP7B have been associated with Wilson disease (WD) and mutations of the gene coding for copper transporter ATP7A have been associated with both Menkes disease (MD) and occipital horn syndrome (OHS) (reviewed in Chang and Hahn 2017 and Tümer 2013). Over 600 disease-causing mutations in the *ATP7B* gene are associated with Wilson disease (OMIM #277900) and almost 300 disease-causing mutations in the *ATP7A* gene are associated with Menkes disease or occipital horn syndrome (OMIM #309400).

Wilson disease is inherited in an autosomal recessive manner and is associated with decreased ceruloplasmin-bound copper and increased non-ceruloplasmin-bound copper in the blood. Defects in the ATP7B protein prevent transport of cytosolic copper into the trans-Golgi network, which limits the amount of copper available for incorporation into ceruloplasmin. While there is variation in the clinical severity and age of onset of the disease, symptoms are due to excessive copper accumulations most notably in

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cells of the liver and brain (reviewed in de Bie et al. 2007 and Gitlin 2003). Due to excessive copper accumulation in these organs, patients initially present with clinical signs related to liver disease typically after 3 years of age and/or neurologic disease typically between 10 and 12 years of age. Behavior changes, Parkinson's disease, and psychiatric disorders as well as a distinct Kayser–Fleischer ring due to copper accumulation on the outer edge of the cornea may also be seen. Most patients do well with treatment, which includes copper chelators and avoidance of food and water with high levels of copper.

Menkes disease and OHS are X-linked diseases associated with generalized copper deficiency. Symptoms of these diseases are directly related to the dysfunction of copper-dependent enzymes (reviewed in de Bie et al. 2007 and Kaler 2011). Though there is variation in the severity of symptoms, patients with Menkes disease are typically diagnosed at 2–3 months of age and if left untreated, may result in severe neurologic defects including intellectual disability and seizures in addition to growth retardation, joint laxity, stretchy skin, and distinct, hypopigmented, coarse, kinky hair. OHS is an allelic variant of Menkes disease with milder symptoms and a later age of onset. Patients with OHS typically have hypopigmented, kinky hair, joint laxity, stretchy skin, and skeletal malformations including exostoses of the occipital bone at muscle attachment sites. OHS patients do not display severe neurologic defects. Patients with both Menkes disease and OHS have increased concentrations of copper in the intestine, kidney, spleen, lung, pancreas, muscle, and skin and below normal copper levels in the liver and brain. While patients with Menkes disease do not survive for more than a few years without early treatment, patients with OHS can live normal life spans with copper supplementation.

The clinical manifestations of copper toxicosis in Labrador Retrievers are most similar to Wilson disease, though there is neither apparent neurologic dysfunction, nor are Kayser–Fleischer rings evident (Fieten et al. 2016). Recently, Fieten et al. (2016) performed a genome-wide association study and identified two SNPs within the canine *ATP7A* and *ATP7B* genes associated with variation in hepatic copper concentrations in Labrador Retrievers. The ATP7B:c.4358G > A mutation was correlated with increased hepatic copper levels and appears to be inherited as an incomplete dominant condition, meaning that dogs heterozygous for the mutation are at risk of developing copper toxicosis while dogs homozygous for the mutation are at a higher risk of developing copper toxicosis (Fieten et al. 2016). The ATP7A:c.980C > T mutation was associated with a decrease in hepatic copper levels, suggesting that it provides protection against copper toxicosis in dogs homozygous or heterozygous for the *ATP7B* mutation (Fieten et al. 2016). Female Labrador Retrievers that were homozygous or heterozygous for the *ATP7B* mutation had slightly higher hepatic copper concentrations as compared to males supporting a possible role of the X-linked *ATP7A* mutation in protecting against disease progression. Females could have one or two copies of the protective mutation; whereas, males need only one copy to provide protection against copper toxicosis (Fieten et al. 2016). Additionally, the inheritance of copper toxicosis in Labrador Retrievers is complex with only 12.5% of the total heritability explained by these two mutations (Fieten et al. 2016). The purpose of this study is to identify the genotypes for *ATP7A* and *ATP7B* and compare those to the clinicopathologic phenotypes of Labrador Retrievers and Labrador Retriever–Golden Retriever crosses (LRGR) located at a single guide dog facility.

Materials and methods

The population analyzed in this study consists of 34 Labrador Retrievers and 8 LRGRs that belong to a single guide dog facility. The facility has a breeding colony of approximately 40 female and 20 male Labrador Retrievers, Golden Retrievers, and LRGRs. All breeding dogs are screened for heritable diseases via radiographs, echocardiograms, ophthalmologic exams, and genetic testing. The dogs included in this study ($n = 42$) are breeding dogs ($n = 9$) that carry at least one copy of the *ATP7B* mutation, as well as the progeny ($n = 33$) of breeding dogs who carry at least one copy of the *ATP7B* mutation. DNA was extracted from buccal swabs via routine methods (Shaffer et al. 2015). PCR amplification of the regions surrounding the ATP7B:c.4358G > A and ATP7A:c.980C > T mutations was performed and restriction digests using SacII and Tsp45I enzymes (New England Biolabs, Ipswich, MA), respectively, were used to determine whether the dogs were homozygous normal, heterozygous, or homozygous mutant for the *ATP7B* and/or *ATP7A* mutations. In addition, all study participants had routine blood serum chemistry testing, including serum alanine aminotransferase (ALT) enzyme activity, measured using the Idexx Catalyst Dx (IDEXX, Westbrook, Maine). Five dogs homozygous for the *ATP7B* mutation also had liver biopsies performed for the purposes of copper quantification and histopathology. Four heterozygous dogs and one homozygous normal dog underwent liver biopsies as well, for the purposes of comparison. Upon consent of the guide dog organization, dogs were anesthetized and intubated for ovariohysterectomies and orchiectomies, and liver biopsies were collected during this time. Biopsies were obtained laparoscopically using laparoscopic biopsy forceps. Approximately, 0.5 g of fresh liver were submitted to the California Animal Health and Food Safety Laboratory (Davis, CA) for hepatic copper concentration. An approximately 5 mm × 5 mm sample of liver was placed in 10%

Table 1 *ATP7A* and *ATP7B* genotype frequencies (%) in 42 Labrador Retrievers and LRGR mixes at a single guide dog facility

ATP7A genotypes	ATP7B genotypes			
	GG	GA	AA	Total
CC	2.38	2.38	0.00	4.76
CT	9.52	19.05	7.14	35.71
TT	0.00	4.76	0.00	4.76
CY	7.14	9.52	7.14	23.81
TY	2.38	21.43	7.14	30.95
Total	21.43	57.14	21.43	100

Table 2 *ATP7A* and *ATP7B* genotype frequencies (%) in 1208 Labrador Retrievers genotyped during routine genetic testing

ATP7A genotypes	ATP7B genotypes			
	GG	GA	AA	Total
CC	27.48	4.64	0.50	32.62
CT	20.70	4.80	1.16	26.66
TT	3.89	1.82	0.25	5.96
CY	16.80	7.28	0.25	24.34
TY	7.20	3.06	0.17	10.43
Total	76.08	21.61	2.32	100

neutral buffered formalin and submitted to IDEXX Laboratories (Westbrook, Maine) for histopathological examination. The diets for the ten dogs, for which hepatic copper quantification was evaluated, are available in supplementary materials, Table 1.

Results

Genotypes

We examined the genotypes of five different litters of dogs in which both parents were genotyped as heterozygous for the *ATP7B* mutation plus an additional two dogs for which one parent was genotyped as heterozygous but the status of the other parent was unknown (Supplementary Materials, Table 2). Litters from the genotyped *ATP7B* heterozygous parents varied in size from 5 to 10 offspring. There were a few offspring that were not genotyped because they had left the program or had passed away for a variety of causes unrelated to this study. Every litter had at least one dog that was homozygous for the *ATP7B* mutation. The percentage of offspring that were homozygous for the *ATP7B* mutation ranged from 14 to 43%.

A total of 42 dogs, 9 breeding dogs and 33 of their offspring, were genotyped for the *ATP7B* and *ATP7A* gene mutations. The frequencies of the ATP7B:c.4358G and ATP7B:c.4358A mutations were 50.0% and 50.0%, respectively. The frequencies of the ATP7A:c.980C and ATP7A:c.980T mutations were 47.5% and 52.5% respectively (Table 1). We also genotyped 1208 client-owned Labrador Retrievers for the ATP7B:c.4358G > A and ATP7A:c.980C > T mutations (Table 2). These Labrador Retrievers were primarily screened as part of routine genetic testing for disease-causing genetic mutations identified in the breed. Samples were submitted from North America and Europe with most of the samples being from the United States of America. The frequencies of the ATP7B:c.4358G and ATP7B:c.4358A mutations were 86.9% and 13.1%, respectively. The frequencies of the ATP7A:c.980C and ATP7A:c.980T mutations were 65.6% and 34.3%, respectively. The frequencies of the ATP7B:c.4358G and ATP7B:c.4358A mutations are statistically different between the guide dog facility and the 1208 client-owned dogs [$p(<0.001, df=1)=89.8$].

Clinicopathologic findings

Ten dogs from the guide dog facility underwent liver biopsies for the purposes of copper analysis and histopathology in addition to serum ALT testing (Table 3). Five dogs were homozygous for the *ATP7B* mutation, four were heterozygous for the *ATP7B* mutation, and one was homozygous normal. Of the dogs that are homozygous mutant for the *ATP7B* mutation, the hepatic copper levels ranged from 620 to 2100 ppm (reference range 100–330 ppm dry weight). Of the dogs that are heterozygous for the *ATP7B* mutation, the hepatic copper levels ranged from 219 to 390 ppm. The dog that was homozygous normal had a hepatic copper level of 320 ppm. The mean hepatic copper concentration for homozygous mutant dogs was 1464 ppm versus 328 ppm in homozygous normal and heterozygous dogs. Dogs that are homozygous for the *ATP7B* mutation have significantly higher hepatic copper levels compared to dogs that are heterozygous for the *ATP7B* mutation (Fig. 1). Copper-positive pigment was identified in four of the five dogs for which sections were stained with rhodanine, but histopathologic findings were otherwise unremarkable in all dogs. Two of the dogs (F1, F2) with rhodanine-positive pigment had hepatic copper concentrations within the reference range and one of the dogs without rhodanine-positive pigment (A1) had elevated hepatic copper concentrations. Serum ALT levels were within normal limits for all ten dogs ranging from 45 to 90 U/L (reference range 10–125 U/L).

Discussion

Genetic testing is on the rise in veterinary medicine, but guidance on interpretation of DNA testing results for diseases with complex inheritance is often lacking. The purpose

Table 3 Hepatic copper quantification, ALT activity, and histopathology for ten Labrador Retriever guide dogs

Dog	Gender	Age (year)	ATP7A genotype	ATP7B genotype	Copper quantification (ppm)[a]	ALT (U/L)[b]	Histopathology[c]
A1[d]	Female	1 year	CT	AA	620	65	No significant findings; no rhodanine-positive intracytoplasmic pigment
B1[d]	Female	2 years	CT	AA	2000	62	Marked rhodanine-positive, intracytoplasmic hepatocellular pigment
C1	Female	1 year	CT	AA	2100	75	Not done
C2	Male	1 year	TY	GA	219	65	No significant findings; rhodanine staining not done
D1	Female	10 months	CT	GG	320	45	No significant findings; rhodanine staining not done
E1	Male	1 year	TY	AA	1600	55	No significant findings; rhodanine staining not done
E2	Female	1 year	CT	GA	380	90	No significant findings; rhodanine staining not done
F1	Male	1 year	TY	GA	330	52	Moderate rhodanine-positive, intracytoplasmic hepatocellular pigment
F2	Male	1 year	TY	GA	390	86	Moderate rhodanine-positive, intracytoplasmic hepatocellular pigment
F3	Male	1 year	TY	AA	1000	48	Moderate rhodanine-positive, intracytoplasmic hepatocellular pigment

[a]Reference range 100–330 ppm dry weight; >400 ppm is considered elevated (California Animal Health and Food Safety Laboratory, Davis, CA)

[b]Reference range 10–125 U/L (IDEXX Catalyst, Westbrook, Maine)

[c]IDEXX Laboratory (Westbrook, Maine)

[d]Dogs were tested as a part of a screening process for selecting breeders. H1 (sire a, b) is the sire of A1. N1 (sire d) is the sire of B1. The genotype of the dams of these two dogs is unknown

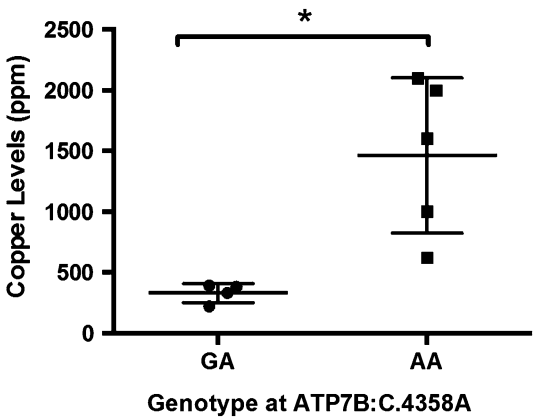

Fig. 1 Copper levels in canine hepatic tissue according to genotype ATP7B:c.4358G > A. Dogs with the homozygous AA genotype had significantly higher copper levels than those with the heterozygous GA genotype (*$p < 0.05$, Mann–Whitney test)

of this study was to evaluate the correlation between genotype and phenotype and to aid in the interpretation of the *ATP7A* and *ATP7B* genetic test results in Labrador Retrievers and Labrador Retriever mixes. Since the Labrador Retriever has recently been identified as the first natural, non-rodent model for ATP7B-related copper toxicosis (Fieten et al. 2016), these findings have implications in human medicine as well.

Copper toxicosis in dogs is diagnosed via a combination of histopathological findings of intracytoplasmic granules that stain positive for copper and elevated hepatic copper concentrations (Washabau and Day 2013). Dogs with inborn errors of copper metabolism will accumulate copper in their liver over time without apparent clinical signs initially (Fieten et al. 2012). In a study by Hoffmann et al. (2006), of 15 Labrador Retrievers with copper-associated chronic hepatitis, the mean age at clinical presentation was 7 years. In the present study, the ages of the dogs ranged from 10 months to 4 years, and dogs were otherwise clinically healthy with normal serum blood chemistry values (Table 3). Fieten et al. (2012) found that over a third of the clinically normal dogs in their study population had elevated hepatic copper concentrations. In our study, there is no correlation between the number of *ATP7B* mutations or the hepatic copper concentrations with serum ALT levels in the population of young dogs in this study. However, there does appear to be a correlation between homozygosity for the *ATP7B* mutations and increased hepatic copper concentrations. A limitation of this study is that hepatic biopsy samples were only collected from dogs undergoing surgery for ovariohysterectomies or orchiectomies. Ideally, we would have preferred to collect a biopsy from at least one *ATP7B* homozygous normal dog from each litter for comparison. Unfortunately, we only had access to one homozygous normal dog, and, therefore, could not perform a statistical comparison to determine if there

is a significant difference in hepatic copper concentrations in *ATP7B* homozygous normal dogs vs heterozygous or homozygous mutant dogs.

In this study, the average hepatic copper concentration in female dogs ($n=5$) was 1084 ppm and in males ($n=5$) was 707.8 ppm. This is in line with previous findings that female Labrador Retrievers are at increased risk for copper toxicosis as compared to males (Hoffmann et al. 2006; Fieten et al. 2012). One possible cause for female predilection could be related to the X-linked *ATP7A* gene mutation, which has been correlated with decreased copper excretion from fibroblasts and overall decreased hepatic copper levels (Fieten et al. 2016). In our study, all the dogs for which hepatic copper values were measured were heterozygous or hemizygous for the *ATP7A* gene mutation (Table 3), which hinders evaluation of whether the presence of this mutation affected hepatic copper concentrations. In other words, because all male and female dogs that were evaluated for the hepatic copper concentrations have one copy of the *ATP7A* mutation regardless of the whether they are homozygous normal, heterozygous, or homozygous mutant for the *ATP7B* mutation, we cannot evaluate how the absence the *ATP7A* mutation would affect the hepatic copper concentrations.

The 50% frequency of the ATP7B:c.4358A mutation in the 42 dogs within the single guide dog facility is significantly much higher than the 13.1% frequency found in the 1208 dogs screened generally for Labrador Retriever disease-associated mutations. This is likely because the focus of this study was to examine the offspring of breeding dogs who carry at least one copy of the *ATP7B* mutation. If all dogs at this facility were genotyped, presumably the percentage of dogs with *ATP7B* mutations would be lower. Although the 1208 Labrador Retrievers screened for this mutation do not represent a randomized sampling of the Labrador Retriever population, a 13.1% gene frequency is higher than, but likely closer to, the true frequency of the *ATP7B* mutation in the breed. Fieten et al. (2016) estimated that 12.5% of the total heritability could be explained by these two mutations with environmental factors such as copper intake in the diet and water playing a large role in the clinical presentation of the disease. Though our dataset is too small to calculate heritability, the presence of two copies of the *ATP7B* mutation is significantly associated with increased hepatic copper concentration in young dogs. Our hope is that with dietary management we can prevent these dogs from developing clinical signs of copper toxicosis. The most significant histopathological finding was cytoplasmic accumulation of rhodanine-positive pigment within hepatocytes, but it was not identified in all dogs with increased hepatic copper concentrations. Other histopathological findings were mild, and as the slides were reviewed by multiple pathologists without a specified grading scale, we do not feel comfortable concluding that these changes are related to increased copper stores in the liver. For future liver biopsies, all slides will be evaluated by a single veterinary pathologist, stained with rhodanine to evaluate for cytoplasmic accumulations of copper, and scored via the grading scale described in Fieten et al. (2012).

A hepatic copper concentration of 400 ppm or more is considered elevated per the California Animal Health and Food Safety Laboratory (Davis, CA). The dogs in this current study, with hepatic copper levels of 400 ppm or more, were started on a copper-restricted commercial dog food, in addition to vitamin E, Marin, and Denosyl supplementation. There is currently no established treatment recommendation for dogs with excessive hepatic copper accumulation without cirrhosis. The treatment regiment provided to the dogs in this study was extrapolated from current recommendations for dogs with cirrhosis. D-penicillamine can be used to chelate copper in patients with excessive hepatic copper. In the dogs of the present report, this treatment was found to be cost prohibitive, and since many of these dogs were released from the guide dog facility, routine monitoring and follow-up would not be possible during and after administration of this treatment. Although follow-up biopsies would be ideal, as not all dogs normalize in hepatic copper upon change to a low copper diet, this may not be feasible in this population of dogs (Fieten et al. 2015). Dogs with the higher copper levels were released from the training program and adopted. Many have been lost to follow-up. A longevity study of these dogs compared to dogs that are diagnosed with copper toxicosis at an older age would be useful for determining the significance of managing this disease early. Additionally, we would like to expand our study size, to further evaluate the positive predictive value of the *ATP7B* DNA test when assessing a dog's risk for developing copper-associated hepatitis. If dogs at an increased risk for copper toxicosis can be identified and medically managed prior to the development of hepatitis, perhaps the onset of clinical signs can be delayed. In humans, the age of onset and clinical manifestations of Wilson disease vary greatly between affected individuals (reviewed in de Bie et al. 2007 and Gitlin 2003). The human–animal model for ATP7B-associated copper toxicosis may yield encouraging results for early medical intervention of this disease based on genotyping. Based on these preliminary results in dogs, human clinicians may want to consider early nutritional modification in children with *ATP7B* mutations to slow the progression of disease onset in children.

A larger sample size is needed to determine the significance of these findings, but our preliminary study shows that young Labrador Retrievers show evidence of increased hepatic copper concentrations prior to elevation of serum ALT. We are not recommending removing dogs from breeding programs that are heterozygous or homozygous for the *ATP7B* mutation based on these results alone. Due to

the high frequency of this mutation in the 1208 Labrador Retrievers we screened, removing all dogs with one or two copies of the *ATP7B* mutation could have detrimental effects on the genetic diversity of the breed. Currently, we recommend not to breed dogs with the mutation together as this preliminary study shows that there is evidence of hepatic copper accumulation at an early age in homozygous mutant dogs. This information may help veterinarians determine whether dietary management should be implemented in a young, clinically normal Labrador Retriever patient that is homozygous for the *ATP7B* mutation.

Compliance with ethical standards

Conflict of Interest On behalf of all authors, the corresponding author states that there is no conflict of interest. Christina Ramirez is an employee of Paw Print Genetics, which provides genetic testing for dogs on a fee-for-service basis.

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