Molecular study on GM1 gangliosidosis, Canavan disease and Krabbe disease in dogs and cats

(犬および猫の **GM1** ガングリオシドーシス、カナバン病およびクラッベ病に関する分子的研究)

The United Graduate School of Veterinary Science
Yamaguchi University

Mohammad Mejbah UDDIN

March 2014

MOLECULAR STUDY ON GM1 GANGLIOSIDOSIS, CANAVAN DISEASE AND KRABBE DISEASE IN DOGS AND CATS

犬および猫の **GM1** ガングリオシドーシス、カナバン病およびクラッベ病に関する分子的研究

ACADEMIC DISSERTATION

Presented to
The United Graduate School of Veterinary Sciences
Yamaguchi University, Japan

By

MOHAMMAD MEJBAH UDDIN

Laboratory of Veterinary Clinical Pathology

Joint Faculty of Veterinary Medicine

Kagoshima University, Japan

In partial fulfillment of requirements for the degree of

Doctor of Philosophy

In

Veterinary Medicine

March 2014

Major supervisor

Osamu YAMATO, DVM, PhD

Professor

Laboratory of Veterinary Clinical Pathology

Joint Faculty of Veterinary Medicine

Kagoshima University, Japan

Co-supervisors

Yasuaki KAWASAKI, DVM, PhD

Professor

Laboratory of Behavioral Physiology and Ecology

Joint Faculty of Veterinary Medicine

Kagoshima University, Japan

Yoshinao HOSAKA, DVM, PhD

Associate Professor

Laboratory of Veterinary Anatomy

School of Veterinary Medicine

Tottori University, Japan

Akira YABUKI, DVM, PhD

Associate Professor

Laboratory of Veterinary Clinical Pathology

Joint Faculty of Veterinary Medicine

Kagoshima University, Japan

Shiho FUJITA, DVM, PhD

Associate Professor

Laboratory of Behavioral Physiology and Ecology

Joint Faculty of Veterinary Medicine

Kagoshima University, Japan

THESIS COPYRIGHT DECLARATION

I, **Mohammad Mejbah Uddin**, hereby certify that this thesis has been written by me, that it is the record of work carried out by me (unless where stated), and that it has not been submitted in any previous application for a higher degree.

Signature of the author: -------------------------------------

Date: 13th March 2014

DEDICATION

This thesis is dedicated to my beloved mother, uncles, wife, daughter (Neesa Mejbah) and departed soul of my father and grandparents.

ABSTRACT

In human medicine, the molecular bases of many inherited disorders have been clarified, and furthermore, the number of molecularly defined disorders continues to increase. In veterinary medicine, many naturally occurring, inherited disorders have been reported, but the molecular bases of many such disorders have yet to be defined. Rapid and accurate genotyping of inherited disorders is very important for reaching swift diagnoses and improving patient outcomes, as well as the control and prevention of the disease in populations of pure-bred animals. In the present study, molecular investigations of GM1 gangliosidosis, Canavan disease, and Krabbe disease were performed in dogs and cats. The objectives of my thesis are to formulate effective strategies for the control and prevention of GM1 gangliosidosis in the Shiba Inu breed of dogs (Chapter 1) and discuss the significance and distribution of feline GM1 gangliosidosis caused by the c.1448G>C mutation of the β-galactosidase (*GLB1*) gene (Chapter 2), and in addition to clarifying the molecular bases of canine Canavan disease (Chapter 3) and feline Krabbe disease (Chapter 4).

Chapter 1: GM1 gangliosidosis is a fatal, progressive neurodegenerative lysosomal storage disease caused by mutations in the *GLB1* gene. A real-time PCR-based genotyping assay has been developed for detecting the pathogenic mutation c.1647delC in the canine *GLB1* gene in the Shiba Inu dogs with GM1 gangliosidosis. In the present study, a molecular epidemiological survey was conducted using a genotyping assay among 590 clinically unaffected Shiba Inu dogs from all over Japan. The number and native district of the affected dogs identified between 1997 and 2013 were also surveyed retrospectively. Of the 590 dogs examined, 6 dogs (1.02%, 6/590) were carriers: 3 dogs (2.27%, 3/132) were from the Kinki district, while the other 3 dogs from the Hokkaido, Kanto, and Shikoku districts. The retrospective survey revealed 23 affected dogs,

among which 19 dogs (82.6%) were born within the last 7 years. Of the 23 affected dogs, 12 dogs (52.2%) were from the Kinki district. Pedigree analysis demonstrated that all the affected and carrier dogs for which pedigree information was available had a close blood relationship. The high carrier frequency in the Kinki district may be related to the high prevalence observed over the past 16 years in this region. Therefore, for the effective control and prevention of the disease, it is necessary to examine as many breeding dogs as possible from all regions of Japan, especially those from kennels located in areas with high prevalence and carrier frequency.

Chapter 2: In feline GM1 gangliosidosis, a pathogenic mutation c.1448G>C in the feline *GLB1* gene has previously been identified in Siamese and Korat cats in Western countries. In the present study, a mutation analysis was performed on a Siamese cat diagnosed histopathologically and biochemically with the disease in Japan in the 1960s and 2 domestic cats suspected of being affected with a lysosomal disease in Bangladesh in 2009. The presence of the c.1448G>C mutation was examined using a direct sequencing method and a PCR-restriction fragment length polymorphism assay with the restriction endonuclease *Hae*III. This analysis demonstrated that the 3 cats were homozygous for the c.1448G>C mutation. Identification of the mutation in a Siamese cat in Japan and in native domestic cats in Bangladesh, in concert with the history of cat domestication, suggests that the c.1448G>C mutation may have been transferred from native domestic cats to Siamese and Korat breeds through the process of breed establishment in Southeastern Asia.

Chapter 3: Canavan disease is an autosomal recessive neurodegenerative leukodystrophy that is caused by mutations in the aspartoacylase (*ASPA*) gene. In order to clarify the molecular basis of a mixed-breed dog affected with Canavan disease, the canine *ASPA* gene was analyzed using a direct DNA sequencing method. As a result, a homozygous deletion-duplication mutation,

c.[535_540delGTTGGT]+[527_534dupGTATAGAA], in exon 4 of the canine *ASPA* gene was identified as a candidate pathogenic mutation for the disease. A genotyping survey was conducted using a real-time PCR-based assay for the mutation among 1,508 dogs. The survey demonstrated that all dogs examined were of the wild-type genotype, suggesting that the mutation is very rare, and therefore, pathogenic.

Chapter 4: Krabbe diseaseis an autosomal recessive neurodegenerative leukodystrophy that is caused by mutations in the galactocerebrosidase (*GALC*) gene. In order to clarify the molecular basis of a mixed-breed cat affected with Krabbe disease, the feline *GALC* gene was analyzed using a direct DNA sequencing method. As a result, a homozygous missense mutation, c.1945G>A (p.A649T), in exon 17 of the feline *GALC* gene was identified as a candidate pathogenic mutation for the disease. A genotyping survey was conducted using a real-time PCR-based assay for the mutation among 1,060 cats. The survey demonstrated that all cats examined were of the wild-type genotype, suggesting that the mutation is very rare, and therefore, pathogenic.

In conclusion, the molecular epidemiological information for GM1 gangliosidosis in the Shiba Inu breed in Japan will contribute extensively to the control and prevention of the disease. A newly developed PCR-based genotyping assay for feline GM1 gangliosidosis will be important for rapid differential diagnosis in cats suspected of having the disease and for genotyping in purebreeds related to Siamese and Korat cats. The molecular bases of canine Canavan disease and feline Krabbe disease are the first to be defined. Therefore, these findings will contribute to future studies in the attempts to better understand the pathogenesis and to develop potential therapeutic methods in both human and veterinary medicine.

TABLE OF CONTENTS

LIST OF TABLES..........x

LIST OF FIGURES..........xi

FREQUENTLY USED ABBREVIATIONS..........xiii

PREFACE..........1

CHAPTER 1: Molecular epidemiology of GM1 gangliosidosis in the Shiba Inu breed dogs in Japan..........4

1.1. ABSTRACT..........5

1.2. INTRODUCTION..........6

1.3. MATERIALS AND METHODS..........8

1.4. RESULTS..........9

1.5. DISCUSSION..........11

CHAPTER 2: Mutation analyses of feline GM1 gangliosidosis..........18

SECTION-A: Mutation analysis of GM1 gangliosidosis in a Siamese cat in Japan..........18

2A.1. ABSTRACT..........19

2A.2. INTRODUCTION..........20

2A.3. MATERIALS AND METHODS..........22

2A.5. RESULTS..........24

2A.6. DISCUSSION..........25

SECTION-B: Mutation analysis of GM1 gangliosidosis in Bangladeshi domestic cats..........28

2B.1. ABSTRACT..........29

2B.2. INTRODUCTION……………………………………………………………………...30

2B.3. MATERIALS AND METHODS……………………………….……………..32

2B.4. RESULTS………………………………………………………………….....34

2B.5. DISCUSSION……………………………………………………….…....35

CHAPTER 3: Molecular basis of Canavan disease in a mixed-breed dog……………….....37

3.1. ABSTRACT………………………………………………………………...38

3.2. INTRODUCTION……………………………………………………….....39

3.3. MATERIALS AND METHODS……………………………………………...41

3.4. RESULTS…………………………………………………………......…43

3.5. DISCUSSION…………………………………………………….....……..44

CHAPTER 4: Molecular basis of Krabbe disease in a mixed-breed cat……………..…..53

4.1. ABSTRACT…………………………………………………….………..54

4.2. INTRODUCTION……………………………………………….……....55

4.3. MATERIALS AND METHODS…………………………………….……..57

4.4. RESULTS…………………………………………………………………......59

4.5. DISCUSSION…………………………………………………….…..…....60

CONCLUSION…………………………………………………………………………72

REFERENCES…………………………………………………………………………..74

ACKNOWLEDGEMENTS……………………………………...……………..…85

LIST OF TABLES

Table 1. Results of the nation-wide genotyping survey of GM1 gangliosidosis in Shiba Inu dogs in Japan.

Table 2. The c.[535_540delGTTGGT]+[527_534dupGTATAGAA] mutation screening of the canine *ASPA* gene with Canavan diseasein dogs by the real-time PCR genotyping assay.

Table 3. Characteristics of primers and amplified fragments in the direct sequencing analysis of the canine *ASPA* gene.

Table 4. Primers and TaqMan probes used in the real-time PCR genotyping assay for the mutation screening of canine Canavan disease.

Table 5. Results of mutational analysis in the exons and flanking intronic regions of the canine *ASPA* gene.

Table 6. The c.1945G>A mutation screening of the feline *GLAC* gene with Krabbe disease in domestic cats by the real-time PCR genotyping assay.

Table 7. Characteristics of primers and amplified fragments in the direct sequencing analysis of the feline *GALC* gene.

Table 8. Primers and TaqMan probes used in the real-time PCR genotyping assay for the mutation screening of feline Krabbe disease.

Table 9. Results of mutational analysis in the exons and flanking intronic regions of the feline *GALC* gene.

LIST OF FIGURES

Figure 1. Map of Japan showing the districts surveyed in the present study.

Figure 2. Change in the number of Shiba Inu dogs with GM1 gangliosidosis from 1977 to 2012.

Figure 3. The pedigree of ShibaInu dogs with GM1 gangliosidosis.

Figure 4. Partial genomic sequence electropherograms of exon 14 in the feline *GLB1* gene from the wild-type and affected cats.

Figure 5. PCR-RFLP assay using agarose gel electrophoresis in the wild-type and affected cats.

Figure 6. PCR-RFLP assay using agarose gel electrophoresis to analyze a wild-type cat and the 2 affected cats (animals 1 and 2).

Figure 7. Partial genomic sequence electropherograms of exon 4 in the canine *ASPA* gene from wild-type and affected dogs.

Figure 8. Real-time PCR amplification plots of wild-type and mutant alleles in canine Canavan disease.

Figure 9. Allelic discrimination plot of end-point fluorescence real-time PCR data showing the 2 genotypes of canine Canavan disease.

Figure 10. Partial genomic sequence electropharograms of exon 17 in the feline *GALC* gene from wild-type and affected cats.

Figure 11. Evolutionary comparison of sequences in the vicinity of GALC Thr.649 (arrow) from various species.

Figure 12. SIFT analysis showing all substitution at p.649 A in the feline GALC enzyme

Figure 13. Probability matrix of SIFT analysis showing the score of the p.A649T

Figure 14. Real-time PCR amplification plots of wild-type and mutant alleles in feline Krabbe disease.

Figure 15. Allelic discrimination plot of end-point fluorescence real-time PCR data showing the 2 genotypes of feline Krabbe disease.

FREQUENTLY USED ABBREVIATIONS

A	-	adenine
A	-	alanine
ASPA	-	aspartoacylase
bp	-	base pair
C	-	cytosine
c.	-	coding DNA sequence
cDNA	-	complementary deoxyribonucleic acid
del	-	deletion
DNA	-	deoxyribonucleic acid
EC	-	Enzyme Commission
fs	-	frameshift
FTA	-	Flinders Technology Associates
G	-	guanine
G	-	glycine
GALC	-	galactocebrosidase
GLB1	-	β-galactosidase
GM	-	monosialic ganglioside
NAA	-	*N*-acetyl-aspartic acid
OMIM	-	Online Mendelian Inheritance in Man
p.	-	protein sequence
PCR	-	polymerase chain reaction
RFLP	-	restriction fragment length polymorphism
T	-	threonine
T_m	-	melting temperature
UTR	-	untranslated region
X	-	translation termination (stop codon)

PREFACE

Inherited disorders are a group of over 200 single gene disorders primarily inherited as autosomal recessive traits. Many of these disorders carry serious clinical consequences to the affected neonate or young infant, which include mild or severe irreversible mental retardation, physical handicaps or even fatality. An accurate diagnosis of inherited disorder is very important for reaching a rapid and favorable patient outcome (Rashed 2001). Some of the more common and important inherited disorders include GM1 gangliosidosis, Krabbe disease and Canavan disease.

GM1 gangliosidosis (OMIM #230500), a lysosomal storage disease that affects the brain, is caused by an autosomal recessive deficiency in acid β-galactosidase (EC 3.2.1.23), which is encoded by the *GLB1* gene (Suzuki et al., 2001). Lysosomal β-galactosidase is required for the degradation of GM1 ganglioside, other glycolipids, and glycoproteins that contain a terminal galactose moiety. Deficiency of this enzyme leads to the storage of massive amounts of GM1 ganglioside and related glycoconjugates in tissues, particularly in the central nervous system, resulting in progressive neurodegeneration and premature death. In domestic animals, naturally occurring GM1 gangliosidosis has been reported in cats, dogs, calves, and sheep (Suzuki et al., 2001; Sewell et al., 2007).

Canavan disease (OMIM #271900) is an autosomal recessive leukodystrophy caused by deficiency of aspartoacylase (EC 3.5.1.15) enzyme due to mutations in the aspartoacylase (*ASPA*) gene (Kaul et al., 1993). Aspartoacylase catalyses the deacetylation of *N*-acetyl-aspartic acid (NAA) into acetate and aspartate in oligodendrocytes. The enzyme actively participates in myelin synthesis by providing NNA-derived acetate for acetyl coenzyme A synthesis, which in turn is used for synthesis of lipid portion of myelin. Consequently, Canavan disease results from

defective myelin synthesis due to a deficiency in the supply of the NAA-derived acetate (Namboodiri et al., 2006). Though the exact role of NAA in the brain remains a matter of investigations (Namboodiri et al., 2006), but the proper metabolism of this compound in the brain is important for correct development and maintenance of the white matter (Matalon et al., 2000). Neurological symptoms of Canavan disease include progressive psychomotor retardation, ataxia, seizures, hypotonia and spasticity. In the veterinary literature, naturally occurring leukodystrophies have been described in cattle, sheep, cats, rabbits, foxes and dogs (Wood and Patterson 2001).

Krabbe disease (OMIM #245200) or globoid cell leukodystrophy is a rapidly progressive demyelinating disease, is caused by mutations in the gene coding for the lysosomal enzyme galactocerebrosidase (GALC; EC 3.2.1.46), which is responsible for the hydrolysis of the galactosyl moiety from several galactolipids, mostly galactosylceramide and galactosylsphingosine (psychosine). These galactsphingolipids are normally synthesized during active myelination and psychosine accumulates in the nervous system with very low GALC activity. Psychosine is highly toxic to the myelin-forming cells, causing their death and the paucity of myelin (Wenger et al., 2001). The excess of galactosylceramide elicits the formation of multinucleated macrophages, the globoid cells found around cerebral blood vessels in the white matter of affected human patients and animal models (Suzuki and Grover, 1970). In veterinary species, naturally occurring Krabbe have been reported in dogs, cats, sheep, mouse (Suzuki and Suzuki, 1985), rhesus monkey (Luzi et al., 1997) and rhesus macaques (Borda et al., 2008).

In the present study, GM1 gangliosidosis, Canavan disease and Krabbe disease in dogs and cats were investigated molecularly. The objectives of my thesis are to formulate an effective

strategy for control and prevention of GM1 gangliosidosis in Shiba Inu breed dogs in Japan, and to discusses the distribution and significance of feline GM1 gangliosidosis caused by the c.1448G>C mutation of the feline *GLB1* gene. Furthermore, the thesis clarifies the molecular basis of Canavan disease and Krabbe disease in a dog and a cat respectively, and develops new molecular diagnostic methods for diagnosis and/or genotyping.

The study undertaken is reported in four chapters, two of which are published in peer-reviewed journals, and two other are manuscripts in preparation for publication. In the chapter 1, molecular epidemiology of canine GM1 gangliosidosisin Shiba Inu breed dogs was investigated in Japan. In chapter 2, mutation analyses of feline GM1 gangliosidosis were studied in a Siamese cat in Japan and two native domestic cats in Bangladesh. In the chapter 3 and 4, molecular bases of the Cnavan disease and Krabbe disease were studied in a mixed-breed dog and a mixed-breed cat respectively.

CHAPTER 1

Molecular epidemiology of GM1 gangliosidosis in the ShibaInu breed in Japan

The above-title work originally appeared in"*BMC Veterinary Research* (Uddin et al., 2013)" as ***Molecular epidemiology of canine GM1 gangliosidosis in the ShibaInu breed in Japan: relationship between regional prevalence and carrier frequency*** authored by:

Mohammad Mejbah UDDIN [1,2], Sayaka ARATA [3], Yukari TAKEUCHI [3], Hye-Sook CHANG [1], Keijiro MIZUKAMI [1], Akira YABUKI [1], Mohammad Mahbubur RAHMAN [1,2], Moeko KOHYAMA [1], Mohammad Alamgir HOSSAIN [2], Kenji TAKAYAMA [4], Osamu YAMATO [1]*

[1] Laboratory of Clinical Pathology, Joint Faculty of Veterinary Medicine, Kagoshima University, Kohrimoto, Kagoshima, Japan.

[2] Faculty of Veterinary Medicine, Chittagong Veterinary and Animal Sciences University, Chittagong, Bangladesh.

[3] Laboratory of Veterinary Ethology, Department of Animal Resources Science, The University of Tokyo, Tokyo, Japan.

[4] Hayashiya Animal Medical Center, Rokujizonara-cho, Uji-shi, Kyoto, Japan.

1.1. ABSTRACT

Canine GM1 gangliosidosis is a fatal disease in the ShibaInu breed, which is one of the most popular traditional breeds in Japan and is maintained as a standard breed in many countries. Therefore, it is important to control and reduce the prevalence of GM1 gangliosidosis for maintaining the quality of this breed and to ensure supply of healthy dogs to prospective breeders and owners. This molecular epidemiological survey was performed to formulate an effective strategy for the control and prevention of this disease. The survey was carried out among 590 clinically unaffected Shiba Inu dogs from the 8 districts of Japan, and a genotyping test was used to determine nation-wide and regional carrier frequencies. The number and native district of affected dogs identified in 16 years from 1997 to April2013 were also surveyed retrospectively. Of the 590 dogs examined, 6dogs (1.02%, 6/590) were carriers:3 dogs (2.27%, 3/132) from the Kinki district and the other 3 dogs from the Hokkaido, Kanto, and Shikoku districts. The retrospective survey revealed 23 affected dogs, among which, 19 dogs (82.6%) were born within the last 7 years. Of the 23 affected dogs, 12 dogs (52.2%) were from the Kinki district. Pedigree analysis demonstrated that all the affected dogs and carriers with the pedigree information have a close blood relationship. Our results showed that the current carrier frequency for GM1 gangliosidosis is on the average 1.02% in Japan and rather high in the Kinki district, which may be related to the high prevalence observed over the past 16 years in this region. This observation suggests that carrier dogs are distributed all over Japan; however, kennels in the Kinki district may face an increased risk of GM1 gangliosidosis. Therefore, for effective control and prevention of this disease, it is necessary to examine as many breeding dogs as possible from all regions of Japan, especially from kennels located in areas with high prevalence and carrier frequency.

1.2. INTRODUCTION

GM1 gangliosidosis, a lysosomal storage disease that affects the brain and multiple systemic organs, is caused by an autosomal recessively inherited deficiency in acid β-galactosidase, which is encoded by the *GLB1* gene (Suzuki et al., 2001). GM1 gangliosidosis in ShibaInu dogs was first reported in 2000 (Yamato et al., 2000). The causative mutation has been identified as a deletion of the cytosine in exon 15 at nucleotide position 1647 in the putative coding region (c.1647delC) of the canine *GLB1*gene (Yamato et al., 2002), thereby enabling molecular diagnosis and/or genotyping with PCR-based DNA tests (Chang et al., 2010; Yamato et al., 2004). Affected dogs manifest neurological signs of progressive motor dysfunction from 5–6 months of age and die at 12–18 months after a clearly defined clinical course (Hasegawa et al., 2012; Yamato et al., 2003), which is associated with progressive accumulation of GM1 ganglioside and the subsequent neuronal damage in the central nervous system (Yamato et al., 2002; Satoh et al., 2007; Hasegawa et al., 2012;).

The Shiba Inu breed is indigenous to and one of the most popular breeds in Japan, where it has been designated as a protected species since 1936 (http://www.nihonken-hozonkai.or.jp/). Thirty to forty thousand puppies are registered every year in Japan (Yamato et al., 2008). Shiba Inu dogs have been transported worldwide and are bred and maintained as a standard breed in many countries. Therefore, it is necessary to control and reduce the prevalence of GM1 gangliosidosis to maintain the quality of this traditional breed and to ensure supply of healthy dogs to prospective breeders and owners in Japan and other countries.

Previously, a preliminary genotyping survey of the disease was carried out on 68 Shiba Inu dogs from only the Hokkaido and the Tohoku districts (Figure 1) in northern Japan (Yamato et al., 2008). This survey revealed 2 carriers among the 68 dogs. Although, carriers appear to be

distributed all over Japan, specific areas and kennels may show high prevalence. Therefore, a large-scale and nationwide survey is necessary to know the current overall and regional prevalence and carrier frequency in Japan, as well as to formulate an effective strategy for the control and prevention of this disease.

In the present study, a large-scale molecular epidemiological survey of GM1 gangliosidosis in the Shiba Inu breed was carried out among 590 dogs from all the districts of Japan by using a genotyping test to determine the current overall and regional carrier frequency. The number and native district of affected dogs identified in 16 years from 1997 to May 2013 were also surveyed retrospectively. The pathway of transmission and distribution of the mutant allele were analyzed on the basis of pedigree information of the affected dogs and carriers identified previously and in this study. In this report, we also discuss the control and prevention of the disease on the basis of the results of these analyses.

1.3. METHODS AND MATERIALS

Sample collection and genotyping

Blood samples were collected randomly from 590 client-owned Shiba Inu-breed dogs with the cooperation of their owners and veterinarians from different animal hospitals in Japan. These dogs were clinically unaffected for GM1 gangliosidosis. The dogs were native to the Japanese districts shown in Table 1 and Figure 1. The genotypes of the dogs were determined using real-time PCR as previously reported (Chang et al., 2010).

Retrospective survey and pedigree analysis

The number and the native district of the affected dogs were surveyed retrospectively based on the records in our laboratory, which has been exclusively supporting the diagnosis of this disease in Japan. Pedigree analysis was performed to elucidate the genetic relationships among the affected dogs and carriers identified previously and in the present study, as well as to deduce the pathway of transmission and distribution of the mutant allele. The genetic relationships among the affected dogs and carriers were analyzed using the pedigree records issued by the Nihon-ken Hozon-kai(http://www.nihonken-hozonkai.or.jp/) and the Japan Kennel Club (http://www.jkc.or.jp/), a kennel club certified by the Federation Cynologique Internationale (http://www.fci.be/).

All the experimental procedures using animals and their specimens were performed in accordance with the guidelines regulating animal use and ethics issued by the Experimental Animals Committee at Kagoshima University.

1.4. RESULTS

Carrier frequency

The results of the nationwide molecular epidemiological survey are shown in Table 1. This survey revealed 584 wild-type dogs, 6 carriers, and no affected dogs in the population of 590 animals. The carrier frequency in the population was 1.02%. Among the 6 carriers identified, 3 dogs were from the Kinki district and the other 3 dogs were from the Hokkaido, Kanto, and Shikoku districts.

Retrospective survey and pedigree analysis

The retrospective survey conducted using information collected over 16 years from 1997 to 2013 revealed 23 affected dogs (A1–A23) and several carriers that were related to these affected dogs. The first affected dog (A1-H), born in the Hokkaido district in 1997, was diagnosed with GM1 gangliosidosis histopathologically and biochemically (Yamato et al., 2000), which was confirmed by molecular analysis using stored DNA and RNA (Yamato et al., 2002). Since this case, other affected dogs with the same molecular defect have been diagnosed sporadically until recently (Figure 2). Of the 23 affected dogs identified, 19 dogs were born within the last 7 years. The pedigree relationships among these affected dogs and the native district of each dog are shown in Figure 3. Although pedigree data were not available for 5 affected dogs, the remaining 18 affected dogs and several related carriers identified previously showed a blood relationship, according to the results of the pedigree analysis. Regarding the native districts, 12 affected dogs were from the Kinki region, accounting for approximately half of all the affected animals.

A pedigree analysis was also performed using the data from 2 carriers (C1-Ka and C2-Ki) identified in the present epidemiological survey. No pedigree data was available for the 4

remaining carriers. C1-Ka and C2-Ki also showed a blood relationship with the affected dogs and carriers identified in the retrospective survey (Figure 3).

1.5. DISCUSSION

Recessively inherited disorders such as lysosomal storage diseases are a major problem in the breeding of pure-breed domestic animals (Baker et al., 2001; Yamato et al., 2008; Yamato et al., 2009). Identification of the carriers that have one abnormal allele in the gene pair, but that are normal in clinical appearance, is critical because there are no physical clues to the presence of the disease in these animals; however, the mutation is transmitted to half of their progeny (Yamato et al., 2009). The frequency of carriers in a population substantially exceeds the incidence of the disease; therefore, the presence of carriers makes recessive diseases the most difficult to control in pure-breed animals (Yamato et al., 2009; Mizukami et al., 2011). Canine GM1 gangliosidosis has been reported in other pure-breed dogs including English Springer Spaniels (Alroy et al., 1985), Portuguese Water Dogs (Saunders et al., 1988), and Alaskan Huskies (Müller et al., 1998); however, to the best of our knowledge, a large-scale molecular epidemiological survey has not been performed to establish effective measures for the control and prevention of the disease.

Our results demonstrated that the overall carrier frequency of GM1 gangliosidosis among ShibaInu dogs in Japan is 1.02% (6/590). In this study, regional data for northern (i.e., Hokkaido and Tohoku districts) and western or southern Japan (i.e., Chugoku, Shikoku, and Kyushu districts) were not fully evaluated because of the low number of samples collected from these regions. Among the districts sufficiently surveyed by a large number of samples, Kinki had the highest carrier frequency (2.27%, 3/132), which is more than 5 times that in Kanto (0.44%, 1/227). As the number of carriers is small, the calculated frequency could potentially be changed dramatically by chance. Specific families that include carriers may have also affected the

frequency (i.e., population stratification). Therefore, the limitation of this study is that the calculated disease prevalence and carrier frequency may be influenced by chance.

In the retrospective survey, in the last 16 years, 23 affected dogs were identified, and 19 dogs of these were born within the last 7 years (Figure 2), suggesting a recent increase in the prevalence of the disease. Of the 23 affected dogs identified, 12 dogs were from the Kinki district. Of the 19 affected dogs identified in the last 7 years, 11 dogs were from the Kinki district, suggesting a recent increase in the prevalence in Japan, probably due to the high incidence of affected dogs in the Kinki district. The high incidence of affected dogs in the Kinki district seems to be attributable to a small number of popular carrier sires (shown by an asterisk in Figure 3) in the same region.

There are 8 district affiliates in Nihon-ken Hozon-kai, a major kennel club specializing in Japanese traditional breeds (http://www.nihonken-hozonkai.or.jp/). Furthermore, each district affiliate has several regional branch divisions with many kennels. Most kennels are small with only a few breeding dogs. Regional activities such as local dog-shows are carried out in each branch division before the district and nationwide activities. Therefore, there is a strong relationship among the kennels belonging to the same branch division. Mating also tends to be among dogs in the same branch division, resulting in potential inbreeding and homozygosity responsible for inherited disorders.

In the present study, a kennel with a very high prevalence of the disease was found in the Kinki district by pedigree analysis. This kennel housed 2 carriers, who were divisional champion dogs (shown by an asterisk in Figure 3), which produced many of the affected and carrier offspring in the Kinki district. Such kennels may be producing affected dogs sporadically by unknowingly mating carriers. It is thought that affected dogs are produced in a small proportion

of kennels with high prevalence, and the mutant allele is then distributed to the surrounding areas from those kennels. This may be the reason behind the relationship between regional prevalence and carrier frequency in GM1 gangliosidosis in Shiba Inu dogs. A similar trend was observed in a survey of neuronal ceroid lipofuscinosis in the Border Collie breed in Japan (Mizukami et al., 2012). Therefore, for the effective control and prevention of these fatal inherited diseases, it is necessary to examine as many breeding dogs as possible, especially from kennels located in areas with high prevalence and carrier frequency. In addition, genotyping of specimens from a random population of breeding dogs should be continued to detect sporadic carriers and prevent their use from being used as breeding dogs (Mizukami et al., 2011). These approaches will also gradually decrease the number of dogs carrying the mutant allele. These active and continuous preventive measures may be necessary to eliminate GM1 gangliosidosis in Shiba Inu dogs.

Table 1. Results of the nation-wide genotyping survey of GM1 gangliosidosis in Shiba Inu dogs in Japan

District	Examined dogs	Genotypes	
		Wild-type	Carrier
Hokkaido	23	22	1
Tohoku	17	17	0
Kanto	227	226	1
Chubu	135	135	0
Kinki	132	129	3
Chugoku	9	9	0
Shikoku	2	1	1
Kyushu*	42	42	0
Unknown	3	3	0
Total	590	584	6

* Including Okinawa prefecture, the small islands south of Kyushu Island

Figure 1. Map of Japan showing the districts surveyed in the present study.

Bold lines show district boundaries on the main island, and dashed lines show prefectural boundaries.

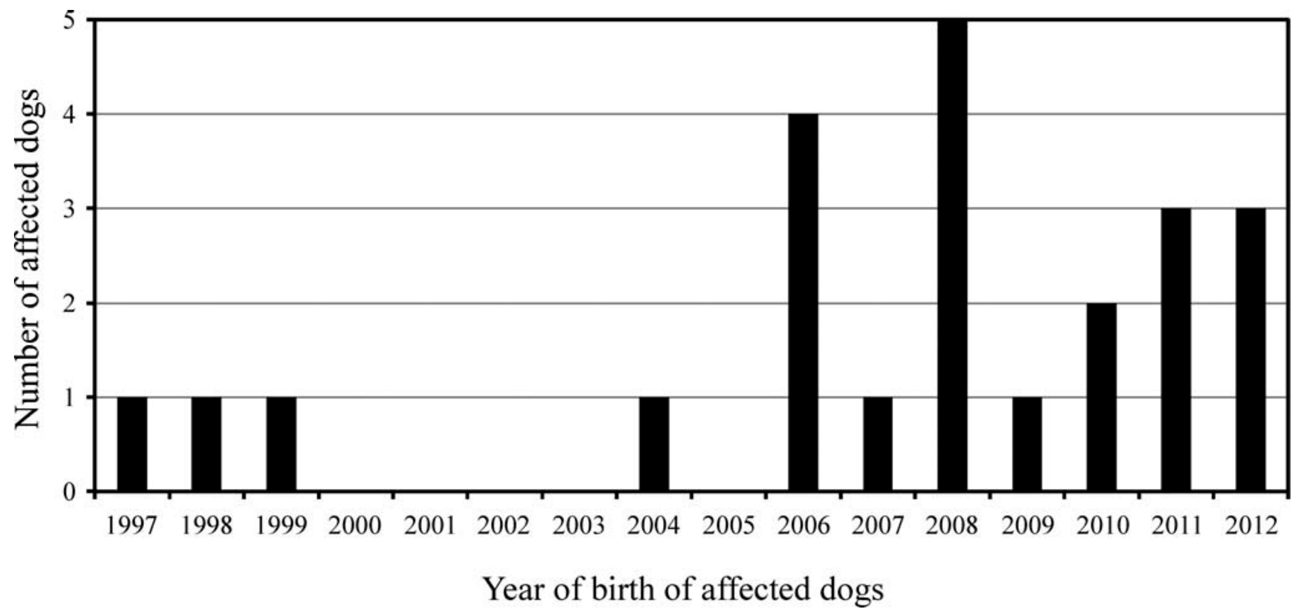

Figure 2. Change in the number of Shiba Inu dogs with GM1 gangliosidosis from 1977 to 2012. The 23 affected dogs (A1–A23) were plotted in the order in which they were born.

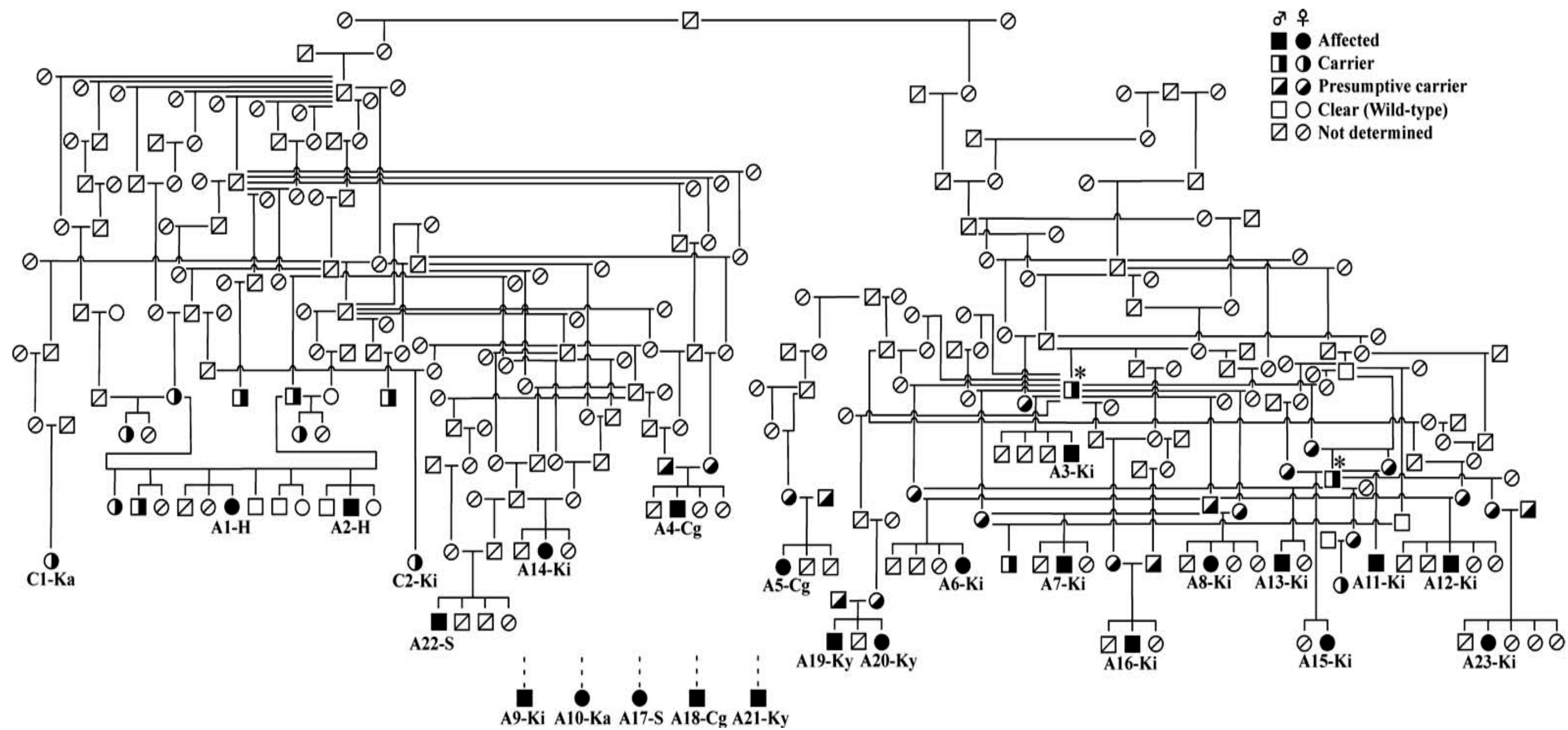

Figure 3. The pedigree of Shiba Inu dogs with GM1 gangliosidosis.

The 23 affected dogs (A1–A23) are from the Hokkaido (-H), Kinki (-Ki), Chugoku (-Cg), Kanto (-Ka), Shikoku (-S), and Kyushu (-Ky) districts. Pedigree information was not available for 5 affected dogs (A9, A10, A17, A18, and A21). Two carriers (C1-Ka and C2-Ki) were identified in the Kanto and Kinki districts, respectively, in the molecular epidemiological survey of this study. * Carriers from a specific kennel, which are related to a number of affected and carrier offspring in the Kinki district.

CHAPTER 2

Mutation analyses of feline GM1 gangliosidosis

SECTION-A

Mutation analysis of GM1 gangliosidosis in a Siamese cat in Japan

The above-title work originally appeared in "*Journal of Feline Medicine and Surgery* (Uddin et al., 2012)" as ***Mutation analysis of GM1 gangliosidosis in a Siamese cat from Japan in the 1960s*** authored by:

Mohammad MejbahUDDIN [1,2], Takeshi TANIMOTO [1], Akira YABUKI[1], Takao KOTANI[3], Mitsuru KUWAMURA [3], Hye-Sook CHANG [1], Osamu YAMATO[1,*]

[1] Laboratory of Clinical Pathology, Joint Faculty of Veterinary Medicine, Kagoshima University, Japan.

[2] Faculty of Veterinary Medicine, Chittagong Veterinary and Animal Sciences University, Bangladesh.

[3] Laboratory of Veterinary Pathology, Graduate School of Life & Environmental Sciences, Osaka Prefecture University, Japan.

2A.1. ABSTRACT

GM1 gangliosidosis is a fatal, progressive neurodegenerative lysosomal storage disease caused by mutations of the β-galactosidase (*GLB1*) gene. In feline GM1 gangliosidosis, a pathogenic mutation (c.1448G>C) of the feline *GLB1* gene was identified in Siamese and Korat cats previously diagnosed with the disease in the United States and Italy, respectively. The present study demonstrated the same mutation in a Siamese cat that had been diagnosed with GM1 gangliosidosis in Japan in the 1960s. The mutation was confirmed using DNA extracted from stored paraffin-embedded brain tissue by a direct sequencing method and a polymerase chain reaction-restriction fragment length polymorphism assay. This pathogenic mutation seems to have been distributed around the world.

2A.2. INTRODUCTION

GM1 gangliosidosis is a fatal, progressive neurodegenerative lysosomal storage disease caused by mutations of the β-galactosidase (*GLB1*) gene (Suzuki et al., 2001). Feline GM1 gangliosidosis was first reported in Siamese cats in 1971 in Japan (Handa and Yamakawa 1971) and the United States (Baker et al., 1971) individually as different occurrences at the respective locations. Subsequently, the disease was reported in mixed-breed cats in the United Kingdom (Blakemore 1972; Barker et al., 1986) and Korat cats in Italy (De Maria et al., 1998). In general, affected cats manifest neurological symptoms of progressive motor dysfunctions starting from 4 to 6 months of age and die prematurely by approximately 1 year of age. A pathogenic mutation has been identified as a single nucleotide substitution from guanine to cytosine in exon 14 at nucleotide position 1448 (c.1448G>C) in the coding region of the feline *GLB1* gene, resulting in the substitution of arginine with proline at amino acid position 483 (p.R483P), in Siamese cats in the United States (Martin et al., 2008). The disease in Korat cats has been demonstrated to be caused by the same mutation (Baker et al., 2001; Wang et al., 2007).

Among Siamese cats with GM1 gangliosidosis diagnosed in Japan, several affected cats from one family were reported in 1969 as having cerebral lipidosis similar to human Tay-Sachs disease, a variant of GM2 gangliosidosis (Kuruhara et al., 1969). This feline disease was subsequently revealed to be GM1 gangliosidosis in 1970 (Kuruhara and Mochizuki 1970). As mentioned, a definitive biochemical diagnosis established by a deficiency of β-galactosidase activity with the accumulation of GM1 ganglioside in brain tissue was described in 1971 (Handa and Yamakawa 1971), but the pathogenic mutation(s) in this family had yet to be determined.

In the present study, genomic DNA extracted from stored brain tissue of a Siamese cat with GM1 gangliosidosis, born in Japan in the 1960s, was analyzed to determine whether it has the

same pathogenic mutation as that (c.1448G>C) previously identified in Siamese and Korat cats with GM1 gangliosidosis in the West. This paper also discusses the significance of the mutation.

2A.3. METHODS AND MATERIALS

Genomic DNA was extracted from paraffin-embedded cerebral tissue of an 8-month-old Siamese cat with GM1 gangliosidosis. The affected cat was one of 3 animals diagnosed with the disease histopathologically and biochemically, which belonged to a family of Siamese cats with 6 clinically-affected members born between 1966 and 1967 after the backcross mating of a pair of cats (Kuruhara et al., 1969; Kuruhara and Mochizuki 1970; Handa and Yamakawa 1971).Stored genomic DNA from whole blood of a mix-breed, clinically healthy adult cat was used as a control.

DNA was used for polymerase chain reactions (PCRs) in direct DNA sequencing and the PCR-restriction fragment length polymorphism (RFLP) assay. Direct sequencing of a 106-base pair (bp) DNA fragment including position 1448 in exon 14 of the feline *GLB1*gene was performed using forward (5’-AGC GAA GTT ACG TGA TCA CTC T-3’, c.1370–1391) and reverse (5’-CTA TGG CAG ATA CAT CAA TGA-3’, c.1445–1475) primers designed based on the exonic sequence of the feline *GLB1*gene (GenBank accession no. AF029974). The PCR fragment was purified by agarose gel electrophoresis and sequenced in the forward and reverse direction.

The PCR-RFLP assay was performed to confirm the presence of c.1448G>C in genomic DNA of the affected cat. The PCR was carried out targeting an 82-bp sequence around the mutation site using forward (5’-GAG CCA CTC TGG ACC TTC TG-3’, c.1412–1431) and reverse (5’-TCA ATG ATT TCA AGG TAG GAC CAG C-3’, c.1469–1482+11) primers designed based on the exonic and intronic sequences of the feline *GLB1*gene (AF029974 and ACBE01328632, respectively). To detect c.1448G>C, the amplification product was digested by a restriction endonuclease, *Hae*III (New England Biolabs, Ipswich, MA, USA). Both the PCR

and digested products were subjected to electrophoresis in 4% agarose (Agarose 21; Nippon Gene). The mutation was judged based on two, 47- and 35-bp, fragments due to the digestion site (GG|CC) in the mutant sequence, whereas the control cat had a single 82-bp band which was undigested due to the wild-type sequence (GGCG).

2A.4. RESULTS

The direct sequencing data for the affected Siamese cat and control mixed-breed cat were compared to those in the GenBank database, i.e., the wild-type (AF006749) and mutant (AF029974) sequences. The affected Siamese cat was found to be homozygous for the c.1448G>C mutation whereas the control cat was homozygous for the wild-type sequence at position 1448 (Figure 4). The PCR-RFLP assay also demonstrated that the affected cat was homozygous for the mutant sequence (GGCC) digested by *Hae*III, but the control cat was not (Figure5).

The results of direct DNA sequencing and a PCR-RFLP assay demonstrated the pathogenic mutation in a Siamese cat diagnosed with GM1 gangliosidosis in Japan in the 1960s to be c.1448G>C, the same as that previously identified in Siamese and Korat cats with the disease in the United States and Italy, respectively (Baker et al., 1971; De Maria et al., 1998; Martin et al., 2008). Only a specimen from one of several affected Siamese cats was available, but it is strongly suspected that the GM1 gangliosidosis in these animals was caused by the common c.1448G>C mutation because all the affected cats were born to the same parents (Kuruhara et al., 1969).

2A.5. DISCUSSION

Cats had been thought to be domesticated in ancient Egypt, where the animal was considered sacred, some time before 1600 BC (Vella et al., 1999), but recent genetic data points to multiple domestication events in separate locations in the Near East 10000 years ago (O'Brien et al., 2008). Subsequent gradual human migration then spread domestic cats across the globe. Modern cat pure-breeds range from the earliest fancy breeds including Egyptian Mau, Persian, Siamese, and Korat established around the 17th century to the most recent breeds such as American Curl and Selkirk Rex established during the late 20th century (Vella et al., 1999; O'Brien et al., 2008). The Siamese and Korat breeds originate from Thailand (Vella et al., 1999).

Based on the history of cat domestication and breed establishment, the occurrence of the same pathogenic mutation at distant locations in the Siamese and Korat breeds suggests that the mutation originates from the Southeast Asian Siamese region and has been distributed in the population of these breeds around the world. Actually, the carrier frequency is high (16.7%, 38/227) in Korat cats from a number of countries (Kuruhara et al., 1969). The results of the present study suggest that there may be the mutation in Siamese cats in the world. Furthermore, since one of the large groups of breeds is derived completely or in part from Southeast Asian ancestors (Vella et al., 1999), GM1 gangliosidosis caused by the same mutation may also occur in those breeds (Oriental Shorthair, Balinese, Havana Brown, Birman, Burmese, Singapura, and others). Thus, since c.1448G>C has possibly spread to related pure-breeds around the world, a PCR-based diagnostic test is important for rapid differential diagnosis in cats suspected of having the disease and/or for genotyping to control and prevent the mutation in pure-breeds related to Siamese and Korat cats. The PCR-RFLP assay developed in the present study and a real-time PCR assay reported previously (Wang et al., 2007) can assist with this.

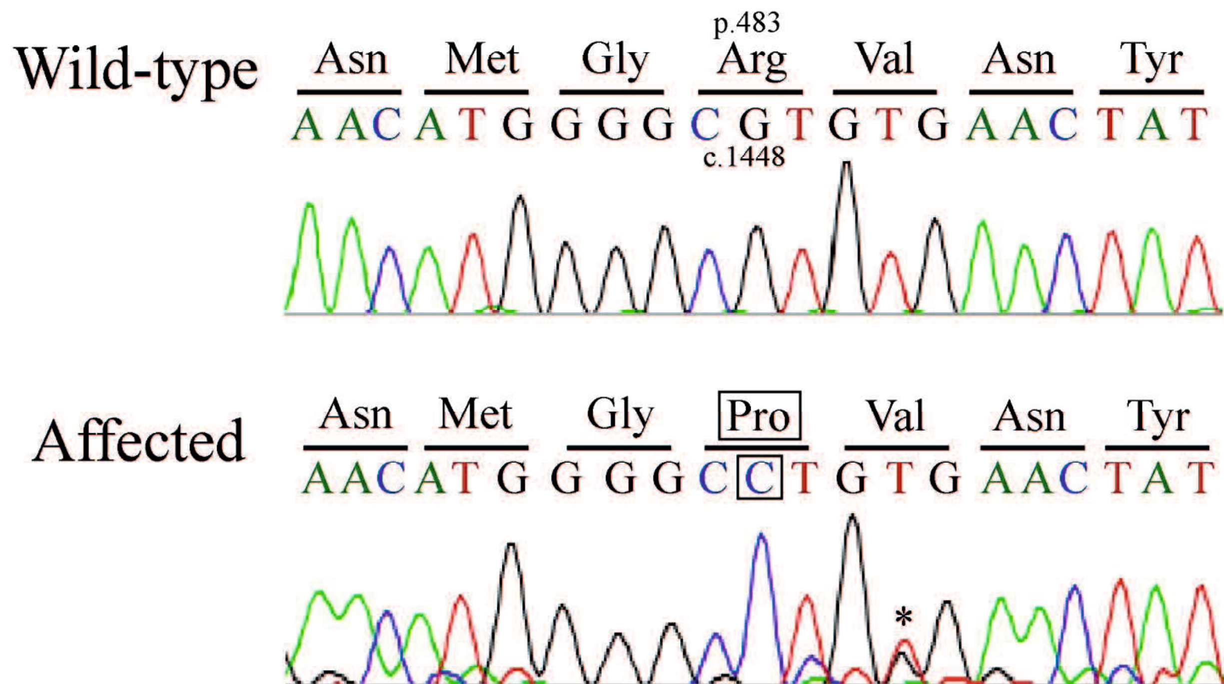

Figure 4. Partial genomic sequence electropherograms of exon 14 in the feline *GLB1* gene from the wild-type and affected cats. The guanine at the coding nucleotide position 1448 is substituted with a cytosine in the affected cat (c.1448G>C). The nucleotide substitution causes the substitution of an arginine at amino acid position 483 with a proline (p.R483P). * This signal was estimated as a thymine although noise from guanine-like signal interfered with the reading.

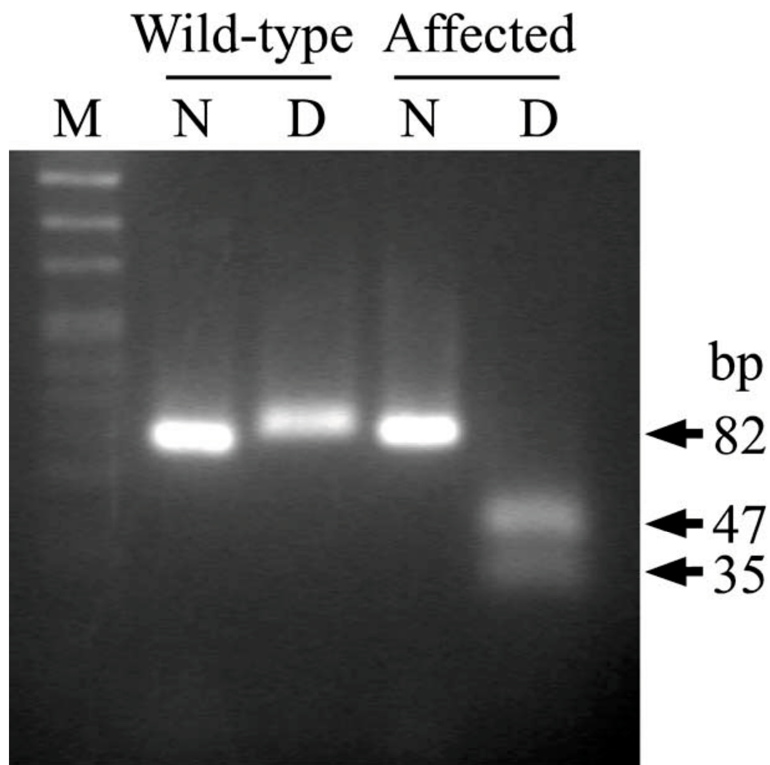

Figure 5. Polymerase chain reaction-restriction fragment length polymorphism assay using agarose gel electrophoresis in the wild-type and affected cats. Amplified DNA without digestion (N) and DNA digested with the restriction endonuclease *Hae*III (D) were analyzed simultaneously. Lane M shows molecular size markers. bp: base pairs.

SECTION-B

Mutation analysis of GM1 gangliosidosis in Bangladeshi domestic cats

The above-title work originally appeared in "*TheJournal of Veterinary Medical Science* (Uddin et al., 2012)" as ***Identification of Bangladeshi domestic cats with GM1 Gangliosidosis caused by the c.1448G>C mutation of the feline GLB1 gene: case study*** authored by:

Mohammad Mejbah UDDIN[1,2], Mohammad Alamgir HOSSAIN[1,2], Mohammad Mahbubur RAHMAN[1,2], Morshedul Alam CHOWDHURY[2], Takeshi TANIMOTO[1], Akira YABUKI[1], Keijiro MIZUKAMI[1], Hye-Sook CHANG[1], Osamu YAMATO[1,*]

[1]Laboratory of Clinical Pathology, Joint Faculty of Veterinary Medicine, Kagoshima University, Kohrimoto, Kagoshima, Japan

[2]Faculty of Veterinary Medicine, Chittagong Veterinary and Animal Sciences University, Chittagong, Bangladesh

2B.1. ABSTRACT

GM1 gangliosidosis is a fatal, progressive neurodegenerative lysosomal storage disease caused by mutations in the β-galactosidase (*GLB1*) gene. In feline GM1 gangliosidosis, a pathogenic mutation (c.1448G>C) in the feline *GLB1* gene was identified in Siamese cats in the United States and Japan and in Korat cats in Western countries. The present study found the homozygous c.1448G>C mutation in 2 apparent littermate native kittens in Bangladesh that were exhibiting neurological signs. This is the first identification of GM1 gangliosidosis in native domestic cats in Southeast Asia. This pathogenic mutation seems to have been present in the domestic cat population in the Siamese region and may have been transferred to pure breeds such as Siamese and Korat cats originating in this region.

2B.2. INTRODUCTION

GM1 gangliosidosis, a lysosomal storage disease that affects the brain, is caused by an autosomal recessive deficiency in acid β-galactosidase (EC 3.2.1.23), which is encoded by the *GLB1* gene (Suzuki et al., 2001). Lysosomal β-galactosidase is required for the degradation of GM1 ganglioside, other glycolipids, and glycoproteins that contain a terminal galactose moiety. Deficiency of this enzyme leads to the storage of massive amounts of GM1 ganglioside and related glycoconjugates in tissues, particularly in the central nervous system, resulting in progressive neurodegeneration and premature death. In domestic animals, naturally occurring GM1 gangliosidosis has been reported in cats, dogs, calves, and sheep (Sewell et al., 2007; Suzuki et al 2001)

Feline GM1 gangliosidosis has been reported in Siamese cats in Japan (Handa and Yama kawa 1971) and the United States (Baker et al., 1971) and in Korat cats in Italy (De Maria et al.,1998). The disease has also been reported in non-pure breed domestic cats in the United Kingdom (Baker et al., 1986; Blakemore et al., 1972) and Japan (Kawasaki et al., 2009; Mochizuki et al., 1977). In general, affected cats manifest neurological signs of progressive motor dysfunctions starting from 4 to 6 months of age and die prematurely by approximately 1 year of age. A pathogenic mutation is a single nucleotide substitution from guanine to cytosine in exon 14 at nucleotide position 1448 (c.1448G>C) in the coding region of the feline *GLB1* gene, resulting in the substitution of arginine with proline at amino acid position 483 (p.R483P). This mutation has been found in Siamese cats in the United States (Martin et al., 2008) and Japan (Uddin et al., 2012) and in Korat cats in the United States, Canada, and several European countries (Baker et al., 2001; Wang et al., 2007). However, to our knowledge, the molecular basis of the disease has yet to be defined for non-pure breed domestic cats.

This case study covers 2 native domestic cats in Bangladesh that were affected by GM1 gangliosidosis caused by the homozygous c.1448G>C mutation. This paper also discusses the meaning of this mutation based on the observations in this study.

2B.3. MATERIALS AND METHODS

In Chittagong, Bangladesh in 2009, 2 stray female shorthair kittens, approximately 6 months old, that appeared to be littermates (animals 1 and 2) exhibiting head and limb tremors and dirty hair coats were brought by a veterinary school student (one of the authors, MAC) to the Veterinary Teaching Hospital, Chittagong Veterinary and Animal Sciences University, Chittagong, Bangladesh. Because the 2 cats were suspected of having an inherited neurodegenerative disorder such as a lysosomal storage disease, DNA from whole blood was transferred to the Laboratory of Clinical Pathology, Kagoshima University, Japan, for genetic tests. Due to the poor predicted prognosis, these cats were taken back to the place where they had been found without any clinical or laboratory examination and were not followed up.

In the Japanese laboratory, molecular diagnostic tests for feline GM1 and GM2 gangliosidoses previously identified and molecularly defined in Japan were carried out using PCR-RFLP methods (Rahman et al 2011;Uddin et al 2012). The PCR-RFLP assay for feline GM1 gangliosidosis was slightly modified in the present study. Briefly, a 326-base pair (bp) DNA fragment including position 1448 in exon 14 of the feline *GLB1*gene was amplified using forward (5'-AGA GCA ATG TCT CCC GAG TCT G-3', c.1351–122 to c.1351–101) and reverse (5'-GAG GAA GTC TTT GTA AAG CCA T-3', c.1482+51 to c.1482+72) primers designed based on the exonic and intronic sequences of the feline *GLB1*gene (GenBank accession nos. AF006749 and ACBE01328632, respectively). To detect c.1448G>C, the amplified product was digested by a restriction endonuclease, *Hae*III (New England Biolabs, Ipswich, MA, USA). Both the unprocessed and digested PCR products were subjected to electrophoresis in 3% agarose (Agarose 21, Nippon Gene, Tokyo, Japan). The presence of the mutation was reflected by the presence of 2 fragments, 218 and 108 bp, due to the restriction site

(GG|CC) in the mutant sequence, whereas the absence of the mutation in the control was reflected by a single 326-bp band that remained undigested due to the wild-type sequence (GGCG). Direct DNA sequencing was also performed to confirm the homozygous c.1448G>C mutation in the genomic DNA of the 2 affected cats using a general sequencing protocol with the same primer pair as for the PCR-RFLP assay.

2B.4. RESULTS

The PCR-RFLP assay for feline GM1 gangliosidosis demonstrated that animals 1 and 2 were homozygous for c.1448G>C, but the control cat was not (Figure 5). The PCR-RFLP assay for feline GM2 gangliosidosis demonstrated that animals 1 and 2 did not have the mutant allele for GM2 gangliosidosis (data not shown). The direct DNA sequencing data confirmed that animals 1 and 2 were homozygous for the c.1448G>C mutation (data not shown). The molecular diagnostic tests demonstrated that animals 1 and 2, both Bangladeshi domestic shorthair cats, had GM1 gangliosidosis caused by the c.1448G>C mutation. This is the first identification of non-pure breed domestic cats with molecularly defined GM1 gangliosidosis. This is also the first report of feline GM1 gangliosidosis in cats in Southeast Asia around the Siamese region. The results of the present study strongly suggest that the c.1448G>C mutation has existed in the domestic cat population in Bangladesh or the Siamese region.

2B.5. DISCUSSION

Cats are thought to have been domesticated in ancient Egypt, where the animal was considered sacred, some time before 1600 BC (Vella et al., 1999), but recent genetic data points to multiple domestication events in separate locations in the Near East 10000 years ago (O'Brien et al., 2008). Subsequent gradual human migration then spread domestic cats across the globe. Modern cat pure breeds range from the earliest fancy breeds including Egyptian Mau, Persian, Siamese, and Korat, which were established around the 17th century, to the most recent breeds, such as American Curl and Selkirk Rex, which were established during the late 20th century (O'Brien et al., 2008; Vella et al., 1999). The Siamese and Korat breeds originated from Southeast Asian ancestors living around the Siamese region (Vella et al., 1999). The c.1448G>C mutation may have been transferred from native domestic cats from the Siamese region to the pure breeds such as Siamese and Korat cats in the process of breed establishment, though the common mutation might have occurred separately in domestic and pure breed cats. As mentioned above, GM1 gangliosidosis has already been reported in non-pure breed domestic cats in the United Kingdom (Baker et al., 1986; Blakemore et al., 1972) and Japan (Kawasaki et al., 2009; Mochizuki et al., 1977), although molecular diagnoses have yet to be made. The disease in these domestic cats might have been caused by the c.1448G>C mutation. Domestic cats carrying this mutation may exist around the world, especially in Southeast Asia. Further studies are required to clarify these important issues for feline clinical genetics.

Figure 6. Polymerase chain reaction-restriction fragment length polymorphism assay using agarose gel electrophoresis to analyze a wild-type cat and the 2 affected cats (animals 1 and 2). Amplified DNA without digestion (N) and DNA digested with the restriction endonuclease *Hae*III (D) were analyzed simultaneously. Lane M shows the molecular size markers. bp: base pairs.

CHAPTER 3

Molecular basis of Canavan disease in a mixed-breed dog

The above-titled manuscript was prepared for submission as: ***A deletion/insertion in the canine aspartoacylase (ASPA) gene causes a frameshift mutation and Canavan disease in a mixed-breed dog*** and authored by:

Mohammad Mejbah UDDIN [1,2], Keijiro MIZUKAMI [1], Mohammad Mahbubur RAHMAN [1,2], Hye-Sook CHANG [1], Mohammad Alamgir HOSSAIN [1,2], Akira YABUKI [1], Kazuya KUSHIDA [1], Moeko KOHYAMA [1], Osamu YAMATO [1, *]

[1] Laboratory of Clinical Pathology, Joint Faculty of Veterinary Medicine, Kagoshima University, Kohrimoto, Kagoshima, Japan.

[2] Faculty of Veterinary Medicine, Chittagong Veterinary and Animal Sciences University, Khulshi, Chittagong, Bangladesh.

3.1. ABSTRACT

Canavan disease, an autosomal recessive neurodegenerative leukodystrophy, is caused by mutations in the aspartoacylase (*ASPA*) gene. Genomic DNA sequence analysis of the canine *ASPA* gene in an affected mixed-breed dog revealed a homozygous 6-base pair (GTTGGT) deletion and 8-base pair (GTATAGAA) duplication in exon 4 at the coding nucleotide position 535 to 540 and 527 to 534 respectively (c.[535_540delGTTGGT]+[527_534dupGTATAGAA]) as the first identification of a pathogenic mutation. This mutation has the potential to cause a frameshift, resulting in the alteration of glycine at amino acid position 180 to a stop codon (p.G180fsX). We implemented a real-time polymerase chain reaction-based assay for diagnosis and carrier screening of the disease in dogs.

3.2. INTRODUCTION

Canavan disease (OMIM #271900) is an autosomal recessive leukodystrophy caused by deficiency of aspartoacylase (EC 3.5.1.15) enzyme due to mutations in the aspartoacylase (*ASPA*) gene (Kaul et al., 1993). Aspartoacylase catalyses the deacetylation of *N*-acetyl-aspartic acid (NAA) into acetate and aspartate in oligodendrocytes. The enzyme actively participates in myelin synthesis by providing NNA-derived acetate for acetyl coenzyme A synthesis, which in turn is used for synthesis of lipid portion of myelin. Consequently, Canavan disease results from defective myelin synthesis due to a deficiency in the supply of the NAA-derived acetate (Namboodiri et al., 2006). Though the exact role of NAA in the brain remains a matter of investigations (Namboodiri et al., 2006), but the proper metabolism of this compound in the brain is important for correct development and maintenance of the white matter (Matalon et al., 2000). Neurological symptoms of Canavan disease include progressive psychomotor retardation, ataxia, seizures, hypotonia and spasticity. Human patients become symptomatic as early as 3 to 6 months of age and death typically by the third decade. The disease is also characterized by dysmyelination and progressive spongiform degeneration in white matter that can be envisioned non-invasively using proton magnetic resonance imaging (MRI) (Baslow and Guilfoyle, 2013). Other clinical diagnostic telltales of Canavan disease include the presence of elevated levels of NAA either in urine/plasma or cerebro-spinal fluid/brain, or enzymatically in cultured skin fibroblast (Baslow and Guilfoyle, 2013). Both methods have drawback as there is a wide variation in the amount of NAA excreted and because the enzyme assay is very sensitive to assay conditions (Sistermans et al., 2000). Therefore, mutational analysis of *ASPA* gene is a reliable alternative for confirmatory diagnosis (Zeng et al., 2006a). To date, more than 70 mutations have been reported in human *ASPA* gene with Canavan disease (Zeng et al., 2006b).

In the veterinary literature, naturally occurring leukodystrophies have been described in cattle, sheep, cats, rabbits, foxes and dogs (Wood et al., 2001). In dog breeds, leukodystrophies resembling human Canavan disease has been described in Dalmatians, Springer Spaniels, Samoyeds, Chow Chows, Weinmaraners, Lurchers, Bernese Mountains, Silkie Terriers, Labrador Retrievers and Shetland Sheepdogs (Wood et al., 2001). Most are considered to be inherited and the molecular defect had not been previously determined in any cases.

In this paper, we describe a molecular defect of Canavan disease in a mixed-breed dog as the first identification of a potentially pathogenic mutation in the canine *ASPA* gene and a rapid and simple genotyping assay for the diagnosis and survey of this mutant allele.

3.3. MATERIALS AND METHODS

Animals and specimens

A sporadic mixed-breed female dog, has been suffering from progressive neurological disorders for 2 years. The affected dog was euthanized at approximately 8years of age due to poor prognosis and owner request. Laboratory investigations of the affected dog revealed elevated level of urinary NAA (Sreenivasan et al., 2013) and brain atrophy with hydrocephalus, observed by gas chromatography-mass spectrometry and MRI respectively (data not shown) (Leone at al., 2012). Blood sample of the affected dog was spotted on Finders Technology Associates filter paper (FTA card, Whatman) for direct DNA sequence analysis. The genotyping survey was carried out using DNA samples from blood-spotted FTA cards of randomly-selected 1,508 dogs including 500 Japanese Shiba Inu breed dogs, other 20 pure breed dogs and 23 mixed-breed dogs (Table 2). From among these dogs, four clinically healthy adults were used as control samples for direct DNA sequence analysis and genotyping tests. All specimens used in the present study were obtained with the consent of dog owners.

Direct DNA sequence analysis

Blood-spotted FTA cards were used directly as a template for polymerase chain reaction (PCR) after quick washing treatment as reported previously (Mizukami et al., 2012). To amplify the sequences of exons 1–6, their exon–intron junctions and 5′-UTR and 3′-UTR regions in the canine *ASPA* gene, 6 sets primers (Table 3) were designed based on GenBank data, i.e., whole genome shotgun sequences (NC_006591 and AAEX03006711) and predicted canine cDNA sequences (XM_849422 and XM_863442). The exon numbering of the canine gene was determined based on information from the human (NM_000049) and feline (XM_003996394)

ASPA genes. Amplified DNA fragments were extracted and sequenced in the forward and reverse direction as described previously (Rahman et al., 2012). The sequencing data from the Canavan disease-affected dog were compared with those from boxer dogs that were previously registered in the GeneBank data (NC_006591, AAEX03006711, XM_849422 and XM_863442) and the same sequence in four healthy control dogs.

Genotyping survey

For genotyping survey, a real-time PCR assay (Applied Biosystems) was designed with primers and probes (Table 4) using same protocol and reagents described previously (Mizukami et al., 2012). The survey was carried out among 1,508 pure breeds and mixed-breed dogs.

3.4. Results

Mutation analysis

A homozygous 6-base pair (bp) (GTTGGT) deletion and simultaneous 8-bp (GTATAGAA) duplication at the coding nucleotide position from 535 to 540 and 527 to 534 respectively in exon 4 was identified, i.e. c.[535_540delGTTGGT]+[527_534dupGTATAGAA] (Figure 7). This mutation has the potential to cause a frameshift, resulting in the alteration of glycine at amino acid position 180 to a stop codon (p.G180fsX). In addition, there were two types of homozygous single missense nucleotide substitutions with an amino acid substitution, c.310G>A (p.D104N) in exon 2 and c.434A>C (p.N145T) in exon 3 (Table 5). However, these substitutions were also observed in the healthy control dogs.

Genotyping survey

Among 1,508 dogs, neither carrier nor affected dogs were found. All dogs were of the wild-type genotype (Table 2 and Figure 8, 9).

3.5. Discussion

In this c.[535_540delGTTGGT]+[527_534dupGTATAGAA] mutation, first 2-bp (GT) are common for both deletion and duplication (Figure 7). In addition, we observed two kinds of homozygous missenes alteration, i.e. c.310G>A (p.D104N) and c.434A>C (p.N145T), but these alleles were also observed in control dogs, suggesting that they were not pathogenic.

The genotyping survey of the c.[535_540delGTTGGT]+[527_534dupGTATAGAA] mutation revealed no animal to have the mutation in a randomly-selected 1,508 dogs, demonstrating that this mutation is extremely rare and rule out the chance of polymorphism. The identified mutation is might be a *de novo* mutation and limited in closely related family members. In human, the severity of Canavan disease symptoms varies significantly on a case by case basis. The course of the disease is relentlessly progressive, although the rate of deterioration is variable (Leone et al., 1999). Life expectancy can vary from less than 1 year to 32 years, in patients with the same genotype (Matalon et al., 2001). Importantly, Canavan disease-associated *ASPA* gene mutation does not appear to correlate with a phenotype. The most common mutation among non-Ashkenazi Europeans, p.A305E, has been implicated in severe, classical and mild Canavan disease cases (Shaag et al., 1995; Sistermans et al., 2000;Yalcinkaya et al., 2005; Janson et al., 2006). Similarly, the most common mutation among Ashkenazim, p.E285A has also been implicated in multiple Canavan disease clinical courses (Shaag et al., 1995). Canine *ASPA* gene contains five introns and six exons coding for 313 amino acids same to those of human and has been conserved (Kaul et al., 1994). The 942-bp open reading frame in dog is 91.2% identical to that in human and the deduced amino acid sequence is 90.6% identical. Canavan disease-associated several mutations (c527del6, c566del7, c527del108, p.P181L and p.P181T) have been reported in exon 4 of the human *ASPA* gene (Zeng et al., 2006a; Shaag et al., 1995). In this report,

the affected dog has a mild clinical course of Canavan disease, which has been reported in human patients with the mutations (Yalcinkaya et al., 2005; Tacke et al., 2005; Janson et al., 2006). Atrophy of brain and hydrocephalus observed in the affected dog may be related with part of the complexity of Canavan disease. The pathology of elevated NAA is likely to be multifactorial, involving osmotic, metabolic, and lipid biosynthetic effects on glia; epileptogenic effects on neurons; and developmental effects from altered induction of the myelination program (Leone et al., 2012; Baslow and Guilfoyle, 2013). The mutation identified in a canine *ASPA* gene will lead to new insights regarding the nature of Canavan disease that may in turn lead to novel therapies using the animal model.

Table 2. The c.[535_540delGTTGGT]+[527_534dupGTATAGAA] mutation screening of the canine *ASPA* gene with Canavan disease in dogs by the real-time genotyping assay

Serial no.	Name of the breed/mixed-breed	No. tested dogs	No. of the carrier/affected
1	Shiba Inu	500	0
2	Welsh Corgi	253	0
3	Toy Poodle	200	0
4	Broder Collie	171	0
5	Dachshund	129	0
6	Jindo	46	0
7	Chihuahua	34	0
8	Saluki	31	0
9	Mixed-breed	23	0
10	Ryukyu	20	0
11	Hokkaido	17	0
12	Beagle	16	0
13	Pappion	11	0
14	Golden Retriever	09	0
15	Wire Fox terrier	09	0
16	Cocker spaniel	09	0
17	Shetland Sheepdog	06	0
18	French Bulldog	06	0
19	German Shepherded	06	0
20	American Cocker Spaniel	05	0
21	Labrador Retriever	04	0
22	Pug	03	0
	Total	1,508	0

Table 3. Characteristics of primers and amplified fragments in the direct sequencing analysis of the canine *ASPA* gene

Exon [a]	Primer	Sequence* 5′→3′ (mers)	Location	Position	T_m (ºC) [b]	Size (bp) [c]
1	Forward	GGCTAAAGAAGGGAGTGTCC (20)	5′-UTR	c.1–187_206	58.4	533
	Reverse	GAATGAGAACATGTATGGGTAGC (23)	Intron 1	c.236+69_91	56.9	
2	Forward	GGCAACTGGCTCCTGATAC (19)	Intron 1	c.237–75_93	58.2	391
	Reverse	GGTCAGAAGAAGGGGTCCA (19)	Intron 2	c.432+84_102	58.2	
3	Forward	CAAAGAGAACAAAGTGTATGGTC (23)	Intron 2	c.433–38_60	55.1	213
	Reverse	ACGTCCTATGTGAAACTCTGAG (22)	Intron 3	c.526+38_59	56.7	
4	Forward	TGCTACATGGTCTACCTTTCTA (22)	Intron 3	c.527–105_126	54.8	319 (321) [d]
	Reverse	GAGCTGGAATGAGCACACATA (21)	Intron 4	c.634+65_85	56.5	
5	Forward	GGTTGCTGTTGTTCCCTAAAG (21)	Intron 4	c.635–66_86	56.5	295
	Reverse	GACTTTATTCATGAGAGACACAG (23)	Intron 5	c.744+77_99	55.1	
6	Forward	GTTATTTGTCTAGAGCCCAACT (22)	Intron 5	c.745–66_87	54.8	383
	Reverse	CAACTACATACCCATAGCACCT (22)	3′-UTR	c.942+77_98	56.7	

[*] Primers were designed based on the GenBank data (XM_849422, XM_863442, NC_006591 and AAEX03006711).

[a] The exon numbering was determined based on information from the human (NM_000049) and feline (XM_003996394) *ASPA* genes; [b] T_m, melting temperature; [c] Amplified DNA fragment size including primer length; [d] Fragment size in the mutant allele including primer length.

Table 4. Primers and TaqMan probes used in the real-time PCR genotyping assay for the mutation screening of canine Canavan disease

Primer/probe	Sequence 5′→3′ (mers)	Position	Reporter (5′)	Quencher (3′)	Final concentration (nM)
Forward primer	GTGACTATTTCCCCTCTGTACCG (23)	c.527–3_25	–	–	450
Reverse primer	GGGTTCTGAGAGCTGATATTTTGGAT (26)	c.553_579	–	–	450
Wild-type probe	AGGTATAGAAGTTGGTCCCC (20)	c.527–2	VIC	NFQ	100
Mutant-type probe	ATAGAACCCCAACCTCA (17)	c.537_553*	FAM	NFQ	100

* Muted sequence; VIC, 6-carboxyrhodamine; FAM, 6-carboxyfluorescein; NFQ, nonfluorescent quencher

Table 5. Results of mutational analysis in the exons and flanking intronic regions of the canine *ASPA* gene

Nucleotide change (exon/intron)	Amino acid change	Genotype					
		Affected	Wild-type				
			GenBank [a]	Control 1	Control 2	Control 3	Control 4
c.310G>A (exon 2)	p.D104N	A/A	G/G	G/G	G/G	A/A	G/G
c.433–30A>G[*] (intron 2)	–	A/A	A/A	G/G	G/G	A/A	A/A
c.434A>C (exon 3)	p.N145T	C/C	A/A	C/C	A/C	A/A	C/C
c.537_540delTGGTinsATAGAA (exon 4)	p.G180fsX	GTATAGAA/ GTATAGAA	GTTGGT/ GTTGGT	GTTGGT/ GTTGGT	GTTGGT/ GTTGGT	GTTGGT/ GTTGGT	GTTGGT/ GTTGGT
c.924T>C[*] (exon 6)	p.(I308=)	T/T	T/T	T/T	T/C	C/C	T/T
c.942+5C>T[*] (3′-UTR)	–	C/C	C/C	T/T	T/T	C/C	C/C

[*] Observed only in some controls; [a] Accession Nos. XM_849422, XM_863442, NC_006591 and AAEX03006711; Control 1 and 2 were Beagle and mixed-breed dogs respectively, Control 3 and 4 were Shiba dogs.

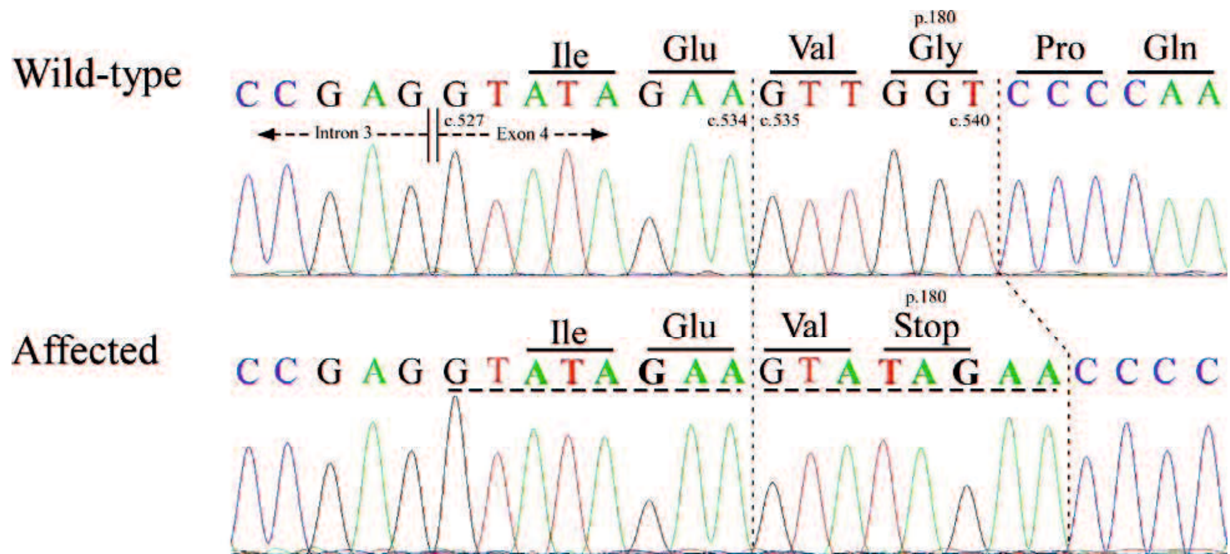

Figure 7. Partial genomic sequence electropherograms of exon 4 in the canine *ASPA* gene from wild-type and affected dogs. A homozygous 6-base pair (GTTGGT) deletion and 8-base pair (GTATAGAA) duplication at nucleotide position 535 to 540 and 527 to 534 respectively in the affected dog. The deletion/duplication may cause a frameshift resulting in the alteration of glycine at amino acid position 180 to a stop codon.

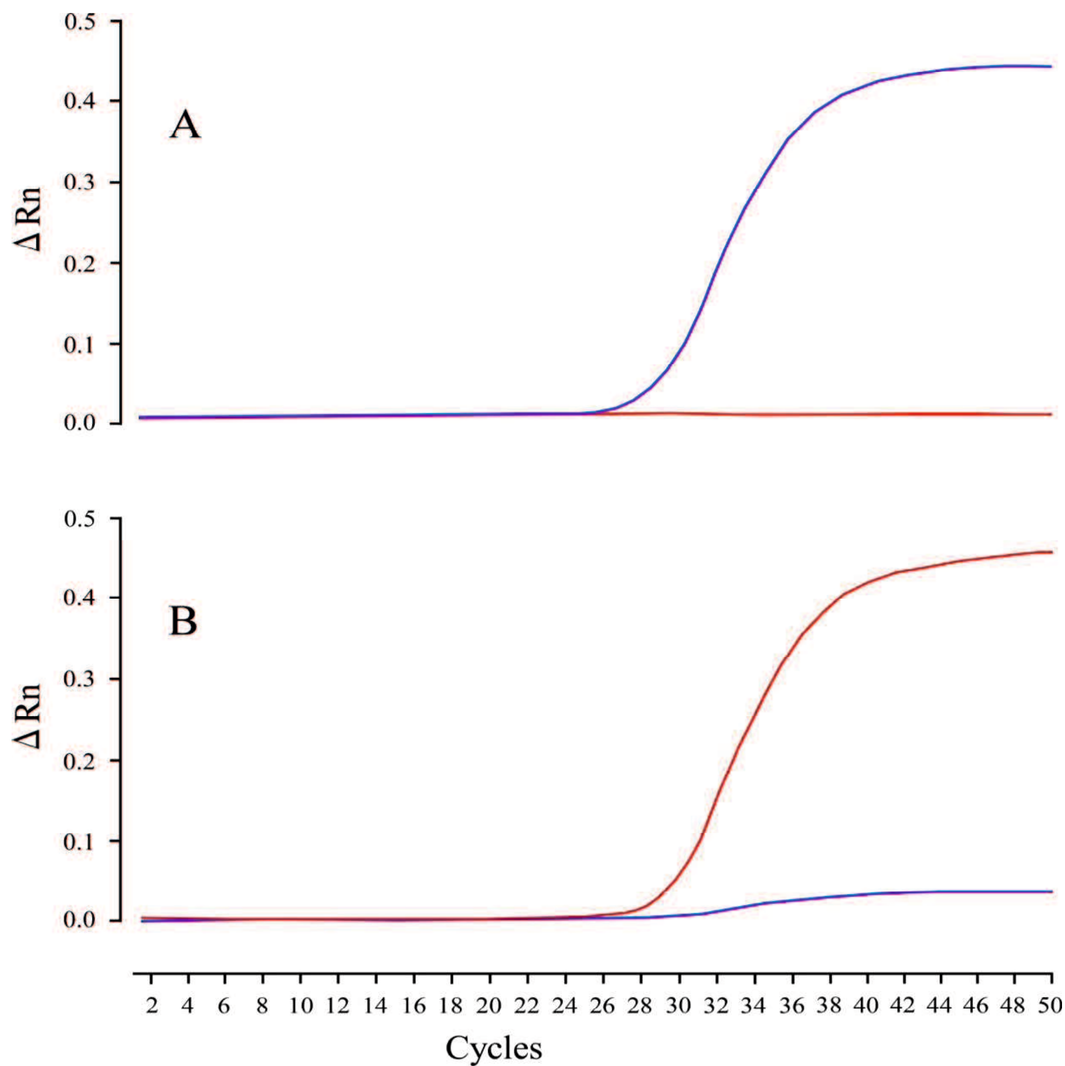

Figure 8. Real-time PCR amplification plots of wild-type and mutant alleles in canine Canavan disease. Amplification was plotted as fluorescence intensity (ΔRn value) against cycle number. The ΔRn value is the reporter dye signal normalized to the internal reference dye and corrected for the baseline signal established in the first few cycles of reaction. Each of 2 amplification plots showed the wild-type (A) and affected (C). Blue and red lines indicate wild-type and mutant alleles, respectively.

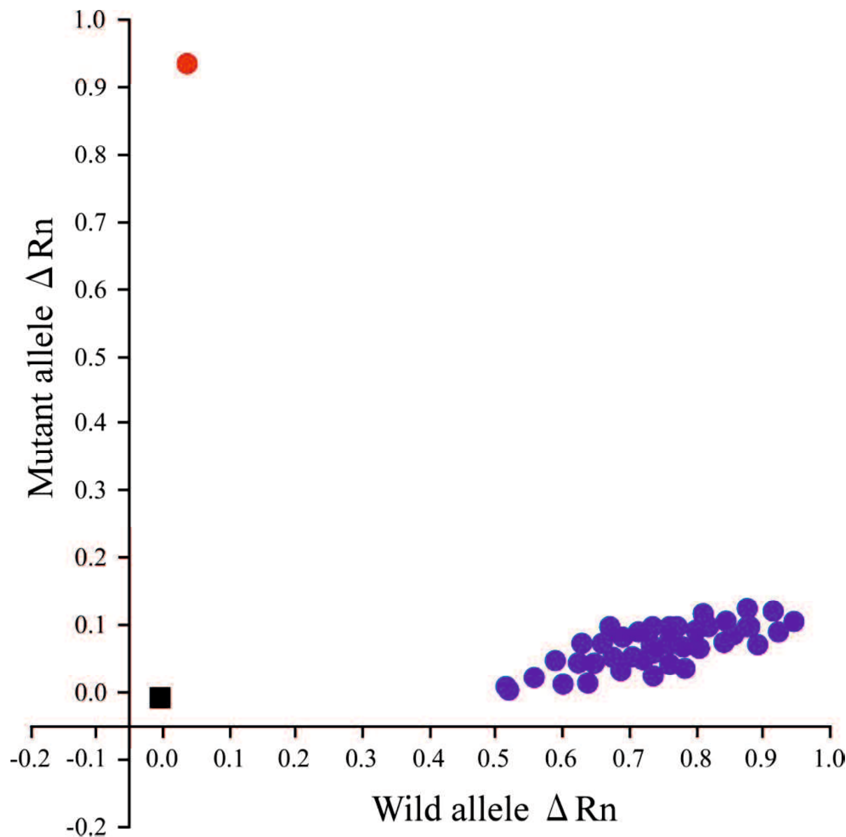

Figure 9. Allelic discrimination plot of end-point fluorescence real-time PCR data showing the 2 genotypes of canine Canavan disease. The allelic discrimination plot was depicted using 47 representative DNA samples in dogs. The plot is expressed as fluorescence intensities (ΔRn values) for each allele at the X- and Y-axes. The ΔRn value in this figure is the end-point reporter dye signal normalized to the internal reference dye and corrected for the baseline signal established in the first few cycles of reaction. ■, no template control; ●, wild-type (46 samples); ●, affected genotype (one sample).

CHAPTER 4

Molecular basis of Krabbe disease in a mixed-breed cat

The above-titled manuscript was prepared for submission as:***A missense mutation in the feline galactocerebrosidase (GALC) gene in a mixed-breed cat with krabbe disease*** and authored by:

Mohammad Mejbah UDDIN [1,2], Keijiro MIZUKAMI [1], Miyoko SAITO [3], Mohammad Mahbubur RAHMAN [1,2], Hye-Sook CHANG [1], Mohammad Alamgir HOSSAIN [1,2], Akira YABUKI [1], Kazuya KUSHIDA [1], Moeko KOHYAMA [1], Osamu YAMATO [1, *]

[1] Laboratory of Clinical Pathology, Joint Faculty of Veterinary Medicine, Kagoshima University, Kohrimoto, Kagoshima, Japan.

[2] Faculty of Veterinary Medicine, Chittagong Veterinary and Animal Sciences University, Khulshi, Chittagong, Bangladesh.

[3] Laboratory of Veterinary Surgery II, School of Veterinary Medicine, Azabu University, Fuchinobe, Kanagawa, Japan.

4.1. ABSTRACT

Globoid cell leukodystrophy (GLD), alias Krabbe disease, is an autosomal recessive, progressive demyelinating disease affecting both the central and peripheral nervous systems, which is caused by deleterious mutations in the galactocerebrosidase (*GALC*) gene encoding a lysosomal enzyme GALC. The present study describes the molecular defect of feline GLD as the first identification of a pathogenic mutation in the feline *GALC* gene. Direct DNA sequence analysis was performed using DNA from a tissue in a mixed-breed cat diagnosed histopathologically with GLD. The analysis demonstrated that the cat was homozygous for a missense mutation as a single nucleotide substitution from guanine to adenine in exon 17 of the feline *GALC* gene at coding nucleotide position 1945 (c.1945G>A), resulting in an amino acid substitution from alanine to threonine at position 649 (p.A649T) of the feline GALC enzyme. Based on the amino acid sequence information in a variety of mammals, the alanine at this position is strongly conserved. Sorting intolerant from tolerant, i.e., SIFT, analysis assessed that the p.A649T substitution is not tolerated and affects the enzyme function. Population screening among 1,060 mixed-breed cats using a real-time PCR assay with TaqMan minor groove binder probes developed for genotyping of c.1945G>A, did not reveal any animal possessing this mutation. These results strongly suggest that c.1945G>A (p.A649T) of the feline *GALC* gene is pathogenic and rare in the cat population. The molecular data in this study will hopefully contribute to the advancement of veterinary clinical genetics.

4.2. INTRODUCTION

Krabbe disease or globoid cell leukodystrophy is a rapidly progressive demyelinating disease with an autosomal recessive inheritance. The disease is caused by mutations in the gene coding for the lysosomal enzyme galactocerebrosidase (GALC; EC 3.2.1.46), which is responsible for the hydrolysis of the galactosyl moiety from several galactolipids, mostly galactosylceramide and galactosylsphingosine (psychosine). These galactsphingolipids are normally synthesized during active myelination in oligodendrocytes and Schwann cells; therefore, these compounds accumulate in the central and peripheral nervous systems with very low GALC activity in the lysosomal degradation pathway of sphingolipids. Psychosine is highly toxic to the myelin-forming cells, causing their death and a paucity of myelin. The excess of galactosylceramide elicits the formation of macrophage-derived globoid cells found around cerebral blood vessels in the white matter of affected human patients and animal models (Suzuki and Grover, 1970). In human, based on the age of onset and clinical progression, Krabbe disease is classified into four types: infantile, late-infantile, juvenile and adult-onset. Most common infantile form Krabbe disease presents with irritability, spasticity and developmental delay before the age of six months, progressing rapidly to severe mental and motor deterioration and death before the age of two. Late-onset form of the disease occurs between the age of 6 months and the fifth decade with slower disease progression (Tappino et al., 2010).

In veterinary species, naturally occurring Krabbe disease have been reported in dogs, cats, sheep, mouse (Suzuki and Suzuki, 1985), rhesus monkey (Luzi et al., 1997) and rhesus macaques (Borda et al., 2008). In dogs, two pathogenic mutations have been reported, i.e., one in West Highland White terrier and Cairn terrier (Victoria et al., 1996) and one in Irish setter (McGraw et al., 2006). Similarly, one mutation in rhesus monkeys (Luzi et al., 1997) and one mutation in

twitcher mice (Sakai et al., 1996) have also been reported. In the feline species, the disease has been described in two inbred domestic shorthaired cats in the USA (Johnson, 1970), one domestic longhaired cat in the USA (Sigurdson et al., 2002) and two domestic shorthaired cats in Italy (Salvadori et al., 2005). In general, affected cats manifest neurological signs mainly characterized by progressive pelvic limb ataxia, paraplegia with loss of deep pain perception in the pelvic limb and intentional tremors of the thoracic limbs starting from one to two months of age and die prematurely at approximately five months of age (Salvadori et al., 2005;Sigurdson et al., 2002). However, to our knowledge, molecular basis of the disease has not yet been described in feline species.

The diagnosis of Krabbe disease may be aided by clinical signs, histopathogical, neuroimaging, neurophysiological and biochemical findings. Molecular analysis of the *GALC* gene is used for definitive diagnosis of the disease (Puckett et al., 2012; Szymańska et al., 2012; Kardas et al., 2013).

In this paper, we describe a molecular defect of Krabbe disease in a Japanese mixed-breed cat as the first identification of a potentially pathogenic mutation in the feline *GALC* gene and a rapid and simple real-time PCR-based genotyping assay for the diagnosis and survey of this mutant allele.

4.3. MATERIALS AND METHODS

Animals and specimens

A male Japanese mixed-breed domestic cat had progressive hind limb paresis followed by tetraparesis and loss of voluntary movement started from 4 months of age. Then the cat showed visual disorder, trismus and cognative impairment and died at 9 months of age. On the basis of histopathological, immunohistichemical and ultrastructural features, the affected cat was diagnosed as feline Krabbe's disease (Ogawa et al., 2013). Purified genomic DNA sample of the affected cat was received from the Laboratory of Veterinary Surgery II, School of Veterinary Medicine, Azabu University, Kanagawa, Japan for molecular studies. A genotyping survey was carried out using DNA samples from blood-spotted FTA cards of randomly-selected 1,060 cats (1,004 mixed-breed domestic and 56 pure breed cats are listed in Table 6). Among mixed-breed cats, five clinically healthy adult cats were used as control samples for direct DNA sequence analysis and genotyping tests. All specimens used in the present study were obtained with the consent of cat owners. All experimental procedures using animals and their specimens were performed in accordance with the guidelines regulating animal use at Kagoshima University, Japan.

Direct DNA sequence analysis

Purified genomic DNA sample of the affected cat was used as a template for PCR. To amplify the sequences of exons 1–17, their exon-intron junctions and 5′-UTR and 3′-UTR regions in the feline *GALC* gene, 17 sets primers (Table 7) were designed based on GenBank data, i.e., whole genome shotgun sequences (NC_018728.1 and AANG02192641.1) and predicted feline cDNA sequence (XM_003987957.1). The exon numbering of the feline gene

was determined based on information from the human (NM_000153) and canine (NM_001003238) *GALC* genes. Amplified DNA fragments were extracted and sequenced in the forward and reverse direction as described previously (Rahman et al., 2012). Direct sequencing data of the feline *GALC* gene of the Krabbe disease-affected cat were registered in the DNA Data Bank of Japan. The sequencing data from the Krabbe disease-affected cat was compared with those from Abyssinian cats that were previously registered in the GeneBank data (XM_003987957.1, NC_018728.1 and AANG02192641.1) and the same sequence in five healthy control cats.

SIFT (sorting intolerant from tolerant) analysis

The identified amino acid substitution in the enzyme was analyzed using website (http://sift.jcvi.org/www/SIFT_seq_submit2.html) to assess wheather the amino acid substitution will have a phenotypic effect or not.

Genotyping survey

For genotyping survey, a real-time PCR assay (Applied Biosystems) was designed with primers and probes (Table 8) using same protocol and reagents described previously (Mizukami et al., 2012). The genotyping survey was carried out among randomly-selected 1,060 cats as mentioned above.

4.4. RESULTS

Mutation analysis

Direct DNA sequencing analysis revealed a total 16 nucleotide changes in the *GALC* gene from the affected cat with Krabbe disease, compared with *GALC* in the GenBank database and/or healthy control cats (Table 9). There was a single nucleotide substitution from guanine to adenine in exon 17 at nucleotide position 1945 (c.1945G>A) in the coding region of the feline *GALC* gene, resulting in the substitution of alanine with threonine at amino acid position 649 (p.A649T) of the feline GALC enzyme in the Krabbe disease-affected cat that was not observed in five control cats examined (Figure 10). In addition, the affected cat had one missense mutation c.58A>G (p.T20A), three silent mutations c.120G>C (p.P145P), c.1335C>T (p.D445D) and c.1992C>T (p.H664H) in exonic regions and eleven variations in intronic regions, are not likely to be pathogenic because these alterations were present in the control cats.

SIFT analysis

SIFT analysis assessed that the p.A649T substitution is not tolerated and affects the enzyme function (Figures 12, 13).

Genotyping survey

Among 1,060 cats, neither carrier nor affected dogs were found for the c.1945G>A (p.A649T) mutation. All cats were of the wild-type genotype (Table 6 and Figure 14, 15)

4.5. DISCUSSION

Diagnosis of the disease can be made by enzyme analysis of GALC activity in leukocytes or cultured skin fibroblasts. However, the enzymatic diagnosis is complicated by the finding of low GALC activity in some healthy individuals (Wenger and Riccardi 1976), inconclusive in many individuals without any of the classical presentations of the disease (Puckett et al., 2012) and highly variable enzyme activities due to very changeable culture conditions (Szymańska et al., 2012). In this Krabbe disease-affected cat, we could not measure the GALC enzyme activities, because unavailable of fresh tissue specimens. Instead, we analyzed the feline *GALC* gene and searched for molecular defects using purified genomic DNA samples. Direct DNA sequencing of the feline *GALC* gene in the affected cat revealed a total 16 nucleotide changes. Among these, fifteen nucleotide alterations are considered to be natural polymorphisms because these were observed in healthy control cats as homozygous or heterozygous pattern (Table 9). One nucleotide change was unique and significant, which is a homozygous missense mutation (c.1945G>A) with an amino acid substitution (p.A649T) (Figure 10).

The feline *GALC* cDNA is composed of 17 exons including 2007 base pair (bp) of a putative open reading frame codes for an enzyme of 669 amino acids, the same length as human (Tappino et al., 2010; Kardas et al., 2013; GenBank NM_000153.3). The nucleotides and amino acids sequences are 89.6% and 88.7% identical to the human, respectively. Based on the amino acid sequence information in a variety of mammals, the alanine at this position 649 is strongly conserved (Figure 11). SIFT analysis assessed that the p.A649T substitution is not tolerated with a probability matrix score of 0.01 where a score below 0.05 is deleterious to functions of the protein (Figures 12 and 13). In addition, population screening among 1,060 mixed-breed cats using a real-time PCR assay developed for genotyping of c.1945G>A (Figures 14 and 15), did

not reveal any animal possessing this mutation. These results strongly suggest that c.1945G>A (p.A649T) of the feline *GALC* gene is pathogenic and rare in the cat population.

The c.1945G>A is the first mutation to be identified in the feline *GALC* gene and is different from mutations that have been identified in humans and dogs. To date, more than 90 different mutations in the human *GALC* gene have been identified as causes of Krabbe disease (Puckett et al., 2012). In the exon 17 of the human *GALC* gene, c.1864G>A (Debs et al., 2012), c.1886T>G (Jardim et al., 1999) and c.1993G>A (Yang et al., 2013) in adult-onset patients and c1873G>A (Kolodny et al., 1995) in a late-onset patient have been reported. But, none of these mutations are at same position, where the feline mutation was identified in the present study. All mutations responsible for late-onset or adult form of Krabbe disease are simple missense mutations mainly located at 5′-end of the gene, whereas most missense mutations responsible for infantile form of the disease are concentrated at the 3′-end of the gene (De Gasperi et al., 1996; Wenger et al., 1997).

In cats, first Krabbe disease has been described in two female kittens with posterior ataxia, ascending incoordination, occasional generalized tremors and cats were humanely destroyed at 6.5 and 8.5 weeks of age (Johnson, 1970). Then, the disease has been reported in a female domestic longhaired cat with initial signs of body tremors and tetraparesis at 8 weeks of age and died at 21 weeks of age having pelvic limb paralysis, muscle atrophy and respiratory distress (Sigurdson et al., 2002). The disease has also been observed in two domestic shorthaired cats with progressive pelvic limb ataxia first noted at 4 and 6 weeks of age and due to poor prognosis euthanized at 3 and 4 months of age respectively (Salvadori et al., 2005). In above mentioned all cats with Krabbe disease have also been characterized by the presence of characteristic leukodystrophy and globoid cells. Clinical and pathomorphological features were also identical

with the infantile form of human patients (Salvadori et al., 2005). Clinicopathological features of the Krabbe disease-affected cat in the present study were similar to previously described all Krabbe disease-affected cats except the onset of clinical signs at 4 months of age with severe disease progression and died at 9 months of age, suggesting the c.1945G>A (p.A649T) mutation of the feline *GLAC* gene may be related with a late-onset form Krabbe disease.

Table 6. The c.1945G>A mutation screening of the feline *GLAC* gene with Krabbe disease in domestic cats by the real-time genotyping assay

Serial no.	Name of the breed/mixed-breed	No. tested cats	No. of the carrier/affected
1	Mixed-breed	1,004	0
2	Abyssinia	5	0
3	American Curl	2	0
3	American Shorthair	8	0
4	Chinchilla	14	0
5	Devon Rex	1	0
6	Himalayan	1	0
7	Japanese cat	5	0
8	Maine Coon Cat	4	0
9	Munchkin	1	0
10	Persian	3	0
11	Russian Blue	2	0
12	Scottish Fold	8	8
13	Somali	2	0
	Total	1,060	0

Table 7. Characteristics of primers and amplified fragments in the direct sequencing analysis of the feline *GALC*gene

Exon [a]	Primer	Sequence * 5′→3′ (mers)	Location	Position	T_m (°C) [b]	Size (bp) [c]
1	Forward	GCAGCCCGTCGGCCAGAG (18)	5′-UTR	c.1–45_62	64.9	279
	Reverse	GGATGCTTCAATGCGGGTTGG (21)	Intron 1	c.147+50_70	60.4	
2	Forward	ACGCCAGGTGGTGCAAGT (18)	Intron 1	c.148–80_97	58.1	234
	Reverse	GCTATGGTGAATTTCAACGGTAG (23)	Intron 2	c.216+46_68	56.9	
3	Forward	CGAAGGCAGTAATTAGTCACTG (22)	Intron 2	c.217–60_81	56.7	197
	Reverse	CCTTCAGCACTTTAATCTCTACC (23)	Intron 3	c.280+30_52	56.9	
4	Forward	GGAACCAGAATACACCTTCTC (21)	Intron 3	c.281–88_108	58.4	352
	Reverse	CTTATCCTACCAGCCGCAAAG (21)	Intron 4	c.394+110_130	58.5	
5	Forward	GATGGTTGCCAGCACTGTAA (20)	Intron 4	c.395–35_54	56.3	246
	Reverse	CTTTCGGACTTTATTTGTAATTTC (24)	Intron 5	c.534+29_52	51.9	
6	Forward	GAGAGCAGTGGCGTCTTAG (19)	Intron 5	c.535–64_82	58.2	200
	Reverse	CGGTACCATGGAAATTAGAAGTAG (24)	Intron 6	c.573+56_79	57.1	
7	Forward	GATACTTCAGCTGTTGTGGTTG (22)	Intron 6	c.574–62_83	56.7	270
	Reverse	CTGATTGCTTGCTTGGGATAC (21)	Intron 7	c.704+36_56	56.5	
8	Forward	GATACAAGTGGCATGTGCTG (20)	Intron 7	c.705–37_56	56.3	292
	Reverse	GTAGAAGGAAAGTACAGTGGGC (22)	Intron 8	c.860+59_80	58.6	
9	Forward	CTACAGTCTTCTAGACCTTTAC (22)	Intron 8	c.861–54_75	54.8	261
	Reverse	CTAGGAGACAAAGCAGGAGAC (21)	Intron 9	c.985+41_61	58.5	
10	Forward	AACCGACTCAGCCACCCA (18)	Intron 9	c.986–83_100	58.1	272
	Reverse	GAGATTAACTGTCTTCGTATTGG (23)	Intron 10	c.1113+22_44	55.1	
11	Forward	GGAGCTTTACTTCTGACTAGG (21)	Intron 10	c.1114–190_210	56.5	378
	Reverse	CACAGTGTAGCCCTTCTCAAG (21)	Intron 11	c.1203+57_77	58.5	
12	Forward	GTTCATTCTTGCTGGTACTTAC (22)	Intron 11	c.1335–37_56	54.8	222
	Reverse	CATATGTTTTAAACACTGCAAACAG (25)	Intron 12	c.1290+19_43	53.9	
13	Forward	CCAAATGAGAGGAGCCCAAC (20)	Intron 12	c.1291–37_56	58.4	298
	Reverse	CTATGTGTCGAACTCGTGCG (20)	Intron 13	c.1441+72_91	58.4	
14	Forward	GAGATTCTGTGAAGACTTTGTAC (23)	Intron 13	c.1442–31_53	55.1	282
	Reverse	GACCTGGTTGAAATAACCTCTC (22)	Intron 14	c.1622+27_48	56.7	
15	Forward	GGCATGTGGCTGTGAAATGAC (21)	Intron 14	c.1623–63_83	58.5	315
	Reverse	GCTGTGAGCTTCTTACTGTGTG (22)	Intron 15	c.1786+47_68	58.6	
16	Forward	CACTGAGCTATGTGATTCTGTGA (23)	Intron 15	c.1787–49_71	56.9	232
	Reverse	TCTGTCCTCAAATGAGAGGG (20)	Intron 16	c.1863+65_84	56.3	
17	Forward	CGGAATAGATTGGCCACACG (20)	Intron 16	c.1864–69_88	58.4	477
	Reverse	ATGGCCCCTAAAGTTCAAGC (20)	3′-UTR	c.2010+223_242	56.3	

[a] The exon numbering was determined based on information from the canine (NM_001003238.1) and human (NM_000153.3)*GALC* gene.

[*] Primers were designed based on the GenBank data (XM_003987957.1, NC_018728.1 and AANG02192641.1).

[b] Tm = melting temperature.

[C] Amplified DNA fragment size including primer length

Table 8. Primers and TaqMan probes used in the real-time PCR genotyping assay for the mutation screening of feline Krabbe disease

Primer/probe	Sequence 5′→3′ (mers)	Position	Reporter (5′)	Quencher (3′)	Final concentration (nM)
Forward primer	CCTGTGAGTTTCCCGAAGAATGG (23)	c.1915_1937	–	–	450
Reverse primer	GAACTCACTCTTTTGAATTTGCACAGT (27)	c.1952_1978	–	–	450
Wild-type probe	CTGGGCCGCGATTG (14)	c.1938_1951	VIC	NFQ	100
Mutant-type probe	CTGGGCCACGATTG (14)	c.1938_1951	FAM	NFQ	100

VIC, 6-carboxyrhodamine; FAM, 6-carboxyfluorescein; NFQ, nonfluorescent quencher

Table 9. Results of mutational analysis in the exons and flanking intronic regions of the feline *GALC* gene

Exon/intron	Nucleotide change	Amino acid change	Genotype						
			Affected	Wild-type					
				GenBank [a]	Control 1	Control 2	Control 3	Control 4	Control 5
Exon 1	c.58A>G	p.T20A	G/G	A/A	G/G	Nd	Nd	Nd	Nd
	c.120G>C	p.P40P	C/C	G/G	C/C	Nd	Nd	Nd	Nd
Intron 4	c.395–34C>T	–	T/T	C/C	T/C	C/C	T/T	Nd	Nd
Intron 6	c.574–41C>T	–	T/T	C/C	T/T	C/T	T/T	Nd	Nd
Intron 7	c.704+30T>C	–	C/C	T/T	C/C	T/C	C/C	Nd	Nd
Intron 10	c.1114–15_16insT	–	T/T	–	T/T	Nd	Nd	Nd	Nd
	c.1114–67T>T/G	–	T/G	T/T	T/G	Nd	Nd	Nd	Nd
Exon 13	c.1335C>T	p.D445D	T/T	C/C	C/C	C/C	C/C	C/C	T/T
Intron 13	c.1441+29_30insT	–	T/T	–	T/T	T/T	T/T	Nd	Nd
	c.1441+40G>C	–	C/C	G/G	C/C	C/C	C/C	Nd	Nd
Intron 16	c.1864–38T>G	–	G/G	T/T	T/T	T/T	G/G	Nd	Nd
Exon 17	c.1945G>A	p.A649T	A/A	G/G	G/G	G/G	G/G	G/G	G/G
	c.1992C>T	p.H664H	T/T	C/C	T/T	T/T	T/T	Nd	Nd
Intron 17	c.2010+68C>A	–	A/A	C/C	A/A	C/A	A/A	Nd	Nd
	c.2010+188G>A	–	A/A	G/G	G/A	G/G	G/A	G/G	A/A
	c.2010+192A>G	–	G/G	A/A	G/G	G/G	G/G	Nd	Nd

[a] Accession Nos. XM_003987957, AANG02192641, NC_018728; Control 1-5 were mixed-breed cats; –, no amino acid change and no data in GenBank; Nd, not determined.

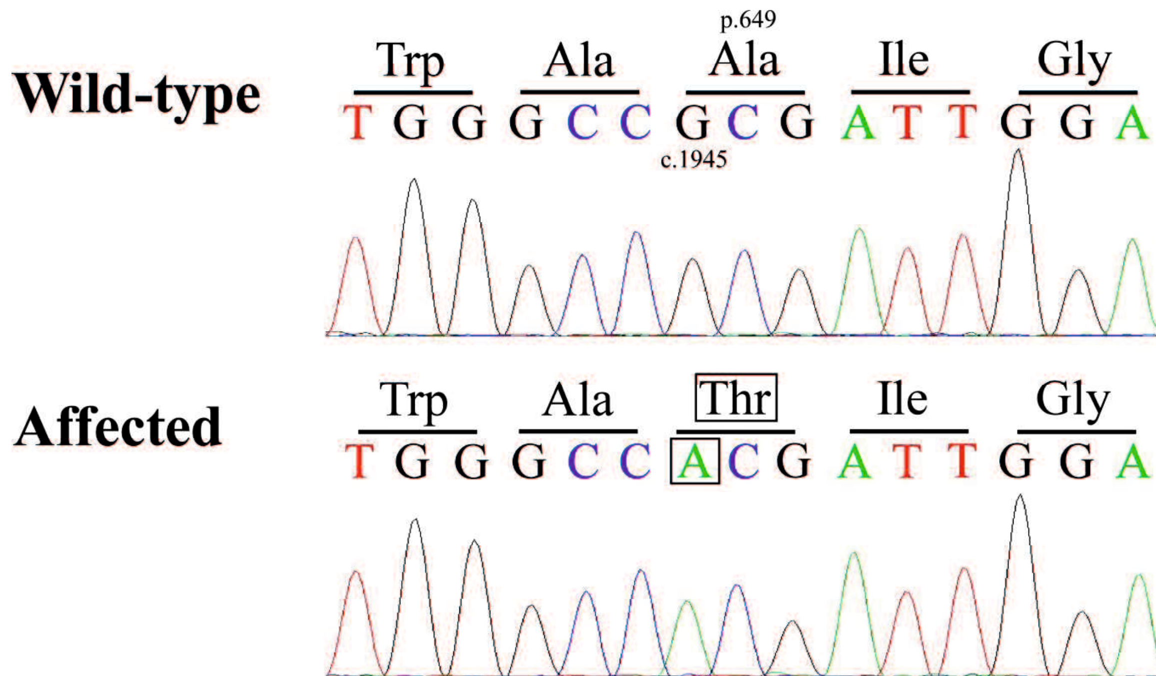

Figure 10. Partial genomic sequence electropharograms of exon 17 in the feline *GALC* gene from wild-type and affected cats. The guanine at nucleotide position 1945 is substituted with an adenine in the affected cat. The nucleotide substitution causes the substitution of an alanine at amino acid position 649 with a threonine.

p.A649T

Affected cat	NGKSVWKNIPVSFPKNGWATIGTHSFEFAQFDNFHVEATS
Homo sapiens	NDKSLWTDIPVNFPKNGWAAIGTHSFEFAQFDNFLVEATS
Canis familiaris	NGKTVWKNIPVSFPKNGWAAIGTHSFEFAQFDNFHVEATS
Pan troglodytes	NDKSLWTDIPVNFPKNGWAAIGTHSFEFAQFDNFLVEATR
Bos Taurus	NGKTLWKNISVNFPKNGWAAIGTHSFEFAQFDNFHVEATH
Mus musculus	NGTILWKNVRVKYPGHGWAAIGTHTFEFAQFDNFRVEAAR
Rattus norvegicus	NGKILWENVPVKYPGHGWAAIGTHTFEFAQFDNFHVEAAR
Equus caballus	NGKPLWTNVPVNFPKNGWAAIGTHFFEFAQFDNFYVEAMG
Gallus gallus	NGYPLWENVTISQPSHGWAAIGTRSFEFAQFDNFHVEAR
Sus scrofa	NGKLLWKNVSVNFPKNGWAAIGTHSFEFAQFDNFRVEATC
Macaca mulatta	NDKSLWTDIPVNFPKNGWAAIGTHSFEFAQFDNFHVEATR

Figure 11. Evolutionary comparison of sequences in the vicinity of GALC Thr.649 (arrow) from various species

Predict not tolerated	Position	Predict tolerated
c h d p n y e s t g r a k m v i	647W	I Q F W
w h y g d n r q f e k s c p m	648A	t l I V A
w h y f i m q r n d e l k c v t p s	649A	G A
h d n r k g e q c p s t a w m v	650I	F L Y I
y w v t s r q p n m l k i h f e d c a	651G	G

Figure 12. SIFT analysis showing all substitution at p.649 A in the feline GALC enzyme

Position	A	C	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y
647W	0.02	0.00	0.01	0.01	0.38	0.01	0.01	0.03	0.02	0.08	0.02	0.01	0.01	0.18	0.02	0.01	0.01	0.02	1.00	0.01
648A	1.00	0.02	0.01	0.02	0.01	0.01	0.00	0.35	0.02	0.09	0.03	0.01	0.02	0.01	0.01	0.02	0.08	0.40	0.00	0.00
649A	1.00	0.00	0.00	0.00	0.00	0.23	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.02	0.02	0.01	0.01	0.00	0.00
650I	0.01	0.00	0.00	0.00	0.17	0.00	0.00	1.00	0.00	0.19	0.02	0.00	0.00	0.00	0.00	0.00	0.01	0.04	0.02	0.21
651G	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Here, score below 0.05 affect protein functions

Figure 13. Probability matrix of SIFT analysis showing the score of the p.A649T

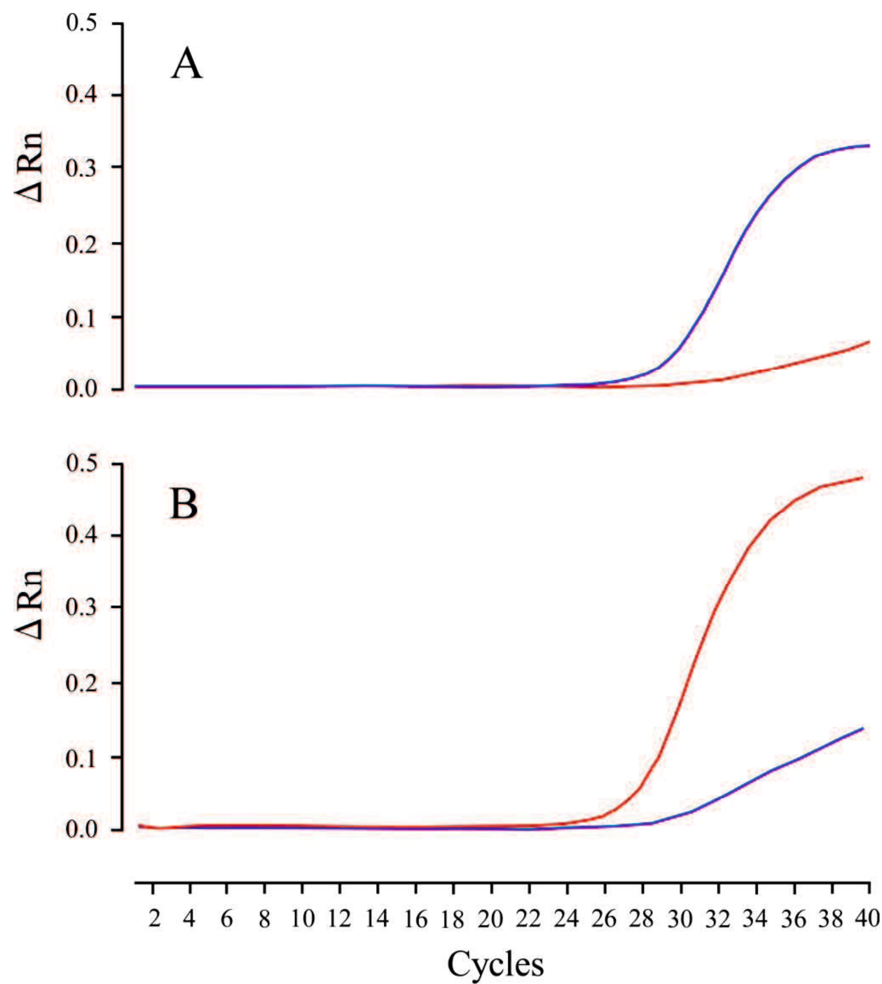

Figure 14. Real-time PCR amplification plots of wild-type and mutant alleles in feline Krabbe disease. Amplification was plotted as fluorescence intensity (ΔRn value) against cycle number. The ΔRn value is the reporter dye signal normalized to the internal reference dye and corrected for the baseline signal established in the first few cycles of reaction. Each of 2 amplification plots showed the wild-type (A) and affected (C). Blue and red lines indicate wild-type and mutant alleles, respectively.

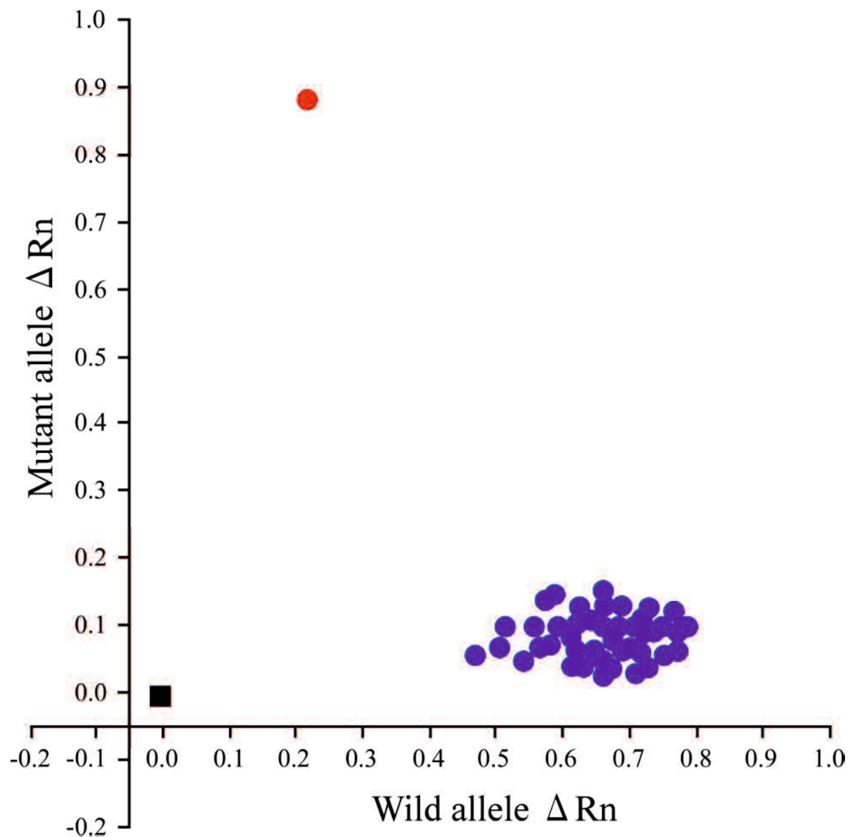

Figure 15. Allelic discrimination plot of end-point fluorescence real-time PCR data showing the 2 genotypes of feline Krabbe disease. The allelic discrimination plot was depicted using 47 representative DNA samples in dogs. The plot is expressed as fluorescence intensities (ΔRn values) for each allele at the X- and Y-axes. The ΔRn value in this figure is the end-point reporter dye signal normalized to the internal reference dye and corrected for the baseline signal established in the first few cycles of reaction. ■, no template control; ●, wild-type (46 samples); ●, affected genotype (one sample).

CONCLUSION

In the present study, a molecular investigation of GM1 gangliosidosis in both dogs and cats, Canavan disease in dogs, and Krabbe disease in cats was conducted.

In Chapter 1, a molecular epidemiological survey was conducted using a real-time PCR-based genotyping assay among 590 clinically unaffected Shiba Inu dogs from all over Japan. The results showed that the current carrier frequency for the disease was 1.02% in the overall Shiba Inu dog population in Japan, and that it was rather high (2.27%) in the Kinki district. The high carrier frequency in the Kinki region seems to be related to the high prevalence in this district. Therefore, for the effective control and prevention of this disease, it is necessary to examine as many breeding dogs as possible from all regions of Japan, especially from kennels located in areas with high prevalence and carrier frequency. The molecular epidemiological information for GM1 gangliosidosis in the Shiba Inu breed in Japan will contribute extensively to the control and prevention of the disease.

In Chapter 2, a mutation analysis was performed for a Siamese cat diagnosed histopathologically and biochemically with the disease in Japan in the 1960s and 2 domestic cats suspected of being affected with a lysosomal disease in Bangladesh in 2009. The results revealed that these 3 cats were homozygous for the c.1448G>C allele of the feline *GLB1* gene. Identification of the mutation in a Siamese cat in Japan and native domestic cats in Bangladesh, making a note of the history of cat domestication, suggests that the c.1448G>C mutation may have been transferred from native domestic cats to Siamese and Korat breeds in the process of breed establishment in Southeastern Asia. A newly developed PCR-based genotyping assay for feline GM1 gangliosidosis will be important for rapid differential diagnosis in cats suspected of having the disease and for genotyping in pure breeds related to Siamese and Korat cats.

In Chapters 3 and 4, the canine *ASPA* gene and the feline *GALC* gene were analyzed using a direct DNA sequencing method in order to clarify the molecular bases of Canavan disease in a mixed-breed dog and Krabbe disease in a mixed-breed cat. As a result, the pathogenic mutations c.[535_540delGTTGGT]+[527_534dupGTATAGAA] (p.G180fsX), for canine Canavan disease, and c.1945G>A (p.A649T), for feline Krabbe disease, were identified. In addition, a real-time PCR-based genotyping assay was developed for the diagnosis and genotyping of each mutation. The molecular bases of canine Canavan disease and feline Krabbe disease were defined for the first time. These findings will contribute to future studies that attempt to understand the pathogenesis of Canavan and Krabbe diseases and develop potential therapeutic methods in both human and veterinary medicine.

REFERENCES

Alroy, J., Orgad, U., Ucci, A. A., Schelling, S. H., Schunk, K. L., Warren, C. D., Raghavan, S.S. and Kolodny, E. H. 1985. Neurovisceral and skeletal GM1 gangliosidosis in dogs with β-galactosidase deficiency. *Science* **229**: 470–472.

Baker, H. J., Lindsey, J. R., McKhann, G. M. and Fallel, A. F. 1971. Neuronal GM1 gangliosidosis in a Siamese cat with β-galactosidase deficiency. *Science* **174**: 838–839.

Baker, H. J., Smith, B. F., Martin, D. R. and Foureman, P. 2001. Molecular diagnosis of gangliosidoses: a model for elimination of inherited diseases in pure breeds. pp. 615–620. *In*: Consultations in Feline Internal Medicine, 4th ed. (August, J. R. ed.) W. B. Saunders, Philadelphia.

Barker, C. G., Blakemore, W. F., Dell, A., Palmar, A., Tiller, P. R. and Winchester, B. G. 1986. GM1 gangliosidosis (type 1) in a cat. *Biochem. J.* **235**: 151–158.

Baslow, M. H. and Guilfoyle, D. N. 2013. Canavan disease, a rare early-onset human spongiform leukodystrophy: insight into its genesis and possible clinical interventions. *Biochimie.* **95**: 946–956.

Blakemore, W. F. 1972. GM1 gangliosidosis in a cat. *J. Comp. Pathol.* **82**:179–185.

Borda, J. T., Alvarez, X., Mohan, M., Ratterree M. S., Phillippi-Falkenstein, K., Lackner A. A. and Bunnell, B. A. 2008. Clinical and immunopathologic alterations in rhesus macaques affected with globoid cell leukodystrophy. *Am. J. Pathol.* **172**: 98–111.

Chang, H.S., Arai. T., Yabuki. A., Hossain. M. A., Rahman, M. M., Mizukami, K. and Yamato O. 2010. Rapid and reliable genotyping technique for GM1 gangliosidosis in Shiba dogs by real-time polymerase chain reaction with TaqMan minor groove binder probes. *J. Vet. Diagn. Invest*. **22**: 234–237.

De Gasperi, R., Sosa, M. A. G., Sartorato, E. L., Battistini, S., MacFarlane, H., Gusella, J. F., Krivit, W. and Kolodny, E. H. 1996. Molecular heterogeneity of late-onset forms of globoid-cell leukodystrophy. *Am. J. Hum. Genet*. **59**: 1233–1242.

De Maria, R., Divari, S., Bo, S., Sonnio, S., Lotti, D., Capucchino, M. and Castagnaro, M. 1998. β-glactosidase deficiency in a Korat cat: a new form of feline GM1 gangliodosis. *Acta Neuropathol.* **96**: 307–314.

Debs, R., Froissart, R., Aubourg, P., Papeix, C., Douillard, C., Degos, B., Fontaine, B., Audoin, B., Lacour, A., Said, G., Vanier, M. T. and Sedel, F. 2012. Krabbe disease in adults: Phenotypic and genotypic update from a series of 11 cases and a review. *J. Inherit. Metab*. Dis. DOI 10.1007/s10545-012-9560-4.

Handa, S. and Yamakawa, T. 1971. Biochemical studies in cat and human gangliosidosis. *J. Neurochem.***18**: 1275–1280.

Hasegawa, D., Yamato, O., Nakamoto, Y., Ozawa, T., Yabuki, A., Itamoto, K., Kuwabara, T., Fujita, M., Takahashi, K., Mizoguchi, S. and Orima, H. 2012. Serial MRI features of canine GM1 gangliosidosis: a possible imaging biomarker for diagnosis and progression of the disease. *Sci. World. J*. **2012**: 250197.

Janson, C. G., Kolodny, E. H., Zeng, B. J, Raghavan, S., Pastores, G., Torres, P., Assadi, M., McPhee, S., Goldfarb, O., Saslow, B., Freese, A., Wang, D.J., Bilaniuk, L., Shera, D.and Leone, P. 2006. Mild-onset presentation of Canavan's disease associated with novel G212A point mutation in aspartoacylase gene. *Ann. Neurol*. **59**: 428–431.

Jardim, L. B., Giugliani, R., Pires, R. F., Haussen, S., Burin M. G, Rafi, M. A. and Wenger, D. A., 1999. Protracted course of Krabbe disease in an adult patient bearing a novel mutation. *Arch. Neurol*. **56**: 1014–1017.

Johnson, K. H. 1970. Globoid leukodystrophy in the cat. *J. Am. Vet. Med. Assoc*. **157**: 2057–2064.

Kardas, F., Uzak, A. S., Hossain, M. A., Sakai, N., Canpolat, M. and Yıkılmaz, A. 2013. A novel homozygous *GALC* mutation: very early onset and rapidly progressive Krabbe disease. *Gene* **517**: 125–127.

Kaul, R., Balamurugan, K., Gao, G. P. and Matalon, R. 1994. Canavan disease: genomic organization and localization of human *ASPA* to 17p13-ter: conservation of the *ASPA* gene during evolution. *Genomics* **21**: 364–370.

Kaul, R., Gao, G. P., Balamurugan, K. and Matalon, R. 1993. Cloning of the human aspartoacylase cDNA and a common missense mutation in Canavan disease. *Nat. Genet*. **5**: 118–123.

Kawasaki, Y., Nagatani, I., Terashima, Y., Koike, H., Miyoshi, N. and Ohishi, A. 2009. Assessment of neurological function in three cats with homozygous or heterozygous GM1 gangliosidosis by brainstem auditory evoked potential. *J. Jpn. Vet. Med. Assoc.* **62**: 148–154 [in Japanese with English summary].

Kolodny, E. H, Sosa, M. A. G., Battistini, S., Sartorato, E. L, Yeretsian, J., MacFarlane, H., Gusella, J. and De Gasperi, R. 1995. Molecular genetics of late-onset Krabbe disease. *Am. J. Hum. Genet.* Supp **l57**: A217.

Kuruhara, Y. and Mochizuki, H. 1970. Electron microscopic studies on feline G1-gangliosidosis with similarities to Tay-Sachs disease. *Adv Neurol Sci (Shinkei Kenkyu No Shimpo)* **14**: 347–357 [in Japanese with English summary].

Kuruhara, Y., Mochizuki, H. and Kobayashi, Y. 1969. Feline cases of cerebral lipidosis with similarities to Tay-Sachs disease. *Adv Neurol Sci (Shinkei Kenkyu No Shimpo)***13**: 260–269 [in Japanese with English summary].

Leone, P., Janson, McPhee, C. G. and During, S. J. M. J. 1999. Global CNS gene transfer for a childhood neurogenetic enzyme deficiency: Canavan disease. *Curr. Opin. Mol. Ther.* **1**: 487–492.

Leone, P., Shera, D., McPhee, S. W., Francis, J. S., Kolodny, E. H., Bilaniuk, L. T., Wang, D. J., Assadi,M.,Goldfarb,O., Goldman,H. W.,Freese,A., Young,D., During M. J., Samulski,R. J., Janson C. G. 2012. Long-term follow-up after gene therapy for canavan disease. *Sci. Transl. Med*. ***4*****(165):** 163.

Luzi, P., Rafi, M. A., Victoria, T., Baskin, G. B. and Wenger, D. A. 1997. Characterization of the rhesus monkey galactocerebrosidase (GALC) cDNA and gene and identification of the mutation causing globoid cell leukodystrophy (Krabbe disease) in this primate. *Genomics* **42**: 319–324.

Martin, D. R., Rigat, B. A., Foureman, P., Varadarajan, G. S., Hwang, M., Krum, B. K., Smith, B. F., Callahan, J. W., Mahuran, D. J. and Baker, H. J. 2008. Molecular consequences of the pathogenic mutation in feline GM1 gangliosidosis. *Mol. Genet. Metab.* **94**: 212–221.

Matalon, R., Rady, P.L., Platt, K. A., Skinner, H. B., Quast, M. J., Campbell, G. A., Matalon, K., Ceci, J. D., Tyring, S. K., Nehls, M., Surendran, S., Wei, J., Ezell, E. L. and Szucs, S. 2000. Knock-out mouse for Canavan disease: a model for gene transfer to the central nervous system. *J. Gene. Med.* **2**: 165–175.

McGraw, R. A. and Carmichael, K. P. 2006. Molecular basis of globoid cell leukodystrophy in Irish setters. *Vet. J.* **171**: 370–372.

Mizukami, K., Chang, H. S., Yabuki, A., Kawamichi, T., Hossain, M. A., Rahman, M. M., Uddin, M. M. and Yamato,O. 2012.Rapid genotyping assays for the 4-base pair deletion of canine *MDR1/ABCB1* gene and low frequency of the mutant allele in Border Collie dogs. *J. Vet. Diagn. Invest.* **24**: 127–134.

Mizukami, K., Chang, H.S., Yabuki, A., Kawamichi, T., Kawahara, N., Hayashi, D., Hossain, M. A., Rahman, M. M., Uddin, M. M.and Yamato, O. 2011. Novel rapid genotyping assays for neuronal ceroidlipofuscinosis in Border Collie dogs and high frequency of the mutant allele in Japan. *J. Vet Diagn. Invest.* **23**: 1131–1139.

Mizukami, K., Kawamichi, T., Koie, H., Tamura, S., Matsunaga, S., Imamoto, S., Saito, M., Hasegawa, D., Matsuki, N., Tamahara, S., Sato, S., Yabuki, A., Chang, H.S. and Yamato, O. 2012. Neuronal ceroidlipofuscinosis in Border Collie dogs in Japan: clinical molecular epidemiological survey (2000–2011). *Sci. World J.* **2012**: 383174.

Mochizuki, H. and Kotani, T. 1977. GM1 gangliosidosis in a Japanese domestic cat. *Exp. Anim.* **26**: 281–282 [in Japanese].

Müller, G., Baumgärtner, W., Moritz, A., Sewell, A. and Kustermann-Kuhn, B. 1998. Biochemical findings in a breeding colony of Alaskan huskies suffering from GM1 gangliosidosis. *J. Inherited.Metab. Dis*. **21**: 430–431.

Namboodiri, A. M. A., Peethambaran, A., Mathew, R., Sambhu, P.A., Hershfield, J., Moffett, J. R. and Madhavarao, C. N. 2006. Canavan disease and the role of *N*-acetylaspartate in myelin synthesis. *Mol. Cell. Endocrinol.* **252**: 216–223.

O'Brien, S. J., Johnson, W., Driscoll, C., Pontius, J., Pecon-Slattery, J. and Menotti-Raymond, M. 2008. State of cat genomics. *Trends Genet.* **24**: 268–279.

Ogawa M, Uchida K, Isobe K, Saito M, Harada T, Chambers JK, Nakayama H. 2013. Globoid cell leukodystrophy (Krabbe‘s disease) in a Japanese domestic cat. *Neuropathology* in press.

Puckett, R. L., Orsini, J. J., Pastores, G. M., Wang, R. Y., Chang, R., Saavedra-Matiz, C. A., Torres, P. A., Zeng B., Caggana, M., Lorey, F. and Abdenur J. E. 2012. Krabbe disease: clinical, biochemical and molecular information on six new patients and successful retrospective diagnosis using stored newborn screening cards. *Mol. Genet. Metab*. **105**: 126–131.

Rahman, M. M., Chang, H. S., Mizukami, K., Hossain, M. A., Yabuki, A., Tamura, S., Kitagawa, M., Mitani, S., Higo, T., Uddin, M. M., Uchida, K. and Yamato, O. 2012. A frameshift mutation in the canine *HEXB* gene in toy poodles with GM2 gangliosidosis variant 0 (Sandhoff disease). Vet. J. **194**: 412–416.

Rahman, M. M., Shoubudani, T., Mizukami, K., Chang, H. S., Hossain, M. A., Yabuki, A., Mitani, S., Higo, T., Arai, T. and Yamato, O. 2011. Rapid and simple polymerase chain reaction-based diagnostic assays for GM2 gangliosidosis variant 0 (Sandhoff-like disease) in Japanese domestic cats. *J. Vet. Diagn. Invest.* **23**: 338–342.

Rashed, M. S. 2001. Clinical application of tandem mass spectrometry: ten years of diagnosis and screening for inherited metabolic diseases. *J. Chromatogr. B***758**: 27–48.

Sakai, N., Inui, K., Tatsumi, N., Fukushima, R., Nishigaki, T., Taniike, M., Nishimoto, J., Tsukamoto, H., Yanagihara, I., Ozono, K. and Okada, S. 1996. Molecular cloning and expression of cDNA for murine galactocerebrosidase and mutation analysis of the twitcher mouse, a model of Krabbe’s disease. *J. Neurochem*. **66**: 1118–1124.

Salvadori, C., Modenato, M., Corlazzoli, D. S., Arispici, M. and Cantile, C. 2005. Clinicopathological features of globoid Cell Leucodystrophy in Cats. *J. Comp. Pathol*. **132**: 350–356.

Satoh, H., Yamato, O., Asano, T., Yonemura, M., Yamauchi, T., Hasegawa, D., Orima, H., Arai, T., Yamasaki, M. and Maede, Y. 2007. Cerebrospinal fluid biomarkers showing neurodegeneration in dogs with GM1 gangliosidosis: possible use for assessment of a therapeutic regimen. *Brain Res*. **1133**: 200–208.

Saunders, G. K., Wood, P. A., Myers, R. K., Shell, L. G. and Carithers, R. 1988. GM1 gangliosidosis in Portuguese water dogs: pathologic and biochemical findings. *Vet. Pathol.* **25**: 265–269.

Sewell, A. C., Haskins, M. E. and Giger, U. 2007. Inherited metabolic disease in companion animals: Searching for nature's mistakes. *Vet. J.* **174**: 252–259.

Shaag, A., Anikster, Y., Glustein, E. J. Z., Fois, A., Michelakakis, H., Nigro, F., Pronicka, E., Ribes, A., Zabot, M. T. and Elpeleg, O. N. 1995. The molecular basis of Canavan (aspartoacylase deficiency) disease in European non-Jewish patients. *Am. J. Hum. Genet.* **57**: 572–580.

Sigurdson, C. J., Basaraba, R. J., Mazzaferro, E. M. and Gould, D. H. 2002. Globoid cell-like leukodystrophy in a domestic longhaired cat. *Vet. Pathol.* **39**: 494–496.

Sistermans, E. A., de Coo, R. F. M., van Beerendonk, H. M., Poll-The, B. T., Kleijer, W.J. and van Oost, B. A. 2000. Mutation detection in the aspartoacylase gene in 17 patients with Canavan disease: four new mutations in the non-Jewish population. *Eur. J. Hum. Genet.* **8**: 557–560.

Sreenivasan, P. and Purushothaman, K. K. 2013. Radiological clue to diagnosis of Canavan disease. *Indian J. Pediatr.* **80**: 75–77.

Suzuki, K. and Grover, W. D. 1970. Krabbe's leukodystrophy (globoid cell leukodystrophy). An ultrastructural study. *Am. J. Obstet. Gynecol*. **106**: 385–396.

Suzuki, k. and Suzuki, k. 1985. Genetic galactosyiceramidase deficiency(globoid cell leukodystrophy, krabbe disease) in different mammalian species. *Neurochem. Pathol.* **3**: 53–68.

Suzuki, Y., Oshima, A. and Nanba, E. 2001. β-Galactosidase deficiency (β-galactosidosis): GM1 gangliosidosis and Morquio B disease. pp. 3775–3809. *In*: The Metabolic and Molecular bases of Inherited Disease, 8th ed. (Scriver, C. R., Beaudet, A. L., Sly, W. S. and Valle, D., eds.), McGraw-Hill, New York.

Szymańska, K., Ługowska, A., Laure-Kamionowska, M., Bekiesińska-Figatowska, M., Szymańska, K., Ługowska, A., Laure-Kamionowska, M., Bekiesińska-Figatowska, M., Gieruszczak-Białek, D., Musielak, M., Eichler, S., Giese, A. K. and Rolfs, A. 2012. Diagnostic difficulties in Krabbe disease: a report of two cases and review of literature. *Folia Neuropathol.* **50**: 346–356.

Tacke, U., Olbrich, H., Sass, J. O., Fekete, A., Horvath, J., Ziyeh, S., Kleijer, W. J., Rolland, M. O., Fisher, S., Payne, S., Vargiami, E., Zafeiriou, D. I. and Omran, H. 2005. Possiblegenotype-phenotypecorrelations in children with mildclinicalcourse of Canavandisease. *Neuropediatrics* **36**: 252–255.

Tappino, B., Biancheri, R., Mort, M., Regis, S., Corsolini, F., Rossi, A., Stroppiano, M., Lualdi, S., Fiumara, A., Bembi, B., Di Rocco, M., Cooper, D.N. and Filocamo, M. 2010.

Identification and characterization of 15 novel *GALC* gene mutations causing Krabbe disease. *Hum. Mutat.* **31**: E1894–1914.

Uddin,M. M., Tanimoto, T., Yabuki, A., Kotani, T., Kuwamura, M., Chang, H. S. and Yamato, O. 2012. Mutation analysis of GM1 gangliosidosis in a Siamese cat from Japan in the 1960s. *J. Feline Med. Surg.* **14**: 900–902.

Vella, C. M., Shelton, L. M., McGonagle, J. J. and Stanglein, T. W. 1999. Robinson's Genetics for Cat Breeds and Veterinarians. 4th ed., Butterworth-Heinemann, Oxford.

Victoria, T., Rafi, M.A. and Wenger, D.A. 1996. Cloning of the canine GALC cDNA and identification of the mutation causing globoid cell leukodystrophy in West Highland White and Cairn Terriers. *Genomics* **33**: 457–462.

Wang, C. Y. and Smith, B. F. 2007. Development of quantitative polymerase chain reaction assays for allelic discrimination of gangliosidoses in cats. *Am. J. Vet. Res.* **68**: 231–235.

Wenger, D. A, Suzuki, K., Suzuki, Y. and Suzuki, K. 2001. Galactosylceramide lipidosis: Globoid cell leukodystrophy (Krabbe disease). pp. 3669–3694. *In*: The Metabolic and Molecular Bases of Inherited Disease, 8th ed. (Scriver, C. R., Beaudet, A.L., Sly, W.S., Valle, D., Childs, B. and Vogelstein, B. eds.) McGraw-Hill, New York.

Wenger, D. A., Rafi, M. A. and Luzi, P. 1997. Molecular genetics of Krabbe disease (globoid cell leukodystrophy): Diagnostic and clinical implications. *Hum. Mutat*. **10**: 268–279.

Wenger, D.A. and Riccardi, V.M. 1976. Possible misdiagnosis of Krabbe disease. *J. Pediatr*. **88**: 76–77.

Wood, S. L.and Patterson, J. S. 2001. Shetland sheepdog leukodystrophy. *J. Vet. Intern. Med.* **15** 486–493.

Yalcinkaya, C., Benbir, G., Salomons, G. S., Karaarslan, E., Rolland, M. O., Jakobs, C. and van der Knaap, M. S. 2005. Atypical MRI findings in Canavan disease: a patient with a mild course. *Neuropediatrics* **36**: 336–339.

Yamato, O., Endoh, D., Kobayashi, A., Masuoka, Y., Yonemura, M., Hatakeyama, A., Satoh, H., Tajima, M., Yamasaki, M. and Maede, Y. 2002. A novel mutation in the gene for canine acid β-galactosidase that causes GM1 gangliosidosis in Shiba dogs. *J. Inherited. Metab. Dis*. **25**: 525–526.

Yamato, O., Funk, H., Kawahara, N., Funková, M., Entlerová, Z., Petrusová, H., Hayashi, D., Satoh, H., Yamasaki, M., Maede, Y., Yabuki, A. and Arai, T. 2009. Mutation screening of canine GM1 gangliosidosis for preventive measures: absence of the mutant allele in breeding Shiba dogs in the Czech Republic. *Jpn. J. Prophylactic Vet. Med.* **1**: 63–68.

Yamato, O., Jo, E. O., Chang, H. S., Satoh, H., Shoda, T., Sato, R., Uechi, M., Kawasaki, N., Naito, Y., Yamasaki, M., Maede, Y. and Arai, T. 2008. Molecular screening canine GM1 gangliosidosis using blood smear specimens after prolonged storage: detection of carriers among Shiba dogs in northern Japan. *J. Vet. Diagn. Invest.* **20**: 68–71.

Yamato, O., Jo, E. O., Shoda, T., Yamasaki, M. and Maede Y. 2004. Rapid and simple mutation screening of GM1 gangliosidosis in Shiba dogs by direct amplification of deoxyribonucleic acid from various forms of canine whole-blood specimens. *J. Vet. Diagn. Invest.* **16**: 469–472.

Yamato, O., Masuoka, Y., Yonemura, M., Hatakeyama, A., Satoh, H., Kobayashi, A., Nakayama, M., Asano, T., Shoda, T., Yamasaki, M., Ochiai, K., Umemura, T. and Maede, Y. 2003. Clinical and clinico-pathologic characteristics of Shiba dogs with a deficiency of lysosomal acid β-galactosidase: a canine model of human GM1-gangliosidosis. *J. Vet. Med. Sci*. **65**: 213–217.

Yamato, O., Ochiai, K., Masuoka, Y., Hayashida, E., Tajima, M., Omae, S., Iijima, M., Umemura, T.and Maede, Y. 2000. GM1 gangliosidosis in Shiba dogs. *Vet. Rec*. **146**: 493–496.

Yang, Y., Ren, X., Xu, Q., Wang, C., Liu, H. and He, X. 2013. Four novel *GALC* gene mutations in two Chinese patients with Krabbe disease. *Gene* **519**: 381–384.

Zeng, B. J., Pastores, G. M., Leone, P., Raghavan, S., Wang, Z. H., Ribeiro, L. A., Torres, P., Ong, E. and Kolodny, E. H. 2006b. Mutation analysis of the aspartoacylase gene in non-Jewish patients with Canavan disease. *Adv. Exp. Med. Biol.* **576**:165–173.

Zeng, B. J., Wang, Z. H., Torres, P. A., Pastores, G. M., Leone, P., Raghavan, S. S. and Kolodny, E. H., 2006a. Rapiddetection of three large novel deletions of the aspartoacylase gene in non-Jewish patients with Canavan disease. *Mol. Genet. Metab.* **89**: 156–163.

ACKNOWLEDGEMENTS

First of all, I would like to give my sincere thankfulness, deepest sense of gratitude to my respected supervisor Professor Dr. Osamu Yamato for scholastic supervision, intellectual guidance, ingenious help and support during the preparation and accomplishment of the present research work, including constant opportunity for critical discussion in every aspect of my study. In addition, I would like to express my immense indebtedness to Dr. Yamato for generous help and support regarding my non-academic issues in Japan.

I would like to express my gratitude to my co-supervisors, Associate Professor Dr. Akira Yabuki and Associate Professor Dr. Hosaka Yoshinao for their helpful suggestions, scientific supports and continuous encouragement during my study. Dr. Yabuki has been very kind to me with whom I was able to discuss openly on academic and non-academic issues. I thank to several current and past fellows, who often helped me in some way, during the course of this work. I am also indebted to the student members at the Laboratory of Veterinary Clinical Pathology, Joint Faculty of Veterinary Medicine, Kagoshima University for their efforts, cooperation, and patience, and for maintaining a pleasant working atmosphere.

I owe special thanks to those who have provided the samples for analysis. Without their help and support this work would never have been possible.

I would also like to thank my colleagues especially from the Department of Anatomy & Histology and Professor Dr. Mohammad Alamgir Hossain, Chittagong Veterinary and Animal Sciences University, Bangladesh, who made possible my further education in The United Graduate School of Veterinary Sciences, Yamaguchi University, Japan. I would also like to express thanks and love to my mother, wife, daughter and all other my family members for their support and encouragement during my stay and study in Japan.