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Molecular epidemiologic study on canine neuronal ceroid lipofuscinosis and GM1 gangliosidosis

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Molecular epidemiologic study on canine neuronal ceroid lipofuscinosis and GM1 gangliosidosis

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DEDICATION

I dedicate this thesis to my beloved father, mother and my husband who provide me tremendous inspiration for higher study.

ABSTRACT

Lysosomal storage diseases are a group of rare, genetic disorders of cellular catabolism. Most of them are inherited as autosomal recessive traits and result from mutations in the coding sequence of one of the acid hydrolases or their activators located in the lysosome. The genetic abnormality results in the reduction or elimination of the catalytic activity of the particular enzymatic reaction, and the reduction in catabolism results in the accumulation of the substrate of that enzymatic reaction within the lysosome. Neurons are affected in most of these diseases because they are postmitotic, permanent cell populations. Thus, many lysosomal storage diseases are manifested as progressive, neurological and eventually lethal disorders. Neuronal ceroid lipofuscinosis (NCL) and GM1 gangliosidosis are also lysosomal storage diseases that occur in several purebred dogs and are problems in producing healthy dogs. In this study, NCL in Chihuahua dogs (Chapter 1) and GM1 gangliosidosis in miniature type of Shiba Inus called Mame Shibas (Chapter 2) were investigated in order to analyze carrier rates and mutant allele frequencies, and to evaluate the necessity of control and prevention.

Chapter 1: NCL is a group of rare lethal neurodegenerative lysosomal storage diseases that occur in a range of dog breeds, including Chihuahuas. Recently, a homozygous single base-pair deletion (c.846delT), which causes a frame shift generating a premature stop codon (p.F282Lfs*13) in the canine *CLN7/MFSD8* gene, has been identified as a causative mutation for NCL in Chihuahuas. The objective of this study was to determine the frequency of the

mutant allele and/or carrier rate of NCL in Chihuahuas in Japan using a newly designed real-time PCR assay. Samples of saliva were randomly collected from 1007 Chihuahua puppies during physical examinations prior to the transportation to pet shops. Screening results revealed a carrier rate of 1.29%, indicating a mutant allele frequency (0.00645) that is considered sufficiently high to warrant measures for the control and prevention of this lethal disease. The genotyping assay designed in this study could make a valuable contribution to the control and prevention of NCL.

Chapter 2: GM1 gangliosidosis is a progressive, recessive, autosomal, neurodegenerative, lysosomal storage disorder that affects the brain and multiple systemic organs due to an acid galactosidase deficiency encoded by the *GLB1* gene. This disease occurs in the Shiba Inu breed, which is one of the most popular traditional breeds in Japan, due to the *GLB1*:c.1649delC (p.P550Rfs*50) mutation. Previous surveys performed of the Shiba Inu population in Japan found a carrier rate of 1.02–2.94%. Currently, a miniature type of the Shiba Inu called “Mame Shiba”, bred via artificial selection to yield smaller individuals, is becoming more popular than the standard Shiba Inu and it is now one of the most popular breeds in Japan and China. The GM1 gangliosidosis mutation has yet to be surveyed in the Mame Shiba population. This study aimed to determine the frequency of the mutant allele and carrier rate of GM1 gangliosidosis in the Mame Shiba breed. Blood samples were collected from 1832 clinically healthy adult Mame Shiba Inus used for breeding across 143 Japanese kennels. The genotyping was

performed using a real-time PCR assay. The survey found nine carriers among the Mame Shibas, indicating that the carrier rate and mutant allele frequency were 0.49% and 0.00246, respectively. This study demonstrated that the mutant allele has already been inherited by the Mame Shiba population. There is a risk of GM1 gangliosidosis occurrence in the Mame Shiba breed if breeders use carriers for mating. Further genotyping surveys are necessary for breeding Mame Shibas to prevent the inheritance of this disease.

In conclusion, on the basis of the results obtained in this study (Chapter 1), it is established that in Japan, the carrier rate of NCL in Chihuahuas is currently 1.29%, and given the lethality of this disease, the corresponding mutant allele frequency (0.00645) is deemed sufficiently high to warrant measures for disease control and prevention. Ideally, in this regard, it is considered important to conduct extensive molecular screening of breeding Chihuahuas in related kennels throughout Japan. To this end, it is believed that the genotyping assay designed in this study will make a potentially valuable contribution to the control and prevention of NCL in Chihuahuas. Furthermore, the results of this study (Chapter 2) show that the carrier rate of GM1 gangliosidosis in the Mame Shiba in Japan is currently 0.49%, and, given the lethality of this disease and popularity of this breed, the corresponding mutant allele frequency (0.00246) is deemed sufficiently high to warrant measures for disease control and prevention. Ideally, continued genotype surveying should be performed on breeding Mame Shibas reared by breeders who undertake appropriate mating management.

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FREQUENTLY USED ABBREVIATION

bp	-	base pair
c.	-	coding DNA sequence
del	-	deletion
DNA	-	deoxyribonucleic acid
dup	-	duplication
FAM	-	6-carboxyfluorescein
FCI	-	federation cynologique international
fs	-	frameshift
FTA	-	flinders technology associates
H	-	histidine
JKC	-	Japan kennel club
KCJ	-	kennel club of Japan
leu	-	leucine
LSDs	-	lysosomal storage diseases
MGB	-	minor groove binder
NA	-	not applicable
NCL	-	neuronal ceroid lipofuscinosis
ND	-	not determine
NFQ	-	non-fluorescent quencher
NIPPO	-	nohon-ken hozonkai
NMSA	-	nihon mame shibaken association
OMIA	-	online mendelian inheritance of animal
OMIM	-	online mendelian inheritance of man
P	-	proline

p.	-	protein sequence
PCR	-	polymerase chain reaction
phe	-	phenylalanine
R	-	arginine
SNP	-	single nucleotide polymorphism
T	-	threonine
VIC	-	6-carboxyrhodamine

CHAPTER 1

Investigation of neuronal ceroid lipofuscinosis in Chihuahua puppies in Japan

The above-titled work originally appeared in "*Animals* (Pervin et al., 2022) as: **Screening and carrier rate of neuronal ceroid lipofuscinosis in Chihuahua dogs in Japan** authored by:

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1.1. ABSTRACT

Neuronal ceroid lipofuscinosis (NCL) is a group of rare lethal neurodegenerative lysosomal storage diseases that occur in a range of dog breeds, including Chihuahuas. Recently, a homozygous single base-pair deletion (c.846delT), which causes a frame shift generating a premature stop codon (p.Phe282Leufs*13) in the canine *CLN7/MFSD8* gene, has been identified as a causative mutation for NCL in Chihuahuas. The objective of this study was to determine the frequency of the mutant allele and/or carrier rate of NCL in Chihuahuas in Japan using a newly designed real-time PCR assay. Samples of saliva were randomly collected from 1,007 Chihuahua puppies during physical examinations prior to the transportation to pet shops. Screening results revealed a carrier rate of 1.29%, indicating a mutant allele frequency (0.00645) considered sufficiently high to warrant measures for the control and prevention of this lethal disease. The genotyping assay designed in this study could make a valuable contribution to the control and prevention of NCL.

1.2. INTRODUCTION

Neuronal ceroid lipofuscinosis (NCL) is a recessively inherited progressive neurodegenerative lysosomal storage disorder detected in both humans and non-human animals, which is characterized by the abnormal accumulation of autofluorescent lysosomal storage bodies in the central nervous system, retina, and other tissues throughout the body (Jolly et al., 1994; Jolly and Palmer, 1995; Chalkley et al., 2014). NCL has been described in many species, including cattle, sheep, pigs, cats, mice, non-human primates, and birds, and most frequently in different breeds of dog, including Chihuahuas (Katz et al., 2017; NCL resource: www.ucl.ac.uk/ncl/)). When occurring in humans and dogs, the disease has similar clinical manifestations, including behavioral abnormalities, visual complications, brain atrophy, seizures, and motor disfunctions, and eventually leads to premature death (Jolly et al., 1994; Jolly and Palmer, 1995).

To date, NCL has been described in numerous different dog breeds, including English Setters (Katz et al., 2005), Dachshunds (Asakawa et al., 2010; Sanders et al., 2010), Chinese Crested dogs (Guo et al., 2015), Salukis (Lingaas et al., 2018), Labrador Retrievers (Rossmeisl et al., 2003), Border Collies (Koie et al., 2004; Mizukami et al., 2011; Mizukami et al., 2012), Cocker Spaniels (Minatel et al., 2000), American Bulldogs (Evans et al., 2005), Australian Shepherds (O'brien and Katzet al, 2008; Katz et al., 2011), Chihuahuas (Nakamoto et al., 2011; Ashwini et al., 2016), Australian Cattle dogs (Kolicheski et al., 2016; Schmutz et al., 2019),

Tibetan Terriers (Drögemüller et al., 2005; Katz et al., 2005; Farias et al., 2011; Wöhlke et al., 2011), Polish Owczarek Nizinny Dogs (Drögemüller et al., 2005), American Staffordshire Terriers (Abitbol et al., 2010), Cane Corso dogs (Kolicheski et al., 2017), Alpenländische Dachsbracke Dogs (Hirz et al., 2017), German Shorthaired Pointers (Guo et al., 2019), Golden Retrievers (Gilliam et al., 2015), Welsh Corgis (Jolly et al., 1994), Yugoslavian Shepherds (Bichsel and Vandevelde, 1982), Dalmatians (Goebel et al., 1998), Shikoku dogs (Tamura et al., 2021), and three types of mixed breed dogs (Australian Cattle Dog-Australian Shepherd mix (Guo et al., 2014), German Shepherd-Australian Cattle dog mix, and a dog with unknown ancestry, although appeared to be a Beagle and Labrador Retriever mix (Villani et al., 2019). Among these breeds, the following nine canine orthologs of the human NCL-associated genes have been identified as causal genes in canine NCL: *CLN1/PPT1* (Sanders et al., 2010; Kolicheski et al., 2017), *CLN2/TPP1* (Drögemüller et al., 2005; Asakawa et al., 2010), *CLN5* (Koie et al., 2004; Mizukami et al., 2011; Mizukami et al., 2012; Kolicheski et al., 2016; Gilliam et al., 2015), *CLN6* (O'brien and Katz, 2008; Katz et al., 2011), *CLN7/MFSD8* (Guo et al., 2015; Ashwini et al., 2016), *CLN8* (Hirz et al., 2017; Guo et al., 2019), *CLN10/CTSD* (Evans et al., 2005), *CLN12/ATP13A2* (Katz et al., 2005; Farias et al., 2011; Wöhlke et al., 2011; Schmutz et al., 2019), and *ARSG* (Abitbol et al., 2010).

The occurrence of NCL in Chihuahuas was reported first in Australia and New Zealand in the 1970s (Rac and Giesecke, 1975; Jolly and Hartley, 1977), and has subsequently been

reported in Japan in 2003 (Kuwamura et al., 2003) and 2011 (Nakamoto et al., 2011). Recently, a homozygous single base-pair deletion (c.846delT; g.1301076delT, CanFam3.1) in exon 8 in the canine *CLN7/MFSD8* gene, which causes a frame shift and thereby generates a premature stop codon (p.Phe282Leufs13*), has been identified in two littermates from Scotland (Faller et al., 2016) and in four dogs from Japan, Italy, and England in 2016 (Ashwini et al., 2016). In the same year, an affected Chihuahua was also reported to be molecularly diagnosed with this mutation in Switzerland (Karli et al., 2016), whereas in 2015, the mutation had been identified for the first time in a Chinese Crested dog with NCL in the USA (Guo et al., 2015). The *CLN7/MFSD8* gene encodes the putative lysosomal transporter that is relevant for lysosomal motility and plays an important role for neuronal cell survival under conditions of starvation (von Kleist et al., 2019). Loss of this transporter may result in enhanced neuronal death through dysfunctions of late endosomes and lysosomes.

According to the Japan Kennel Club (JKC: https://www.jkc.or.jp), certified by the Federation Cynologique Internationale (FCI: http://www.fci.be/), from 2001 to the present (2021), Chihuahuas have been the second most commonly registered breed of dog in Japan. During this period, an average of 64,200 Chihuahua puppies were registered annually by the JKC. The presence of fatal inherited diseases like NCL in such a popular breed is a serious problem if the frequency of the mutant allele is sufficiently high to sporadically give rise to affected dogs.

To the best of our knowledge, neither the mutant allele frequency nor carrier rate of NCL have been determined for Chihuahuas in Japan. Accordingly, we believe it to be of particular importance to establish the genetic status of NCL in Japanese Chihuahuas, which would enable the supply of healthy dogs to breeders and pet owners, as well as indicating the necessity to take preventive measures to eradicate this devastating neurological disorder. In this regard, a simple and reliable genotyping method is deemed necessary for the large-scale screening of dogs. To meet this need, we sought in this study to develop a real-time PCR assay that could be used to determine the mutant allele frequency and/or carrier rate of NCL in Chihuahuas in Japan.

1.3. MATERIALS AND METHODS

The experiments conducted in this study were performed in accordance with the guidelines regulating animal use and ethics at Kagoshima University (No. VM15041; approval date: September 29, 2015), and informed consent was obtained from cooperating breeders.

Sample Collection and Storage

Using sterile cotton swabs, saliva samples were randomly collected from 1,007 clinically healthy Chihuahua puppies at the time of medical checks performed by an animal technician (N.T.) and a veterinarian (T.T.) at a corporate animal hospital (Bio Art) in Tokyo, Japan, with the informed consent of the breeders who owned these puppies. These samples were spotted onto Flinders Technology Associates (FTA) filter papers (Indicating FTA Classic Card; Whatman International Ltd., Piscataway, NJ, U.S.A.) and stored in a refrigerator (4 °C) until used for the extraction of DNA. DNA samples from two healthy (homozygous for the wild-type allele), two heterozygous carrier, and 12 affected (homozygous for the mutant allele) dogs, which had been determined in a previous study (Nakamoto et al., 2011; Ashwini et al., 2016), were used to evaluate the results of the genotyping assay, having initially confirmed these genotypes by Sanger sequencing (Kazusa Genome Technologies Ltd., Kisarazu, Japan) using the specific primers listed in Table 1.

Genotyping of the c.846delT Mutation in the Canine CLN7/MFSD8 Gene

The primers and TaqMan minor groove binder (MGB) probes used for the real-time PCR assay (the sequences of which are listed in Table 1) were designed based on the sequences of the canine CLN7/MFSD8 gene in wild-type dogs (GenBank accession no. NC_006601.3). These primers and probes, each of which was linked to a fluorescent reporter dye (6-carboxyrhodamine or 6-carboxyfluorescein) at the 5′-end and a non-fluorescent quencher dye at the 3′-end, were synthesized by a commercial company (Applied Biosystems, Foster City, CA, USA.). For the preparation of DNA templates, we used a disc punched out of the FTA cards for DNA extraction, as previously described (Mizukami et al., 2011). Real-time PCR amplifications were carried out in a final volume of 5 μL consisting of a 2× PCR master mix (TaqMan GTXpress Master Mix, Applied Biosystems), 80× genotyping assay mix (TaqMan SNP Genotyping Assays, Applied Biosystems) containing the specific primers and TaqMan MGB probes, and template DNA. A negative control containing nuclease-free water instead of DNA was included in each run. The cycling conditions consisted of 20 s at 95 °C, followed by 50 cycles of 3 s at 95 °C and 20 s at 60 °C, with a subsequent holding stage at 25 °C for 30 s. The data obtained were analyzed using StepOne version 2.3 (Applied Biosystems). In addition, an allelic discrimination plot was constructed based on the three types of amplification plots (homozygous wild-type, heterozygous, and homozygous mutant). These data were calculated

using software based on the results obtained using the reference DNA samples from the 45 dogs (20 homozygous wild-type, 13 heterozygous carrier, and 12 homozygous mutant).

Statistical Analysis

The allele frequencies obtained in this study were analyzed using Chi-square tests for Hardy-Weinberg equilibrium. Deviation between the measured and expected values were considered statistically significant at $P<0.05$

1.4. RESULTS

We established that the newly designed real-time PCR assay assessed in this study can be used to clearly distinguish genotypes related to NCL in Chihuahuas after 50 cycles of amplification, in the absence of any non-specific allelic amplification (Figure 1). The real-time PCR genotyping results obtained for the control samples were found to be consistent with those obtained based on Sanger sequencing. Moreover, this assay enabled us to construct allelic discrimination plots based on the amplification plots obtained for the three genotypes using the DNA samples obtained from 45 dogs (20 homozygous wild-type, 13 heterozygous carrier, and 12 affected dogs) (Figure 2).

Genotyping based on the screening of puppies revealed that among the population of 1,007 Chihuahuas surveyed, there were 994 homozygous wild-type dogs, 13 heterozygous carriers, and no affected dogs. On the basis of these observations, we estimated a carrier rate of 1.29%. The corresponding mutant allele frequency was 0.00645, indicating that the expected frequencies of homozygous wild-type, heterozygous carrier, and homozygous mutant genotypes were 0.987, 0.0128, and 0.0000417, respectively. Chi-square test analysis ($\chi 2$ = 0.042503, df = 2, *P* value = 0.979) indicated that these three genotypes were in Hardy-Weinberg equilibrium

1.5. DISCUSSION

The clinical signs and history of NCL in Chihuahuas are particularly severe and devastating (Jolly et al., 1994; Kuwamura et al., 2003; Nakamoto et al., 2011). The onset of neurological signs is typically detected between 13 and 21 months of age, and these grow in severity with increasing age. Requests from owners for euthanasia or sudden death attributable to status epilepticus generally occur at 13 to 24 months of age. The signs include visual defect, a startled response to sound and touch, ataxia, and epileptic seizure (Rac and Giesecke, 1975; Jolly et al., 1994; Kuwamura et al., 2003; Nakamoto et al., 2011; Villani et al., 2019). Long term nursing care for affected dogs with such progressive neurological signs can represent a considerable mental burden for their owners, and consequently, even a few cases of NCL among dogs could have a substantial impact on their owners and breeders.

In the present study, we established that the carrier rate and mutant allele frequency of NCL in the Chihuahua study population were 1.29% and 0.00645, respectively. On the basis of this frequency, the expected number of heterozygous carriers and affected dogs among the mean number of Chihuahua puppies (approximately 64,200/year) registered in recent years are 8,234 and 2.67, respectively, thereby indicating that approximately 8,000 carriers and 2 to 3 affected dogs might be bred annually in Japan. Consistent with these estimates, in the past decade, more than 20 affected dogs were molecularly diagnosed in our laboratory, thereby supporting the diagnosis of this disease in Japan (Ashwini et al., 2016; Kolicheski et al., 2016).

Furthermore, reports of affected dogs in multiple countries, including Australia, New Zealand, Japan, Scotland, Italy, England, and Switzerland (Rac and Giesecke, 1975; Jolly and Hartley, 1977; Kuwamura et al., 2003; Nakamoto et al., 2011, Ashwini et al., 2016; Faller et al., 2016; Karli et al., 2016), provide evidence to indicate that the causative c.846delT mutation is al-ready extensively distributed worldwide. Consequently, this raises concerns regarding the health and breeding ethics of Chihuahuas, one of the most popular canine breeds, not only in Japan but also worldwide. Accordingly, given the established lethality of NCL, the figures obtained in this study for the carrier rate (1.29%) and mutant allele frequency (0.00645) are deemed sufficiently high to warrant measures for the control and prevention of the disease.

Real-time quantitative PCR approaches have been widely used for the detection of mutations in genes causing inherited diseases in both humans and non-human animals (Rahman et al., 2014). A notable advance in this regard has been the development of real-time PCR assays performed in combination with the use of FTA cards for sampling, which has eliminated the need for traditional multi-step extraction and purification procedures, thereby enabling rapid processing and reporting of the results within 2 h after sample collection (Mizukami et al., 2011). Moreover, collection of saliva using FTA cards also contributes to reducing the distress caused when handling animals. Accordingly, we believe that the real-time PCR assay designed in this study would make an important contribution in efforts to control and prevent NCL in Chihuahuas.

In the present study, we surveyed puppies directly in order to determine the carrier rate and mutant allele frequency of NCL in Chihuahuas. Ideally, such screening should be conducted for all dogs bred from kennels to comprehensively establish the risk of producing carriers and affected dogs. However, blanket screening of this nature is considered an ineffective and high-cost approach. In addition, breeders should be notified of the detection of carriers, thereby enabling them to identify parent carrier dogs. Consequently, the comprehensive screening of puppies might not contribute effectively to the prevention and eradication of canine inherited diseases, and with respect to preventing the production of affected dogs and reducing the number of carriers, it would be more productive and less costly to survey only breeding dogs reared by breeders and undertake the appropriate management of mating.

In Japan, genotyping surveys have previously been performed for several canine inherited diseases in order to determine the associated carrier rates and mutant allele frequencies, and thereby evaluate the necessity for preventive measures. Among these diseases, lethal disorders characterized by progressive neurological disfunctions include GM1 gangliosidosis in Shiba Inus (carrier rate: 1.02%) (Uddin et al., 2013), GM2 gangliosidosis variant 0 (Sandhoff disease) in Toy Poodles (0.20%) (Rahman et al., 2014), and NCL in Border Collies (8.11%) (Mizukami et al., 2011).

Similar to Chihuahuas, Shiba Inus and Toy Poodles are also particularly popular canine breeds in Japan, and consequently, underlying lethal diseases in these breeds could be expected to have wide-ranging implications, such as those associated with NCL in Chihuahuas. Indeed, as with NCL in Chihuahuas, Shiba Inus affected with GM1 gangliosidosis have continued to be detected at the rate of 1 to 5 affected dogs per year, not only until 2012 (Uddin et al., 2013) but also up to the present day. In contrast, Toy Poodles affected with Sandhoff disease have not been diagnosed since four affected dogs were reported in 2014 (Rahman et al., 2014), which can probably be attributed to the low mutant allele frequency in the Japanese Toy Poodle population, and thus indicates that urgent preventive measures are currently unnecessary. Furthermore, there has been a high mutant allele frequency and an increasing trend among Border Collies affected with NCL since 2012 (Mizukami et al., 2011; Mizukami et al., 2012). However, appropriate remedial measures, including the examination of all breeding dogs, particularly in kennels with a high prevalence, and subsequent appropriate breeding control, have contributed to a declining trend (Mizukami et al., 2012), with no positive cases being reported recently.

Preventive measures should be undertaken in an appropriate manner in full cooperation with involved breeders and nationwide or specific kennel clubs, having initially assessed the current mutant allele frequencies and population numbers of the implicated dog breeds in each region and country. An assessment of the data obtained from systematic and functional

monitoring surveys indicates that continuous removal of carriers from breeding colonies might be the most effective measure for preventing and eradicating fatal hereditary diseases in purebred dogs.

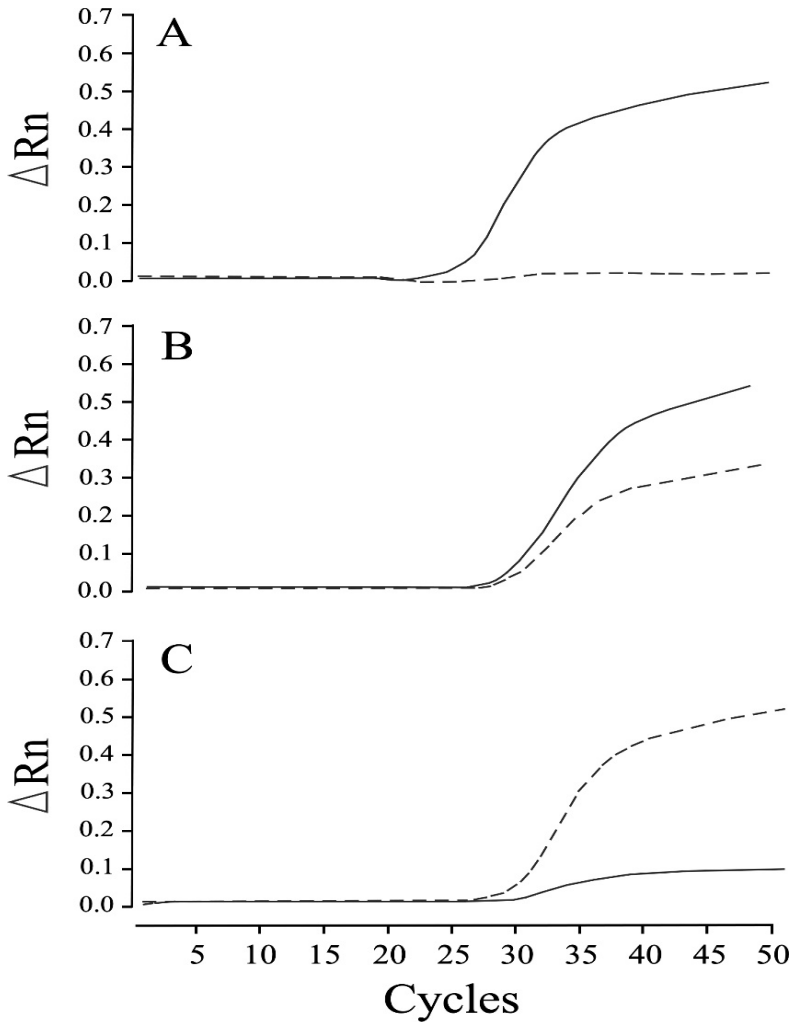

Figure 1. Real-time PCR amplification plots of wild-type and mutant alleles for neuronal ceroid lipofuscinosis in Chihuahuas. Amplification was plotted as fluorescence intensity (ΔRn values) against cycle number. ΔRn values represent the reporter dye signal normalized to that of the in-ternal reference dye and corrected for the baseline signal established in the initial few PCR cycles. The three amplification plots show the homozygous wild-type (A), heterozygous carrier (B), and homozygous mutant (affected) (C) genotypes. The solid and dotted lines indicate amplification in the presence of wild-type and mutant alleles, respectively.

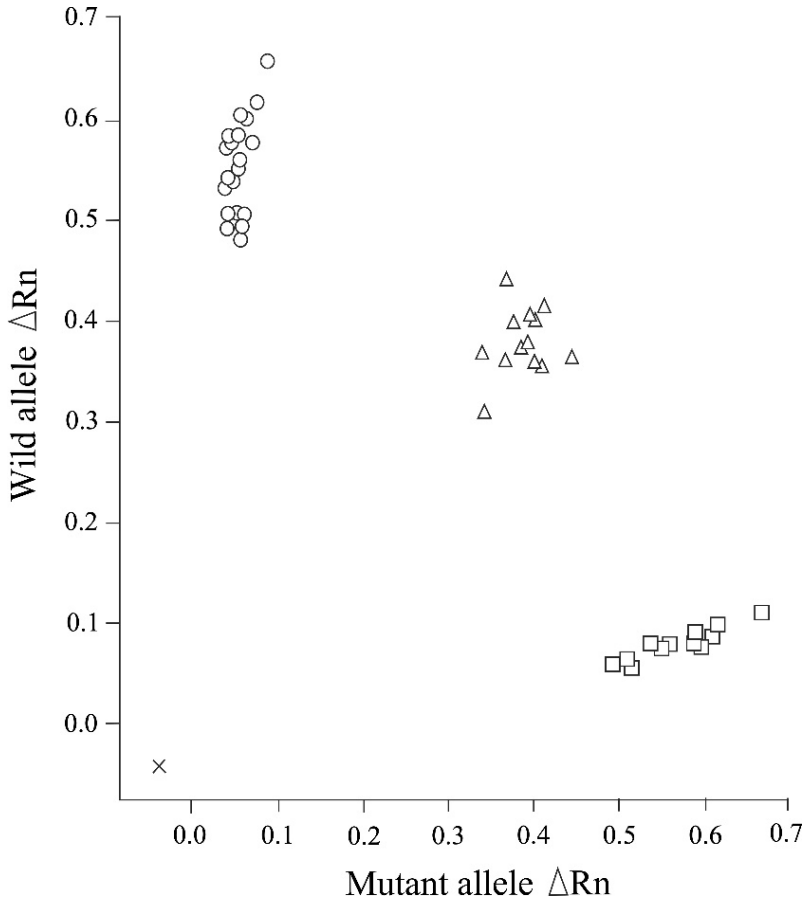

Figure 2. An allelic discrimination plot of end point fluorescence real-time PCR data showing the three genotypes (homozygous wild-type, heterozygous carrier, and homozygous mutant) of neuronal ceroid lipofuscinosis in Chihuahuas. This plot presents the data obtained for 45 representative DNA samples of Chihuahuas that had previously been genotyped. The plot shows the fluorescence intensities (ΔRn values) for each allele. × = no template control; ○ = homozygous wild-type genotype (20 samples); Δ = heterozygous carrier genotype (13 samples); □ = homozygous mutant (affected) genotype (12 samples).

Table 1. Sequences of the primers and probes used in the real-time PCR assay and Sanger sequencing for the c.846delT mutation in the canine *CLN7/MFSD8* gene.

Primer/probe	Sequence 5′ to 3′ (mer)	Position	Reporter (5′)	Quencher (3′)	Tm (°C)	Concentration (nM)
RT-PCR:						
Forward primer	GCTGTTGTGGCCACTAATA TTGTG (24)	c.805_828	NA	NA	66.1	450
Reverse primer	AGAATAAAACTTACGTTTC AAAAAGGGCAA (30)	c.851_866+14	NA	NA	68.1	450
Probe for wild-type allele	TTCGTGATTCTATTTATCT (19)	c.832_850	VIC	NFQ	48.4	100
Probe for mutant allele	TTTTTCGTGATTCTATTATC T (21)	c.829_850	FAM	NFQ	52.3	100

Sanger sequencing:						
Forward primer	GTCATAGAATTTGCTACATATAA TTTC (27)	Intron 7	NA	NA	57.1	NA
Reverse primer	GTTTTGAGAACATTGATATGCTT GAT (26)	Intron 8	NA	NA	62.9	NA

Tm = melting temperature calculated using Oligo Calculator (http://www.ngrl.co.jp/tools/0217oligocalc.htm); NA = not applicable; FAM = 6-carboxyfluorescein; VIC = 6-carboxyrhodamine; NFQ = non-fluorescent quencher

CHAPTER 2

Investigation of GM1 gangliosidosis in miniature Shiba breeding dogs in Japan

The above-titled work originally appeared in "*Animals* (Pervin et al., 2022)" as: **Carrier rate and mutant allele frequency of GM1 gangliosidosis in miniature Shiba Inus (Mame Shiba): population screening of breeding dogs in Japan** authored by:

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2.1. ABSTRACT

GM1 gangliosidosis is a progressive, recessive, autosomal, neurodegenerative, lysosomal storage disorder that affects the brain and multiple systemic organs due to an acid β-galactosidase deficiency encoded by the *GLB1* gene. The disease occurs in the Shiba Inu breed, which is one of the most popular traditional breeds in Japan, due to the *GLB1*:c.1649delC (p.P550Rfs*50) mutation. Previous surveys performed of the Shiba Inu population in Japan found a carrier rate of 1.02-2.94%. Currently, a miniature type of the Shiba Inu called "Mame Shiba", bred via artificial selection to yield smaller individuals, is becoming more popular than the standard Shiba Inu, and it is now one of the most popular breeds in Japan and China. The GM1 gangliosidosis mutation has yet to be surveyed in the Mame Shiba population. This study aimed to determine the frequency of the mutant allele and carrier rate of GM1 gangliosidosis in the Mame Shiba breed. Blood samples were collected from 1832 clinically healthy adult Mame Shiba Inus used for breeding across 143 Japanese kennels. The genotyping was performed using a real-time PCR assay. The survey found nine carriers among the Mame Shibas, indicating that the carrier rate and mutant allele frequency were 0.49% and 0.00246, respectively. This study demonstrated that the mutant allele has already been inherited by the Mame Shiba population. There is a risk of GM gangliosidosis occurrence in the Mame Shiba breed if breeders use carriers for mating. Further genotyping surveys are necessary for breeding Mame Shibas to prevent the inheritance of this disease.

2.2. INTRODUCTION

GM1 gangliosidosis is a progressive, recessive, autosomal, neurodegenerative, lysosomal storage disorder that affects the brain and multiple systemic organs due to an acid β-galactosidase (GLB1, EC 3.2.1.23) deficiency encoded by the *GLB1* gene (Nicoli et al., 2021; Rha et al., 2021). The disease is characterized by an abnormal accumulation of β-linked galactose containing glycoconjugates in the central nervous system, including the glycosphingolipid GM1 ganglioside, resulting in the premature death of affected individuals due to brain damage from progressive neurological malfunctions.

GM1 gangliosidosis occurs in humans [Online Mendelian Inheritance in Man (OMIM) 230500, 230600, and 230650] and several other animal species [Online Mendelian Inheritance in Animals (OMIA) 000402], including dogs, cats, cattle, sheep, emus, American black bears, and murine models (Nicoli et al., 2021). Naturally occurring GM1 gangliosidosis has been reported in many dog breeds, including mixed Beagles (Read et al., 1976), English Springer Spaniels (Alroy et al., 1985), Portuguese Water dogs (Saunders et al., 1988), Alaskan Huskies (Müller et al., 1998), a mixed breed dog (Whitfield et al., 2000), and Shiba Inus (Yamato et al., 2000). To date, three different mutations have been identified in the canine *GLB1* gene that can cause the disease in Portuguese Water dogs (Wang et al., 2000), Shiba Inus (Yamato et al., 2002), and in Alaskan Huskies (Kreutzer et al., 2005). According to the OMIA website (https://www.omia.org/), the causative mutations are located in the canine chromosome 23

(CanFam3.1), specifically, g.3754313G>A (c.179G>A, p.R60H) in Portuguese Water dogs, g.3796317delC (c.1649delC, p.P550Rfs*50) in Shiba Inus, and g.3796356_3796374dup (c.1688_1706dup, p.T570Pfs*22) in Alaskan Huskies.

The Shiba Inu breed, also called the standard Shiba (Table 2 and Figure 1), is an ancient and native basal spitz breed in Japan (Parker et al., 2017), and it was designated as a protected species in 1936 (Uddin et al., 2013). The breed is genetically similar to wolves and differs from European dog breeds (Parker et al., 2004). The breed is indigenous to and has become one of the most popular breeds in Japan, with thirty to forty thousand puppies registered every year in Japan alone (Yamato et al., 2008). Shiba Inus have been transported worldwide and are bred and maintained as a standard breed in many countries. However, in recent years, artificial selection has been used while breeding standard Shiba Inus to produce puppies with a smaller body size, which are called “Mame Shiba”, or the miniature Shiba (Table 2 and Figure 1) (Lyu et al., 2021). “Mame” is a Japanese word that means “bean,” representing “small”. The Mame Shiba is bred from the standard Shiba Inu in order to maintain their purebred status, and both breeds share some common traits, such as coat color. Whole genome sequencing indicates that there may be a link between specific candidate genes body size (Lyu et al., 2021). Although the Shiba Inu and other traditional Japanese breeds, such as the Akita, Kishu, Shikoku, Kai, and Hokkaido (Table 2), are approved by the Japan Kennel Club (JKC), a kennel club certified by the Federation Cynologique Internationale (FCI), and the Nihon-ken Hozonkai (NIPPO), a

kennel club especially for traditional Japanese dog breeds, the Mame Shiba has not been approved by any of these organizations. The Mame Shiba is currently approved and standardized separately by the Kennel Club of Japan (KCJ) and the Nihon Mame Shibaken Association (NMSA), which issue each specific pedigree paper to Mame Shiba Inus. The Mame Shiba is becoming more popular than the standard Shiba Inu, and it is now one of the most popular breeds in Japan and China (Lyu et al., 2021), but the data about the number of registered Mame Shibas is not publicly available.

In 2008, a small-scale molecular survey of GM1 gangliosidosis in 68 standard Shiba Inus was carried out in northern Japan, which found two carrier dogs, indicating a carrier rate of 2.94% (Yamato et al., 2008). Following this, a large scale molecular epidemiological survey was carried out of 590 standard Shiba Inus across all districts of Japan in 2013, which found six carriers, indicating an average carrier rate of 1.02% in Japan and 2.27% in the Kinki district (Uddin et al., 2013). There is a high probability of transferring the mutant allele to the Mame Shiba as it is selected and bred from the standard Shiba Inu. However, the GM1 gangliosidosis mutation has yet to be surveyed in the Mame Shiba population. Therefore, it is necessary to survey the Mame Shiba population to know the current carrier rate and mutant allele frequency, and plan an effective strategy for breeders to control GM1 gangliosidosis in this new, popular canine breed. In this study we performed a large-scale molecular survey of GM1 gangliosidosis in the Mame Shiba population in Japan.

2.3. MATERIALS AND METHODS

The experiments conducted in this study were performed in accordance with the guidelines regulating animal use and ethics at Kagoshima University (no. VM15041; approval date: 29 September 2015), and oral informed consent was obtained from the participating breeders.

Sample Collection and Genotyping

From February 2019 to April 2022, whole blood samples (≦ 0.3 mL) were randomly collected from 1832 clinically healthy adult Mame Shiba Inus (439 males and 1393 females) across 143 kennels in the Kyushu to Kanto districts, Japan, that were born between 2012 and 2021 and are used for breeding. The blood samples were spotted onto Flinders Technology Associates filter papers (FTA card; Whatman International Ltd., Piscataway, NJ, USA) and stored in a refrigerator at 4 °C ready for DNA extraction. DNA was extracted from discs punched out of these FTA cards following appropriate treatment, as previously described (Chang et al., 2010). The genotypes of the dogs were determined using real-time PCR as previously reported (Chang et al., 2010).

Statistical Analysis

The allele frequencies obtained in this study were analyzed using a Chi-square test for Hardy-Weinberg equilibrium. The deviations between the measured and expected values were regarded as statistically significant at $P<0.05$. Differences in the carrier rates between the Mame Shiba population in this study and the standard Shiba Inu populations in previous studies (Yamato et al., 2008; Uddin et al., 2013) were statistically analyzed using Fisher's exact test, with $P<0.05$ considered to be a statistically significant difference. Statistical analyses were performed using R software.

2.4. RESULTS

The real-time PCR clearly determined genotypes of all the samples. Genotyping of Mame Shiba Inus revealed that among the 1832 dogs surveyed, there were 1823 homozygous wild-type dogs, nine heterozygous carriers, and no homozygous mutant dogs. Based on these observations, we estimated a carrier rate of 0.49%. The corresponding mutant allele frequency is 0.00246, indicating expected frequencies of homozygous wild-type, heterozygous carrier, and homozygous mutant genotypes of 0.995, 0.00490, and 0.00000603, respectively. Chi-square test analysis ($\chi 2 = 0.0111$, df = 2, *P* value = 0.995) indicates that these three genotypes were in Hardy-Weinberg equilibrium.

Differences in the carrier rates between the populations of the Mame Shiba in this study (0.49%, 9/1832) and the standard Shiba Inu in our previous studies 2.94%, 2/68 (Yamato et al., 2008); 1.02%, 6/590 (Uddin et al., 2013), were analyzed statistically using Fisher's exact test. There were not significant differences between the carrier rates in the Mame Shiba in this study and the standard Shiba Inu in either study in 2008 ($P = 0.222$) or 2013 ($P = 0.0564$)

2.5. DISCUSSION

Dogs have been associated with humans longer than any other domestic animals, and they play a number of important roles in modern society (enz-Schwarzburg et al., 2020). Many researchers are working to localize genes of interest in dogs, particularly disease-related genes, to understand inherited diseases in humans and to guide dog breeding programs in dogs to improve their health and welfare (Takeuchi et al., 2009). Therefore, knowledge of the inheritance risk for different diseases in a typical dog breed and identifying which diseases spread between dog breeds is valuable for both veterinary care and for breeding healthy dogs; this includes GM1 gangliosidosis in the Shiba Inu and the Mame Shiba.

The clinical signs of GM1 gangliosidosis in the Shiba Inu are very severe with the onset of neurological signs at 5 to 6 months of age, which worsen gradually until death due to the accumulation of GM1 ganglioside in the central nervous system (Yamato et al., 2000; Yamato et al., 2003; Satoh et al., 2007). The age at which euthanasia is requested by the owners or the occurrence of sudden natural death is from 12 to 15 months of age (Yamato et al., 2000), or by 18 months old at the latest. Long-term care for affected dogs with such devastating neurological signs may be a considerable mental and physical burden to their owners. Therefore, even a small number of dogs with GM1 gangliosidosis could have a major impact on owners, breeders, various companies involved in selling dogs, and kennel clubs. Therefore, the appearance of affected dogs and a high frequency of the GM1 gangliosidosis mutant allele are undesirable in

the Mame Shiba breed.

This study revealed that the carrier rate and mutant allele frequency of GM1 gangliosidosis in the breeding population of the Mame Shiba were 0.49% and 0.00246, respectively. They are relatively low compared to the figures form 2008 (2.94% and 0.0147) and 2013 (1.04% and 0.00508) in the standard Shibas (Yamato et al., 2008; Uddin et al., 2013), but the difference is not significant. This suggests that the Mame Shiba has already inherited GM1 gangliosidosis through the establishment of the breed from the standard Shiba Inu. Given the lethality of this disease and the popularity of the Mame Shiba, the corresponding mutant allele frequency (0.00246) is deemed sufficiently high to warrant measures for disease control and prevention, as in the standard Shiba Inu.

In Japan, molecular epidemiological surveys have previously been performed for several canine inherited diseases in order to determine the associated carrier rates and mutant allele frequencies, and thereby evaluate the necessity of prevention measures (Mizukami et al., 2011; Rahman et al., 2014; Pervin et al., 2022). Among these diseases, lethal disorders characterized by progressive neuro-logical disfunctions include Sandhoff disease in Toy Poodles (carrier rate = 0.20%) (Rahman et al., 2014), neuronal ceroid lipofuscinosis (NCL) in Border Collies (8.11%) (Mizukami et al., 2011), and NCL in Chihuahuas (1.29%) (Pervin et al., 2022). Similar to Shiba Inus, Toy Poodles and Chihuahuas are also particularly popular canine breeds in Japan; according to the JKC, from 2008 to 2021, they were the first and second most commonly

registered breeds in Japan, respectively. Consequently, underlying lethal diseases in these popular breeds could be expected to have wide-ranging implications, such as those associated with GM1 gangliosidosis in standard Shiba Inus and Mame Shibas. Indeed, standard Shiba Inus affected by GM1 gangliosidosis have continued to be detected at the rate of 1-5 dogs per year (Uddin et al., 2013). In addition, GM1 gangliosidosis has been diagnosed in two standard Shiba Inus in China and Korea, respectively (personal information, O.Y.). The Mame Shiba is becoming more popular in Japan and China (Lyu et al., 2021), and therefore, the prevention and eradication of GM1 gangliosidosis in Mame Shibas is recommended with respect to preventing the production of affected dogs and reducing the number of carriers. Comprehensive screening of all puppies, for example, blanket screening, is considered ineffective and prohibitively expensive in a large population like Chihuahuas (Pervin et al., 2022). Continued genotyping should be performed on breeding Mame Shibas, and breeders should appropriately manage mating. This prevention strategy would be more effective and lower cost, especially in the large populations of Shiba Inus and Mame Shibas.

Besides GM1 gangliosidosis, a causative mutation for Sandhoff disease has been identified in standard Shiba Inus in the United States (Kolicheski et al., 2017; Wang et al., 2018). The Shiba Inu is genetically predisposed to glaucoma (Kato et al., 2006), which is potentially associated with certain genes (Kanemaki et al., 2013). The Shiba Inu is also predisposed to chronic enteropathy (Ohmi et al., 2011; Ohmi et al., 2017) and atopic dermatitis,

which are related to certain genetic backgrounds (Ohmi et al., 2017; Tanaka et al., 2020). In addition, the Shiba Inu has a special phenotype that usually has no clinical sign, but they are more susceptible to onion-induced hemolytic anemia and one of its causative agents (NPTS) than other at the same dosage (Yamato et al., 1999). It is important for their total health management to clarify whether these genetic characteristics have been transferred to the Mame Shiba. However, body size, inbreeding, and deleterious morphologies should also be considered (Bannasch et al., 2021). It is important not only to monitor inherited or genetic diseases, but also the dis-orders caused by the potentially excessive miniaturization of the Mame Shiba.

Figure 1. Typical appearance of a Shiba Inu (S: standard type) and a Mame Shiba Inu (M: miniature type). The color coat of these dogs is red, the most popular among the four coats found in this breed (red, black-and-tan, sesame, and white).

Table 2. Withers height of Japanese dog breeds.

Body size	Breed*	Club**	Sex	Withers height (cm)		
				Standard/Ideal	Range	Limitation
Large	Akita	NIPPO	Male	67	64–70	ND
			Female	61	58–64	
Middle	Kai, Hokkaido	NIPPO	Male	52	47–55	ND
			Female	49	44–52	
	Kishu, Shikoku	NIPPO	Male	52	49–55	ND
			Female	49	46–52	
Small	Shiba	NIPPO	Male	39.5	38–41	ND
			Female	36.5	35–38	
Miniature	Mame Shiba	KCJ	Male	ND	30–34	≧ 25
			Female	ND	28–32	
		NMSA	Male	30	25–34	Caution (< 25)
			Female	28	25–32	

* The Japanese traditional breeds Akita, Kishi, Shikoku, Kai, Hokkaido, and Shiba are approved by the Nihon-ken Hozonkai (NIPPO: https://www.nihonken-hozonkai.or.jp) and the Japan Kennel Club (JKC: https://www.jkc.or.jp), which is certified by the Federation Cynologique Internationale (FCI: http://www.fci.be/). These two kennel clubs issue pedigree papers to registered dogs, but the Mame Shiba is not approved by either kennel club.

** The standards of withers height for Japanese breeds (except for Mame Shiba) are provided by NIPPO. The Mame Shiba is approved and standardized separately by the Kennel Club of Japan (KCJ: http://www.kcj.gr.jp/index.html) and the Nihon Mame Shibaken Association (NMSA: https://nmsa.jpn.com), which issue specific pedigree papers to registered Mame Shibas. ND: not determine

CONCLUSION

On the basis of the results obtained in this study (Chapter 1), we established that in Japan, the carrier rate of NCL in Chihuahuas is currently 1.29%, and given the lethality of this disease, the corresponding mutant allele frequency (0.00645) is deemed sufficiently high to warrant measures for disease control and prevention. Ideally in this regard, it is considered important to conduct extensive molecular screening of breeding Chihuahuas in JKC-registered kennels throughout Japan. We believe that the genotyping assay designed in this study will make a potentially valuable contribution to the control and prevention of NCL in Chihuahuas.

Furthermore, the results of this study (Chapter 2) show that the carrier rate of GM1 gangliosidosis in the Mame Shiba in Japan is currently 0.49%, and given the lethality of this disease and the popularity of this breed, the corresponding mutant allele frequency (0.00246) is deemed sufficiently high to warrant measures for disease control and prevention. Ideally, continued genotype surveying should be performed on breeding Mame Shibas reared by breeders who undertake appropriate mating management.

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