

Molecular study on the diagnosis, genotyping, and allele  
frequency of multiple inherited disorders in dogs and cats  
(犬および猫の多種遺伝子病の診断法、遺伝子型判定  
ならびにアレル頻度に関する分子的研究)

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## ABSTRACT

At present, inherited disorders in animals are not rare. In the Online Mendelian Inheritance in Animals which is the database of inherited disorders and traits in animals, more than 3,000 disorders/traits have been registered in 243 animal species. The number of identified causative variants for inherited disorders/traits has been increased with the advancement of animal genetics after the canine and feline whole genome sequence projects were completed approximately 15 and 10 years ago, respectively. In such a situation, diagnostic and genotyping methods based on the causative mutations become more important and necessary in veterinary medicine. Molecular diagnosis enables veterinarians to choose the appropriate therapeutic methods and determine the accurate prognosis. The genotyping methods can be used for epidemiological study to prevent and/or control genetic disorders. The study on inherited disorders using animal models would contribute to human medicine as well as clarifying animal disorders. The objectives of this thesis are to identify inherited disorders in animals and their causative mutation, develop their rapid and easy diagnostic and genotyping methods, and perform an epidemiological study to determine the allele frequency in the animal population.

Gangliosidosis is one of the inherited disorders likely to be encountered in the field of veterinary medicine. GM1 and GM2 gangliosidoses are progressive neurodegenerative lysosomal storage diseases resulting from the excessive accumulation of GM1 and GM2

gangliosides in the lysosomes, respectively. The disorders are more likely to occur in many animal species and breeds compared to other lysosomal diseases. Naturally occurring GM1 gangliosidosis has been reported in dogs, cats, ruminants, and wild animals. Naturally occurring GM2 gangliosidosis has been reported in dogs, cats, Yorkshire pigs, Jacob sheep, rabbits, Muntjak deer, and American flamingos. In the first chapter, the case report of GM2 gangliosidosis variant 0 in a mixed-breed dog was described. In this case, the diagnostic methods for GM1 and GM2 gangliosidosis were employed based on comprehensive findings, which included clinical, biochemical, histopathological, and genetic findings using various types of specimens. Subsequently, the auxiliary diagnosis methods for the diagnosis of both gangliosidoses using *in situ* detection of GM1 and GM2 gangliosides were established to diagnose animals suspected to have gangliosidosis. These novel methods were considered as useful for the retrospective diagnosis of suspected cases of all animal species whose paraffin-embedded cerebral tissues are stored.

In the second chapter, an epidemiological study for more common genetic disorders of pure-breed dogs was described. The epidemiological data are important for preventing and/or controlling genetic disorders by establishing breeding strategies. First, canine degenerative myelopathy (DM) in the Collie breed was selected. Canine DM is an adult-onset, progressive neurodegenerative disease that occurs in multiple dog breeds. A DM-associated mutation of the canine superoxide dismutase 1 (*SOD1*) gene, designated c.118G>A (p.E40K), has been

implicated as one of pathogenetic determinants of the disease in many breeds, but it remains to be determined whether the c.118G>A mutation is responsible for development or progression of DM in Collies. Previously, DM was diagnosed clinically and histopathologically in a Rough Collie, in Japan, suggesting the possibility that the Collie breed may be predisposed to DM due to the high frequency of c.118G>A in Japan. The formalin-fixed spinal cord from the dog was further investigated by immunohistochemical analysis and a molecular epidemiological survey of c.118G>A in a population of Collies in Japan was performed to determine the mutant allele frequency (0.138). Secondly, canine progressive rod-cone degeneration (PRCD) was selected. Canine PRCD is a middle- to late-onset, autosomal recessive, inherited retinal disorder caused by a substitution (c.5G>A) in the canine *PRCD* gene that has been identified in 29 or more purebred dogs. A TaqMan probe-based real-time PCR assay was developed and evaluated for rapid genotyping and large-scale screening of this mutation. Furthermore, a genotyping survey was carried out in a population of the three most popular breeds in Japan (Toy Poodles, Chihuahuas, and Miniature Dachshunds) to determine the current mutant allele frequency (0.09, 0.02, and 0.00, respectively).

In conclusion, clinical and immunohistochemical diagnostic methods described in the first chapter will contribute to improving our understanding of the characteristics of gangliosidosis. Furthermore, molecular epidemiological findings and data obtained in the second chapter will

provide the novel and useful information on most popular pure-breed dogs in Japan for veterinary practitioners, researchers, and breeders.

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## FREQUENTLY USED ABBREVIATION

BMDs	-	Bernese Mountain Dogs
DAB	-	3,3'-diaminobenzidine
DAPI	-	4',6-diamidino-2-phenylindole dihydrochloride
DM	-	degenerative myelopathy
HE	-	hematoxylin and eosin
Hex	-	hexosaminidase
LFB	-	Luxol fast blue
MGB	-	minor groove binder
MR	-	magnetic resonance
OMIA	-	Online Mendelian Inheritance in Animals
PBS	-	phosphate-buffered saline
PRA	-	progressive retinal atrophy
PRCD	-	progressive rod-cone degeneration
PWCs	-	Pembroke Welsh Corgis
SOD1	-	superoxide dismutase 1
SD	-	Sandhoff disease
TLC	-	thin-layer chromatography
T <sub>m</sub>	-	melting temperature

## CHAPTER 1.1

### **Case study of GM2 gangliosidosis variant 0 (Sandhoff disease) in a mixed-breed dog**

The above-titled work originally appeared in “*Journal of the American Animal Hospital Association* (Kohyama et al., 2015)” as: **GM2 Gangliosidosis Variant 0 (Sandhoff Disease) in a Mixed-Breed dog** authored by:

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### **1.1.1. ABSTRACT**

GM2 gangliosidosis variant 0 (Sandhoff disease, SD) is a fatal, progressive, neurodegenerative lysosomal storage disease caused by simultaneous deficiencies of acid  $\beta$ -hexosaminidases A and B. Canine SD has so far been identified only in two purebreeds. In this article, we present the case of a 10 mo old, male dog of mixed breed that developed progressive neurological signs including ataxia, postural deficit, and visual deficits and finally died at the age of 21 mo. The dog was diagnosed with SD on the basis of the results of biochemical and histopathological analyses. This is the third report of canine SD and the first time it has been identified in a mixed breed.

### 1.1.2. INTRODUCTION

GM2 gangliosidoses are a group of progressive, neurodegenerative, lysosomal storage diseases resulting from the excessive accumulation of GM2 ganglioside and related glycoconjugates in lysosomes, especially in the neuronal lysosomes (Gravel et al., 2001; Yamato et al., 2004b; Haskins et al., 2008). There are three main variants of GM2 gangliosidoses: 0, B, and AB, caused by defects of different genes. The diseases are inherited in an autosomal recessive manner, and affected individuals die prematurely of brain damage, with progressive neurological signs including motor and psychointellectual dysfunction and visual defects. GM2 gangliosidosis variant 0, called Sandhoff disease (SD), is a variant of GM2 gangliosidosis caused by deleterious mutations of the *HEXB* gene encoding the  $\beta$ -subunit that is a common component of the lysosomal acid  $\beta$ -hexosaminidase (Hex) A and Hex B.

In veterinary species, naturally occurring SD has been reported in dogs and cats. Canine SD has been reported in a golden retriever and in toy poodles (Yamato et al., 2002; Tamura et al. 2010). The mutation causing SD in toy poodles is the only mutation that has been identified in canine SD (Rahman et al. 2012). Here, we report the first case of SD in a mixed-breed dog.

### **1.1.3. CASE REPORT**

A 10 mo old, medium-sized, male dog of mixed breed, weighing 12.2 kg and with a black-and-white, longhaired coat, was referred to the Minamata Animal Hospital with a complaint of hindlimb weakness. The motor disorder gradually progressed to weakness of all four limbs; by the age of 13 mo, the dog could not walk or stand normally. The dog was referred to the Kitano Animal Hospital for further examinations, but hematological examinations such as complete blood count and biochemistry, as well as radiographic examination of the whole body, did not show any abnormality.

By 14 mo old, the dog's weight had been reduced to 9.6 kg, with a body condition score of 1.5 on a 5-point scale, and the dog was therefore referred to the Veterinary Teaching Hospital in Kagoshima University for a complete examination. Neurological examination revealed lethargy, ataxic astasia, decreased proprioception in hindlimbs, bilateral absence of the menace response, and increased reflex response in all four limbs. Although the pupillary light reflex was normal, the dog had a visual deficit as demonstrated by the absence of any reaction in the cotton-ball test. Magnetic resonance (MR) images of the brain obtained using a 0.4 Tesla system (APERTO Inspire; Hitachi Medical Corporation, Tokyo, Japan) did not show any abnormality. The dog's cerebrospinal fluid was transparent, with a normal cell count (1.7 cells/IL) and a normal concentration of total protein (11.9 mg/dL). Serum antibody titer against canine distemper virus was not increased. Hematological examinations such as

complete blood count and biochemistry did not show any abnormality. A blood smear stained with Giemsa stain showed excessive cytoplasmic vacuolation in approximately 30% of the monocytes (Fig. 1A), but there were no abnormal cytoplasmic vacuoles in lymphocytes and other leukocytes.

The progressive neurologic signs and excessive monocyte vacuoles suggested a lysosomal storage disorder. Therefore, leukocytes, separated from heparinized whole blood, were subjected to fluorometric assays for the activity of lysosomal acid hydrolases using artificial substrates for each enzyme (Yamato et al., 2000; Yamato et al., 2002). The assay revealed markedly decreased activities of both Hex A and Hex B; however, the activity of  $\beta$ -galactosidase measured to warrant the quality of the specimen was within the normal range (Table 1). This resulted in the establishment of an antemortem biochemical diagnosis of SD due to simultaneous deficiencies of Hex A and Hex B.

Finally, the dog died of neurological deterioration at the age of 21 mo, and a whole-body autopsy was performed with the owner's permission. Grossly, there were no abnormal lesions in either the brain or the visceral organs. Histopathological examination revealed that the neurons were swollen like balloons throughout the central nervous system, and the number of Purkinje cells was reduced in the cerebellum. These swollen neurons, when stained with hematoxylin and eosin, demonstrated pale-to-eosinophilic granular materials in the cytoplasm (Fig. 1B). This neuronal change was also observed in the spinal ganglia. Slight myelin loss

was observed in grey and white matters in the cerebrum stained with Luxol Fast Blue staining (data not shown), suggesting dysmyelination and/or demyelination. There was no abnormal change in liver, kidney, heart, and testis. Membranous cytoplasmic bodies were observed in transmission electron microscopic images of the cerebral neurons (Fig. 1C), suggesting the accumulation of sphingolipids, such as gangliosides. To confirm GM2 ganglioside accumulation due to Hex deficiency, immunohistochemical examination was performed with biotin-conjugated monoclonal antibody against *N*-acetyl GM2 ganglioside (Biotin conjugated anti-*N*-acetyl GM2 monoclonal antibody; Seikagaku Kogyo, Tokyo, Japan) (1:10 dilution), peroxidase-labeled streptavidin (Peroxidase-labeled streptavidin; KPL, Gaithersburg, MD, USA), and 3,3'-diaminobenzidine (DAB buffer tablet; Merck, Darmstadt, Germany) as a peroxidase substrate. Sections were counterstained with Mayer's hematoxylin stain. This immunohistochemical analysis demonstrated the accumulation of GM2 ganglioside in the cytoplasm of neurons (Fig. 1D). On the basis of the results of clinical, biochemical, morphological, and immunohistochemical analyses, the dog was conclusively diagnosed with SD.

When using a genotyping assay developed previously, DNA from the dog tested negative for the mutation of the canine *HEXB* gene that causes SD in toy poodles (Rahman et al., 2012). Pedigree analysis was performed based on the information obtained from the dog's owner. A clinically healthy dam, a female littermate, and a related bitch were kept in the same

house with the affected dog. The dam and the sire, who had died in a traffic accident at about 1 yr of age, seemed to have originated from the same pedigree in a different house. All the dogs in this pedigree were mixed breed. Lysosomal enzyme activities in the leukocytes from the pedigree members were measured, but no dog was found to have deficient activities of Hex A or Hex B (Table 1).

#### **1.1.4. DISCUSSION**

The present study reports the clinical, biochemical, pathological, and ultrastructural findings in a mixed-breed dog that exhibited progressive motor and visual disorders from 10 to 21 mo of age before dying prematurely of neurological deterioration. The onset and duration of the disease and most of the clinical signs were similar to those of SD previously reported in a golden retriever and in a family of toy poodles, with SD onset between 9 and 12 mo of age and death before completing 2 yr (Yamato et al., 2002; Matsuki et al., 2005; Tamura et al., 2010). The clinical signs common to the present case and previous reported cases are postural deficit, ataxia, decreased menace response, visual deficits, and a decreased level of consciousness. The excessive cytoplasmic vacuolation of monocytes that was observed in the blood smear of the present case (Fig. 1A) has also been observed in toy poodles with SD (Tamura et al., 2010). Enzymatic analysis showed deficient activities of both Hex A and Hex B (Table 1), resulting in the antemortem biochemical diagnosis of SD. Histopathological and ultrastructural analyses suggested sphingolipidosis because of swollen neurons with inclusions that were found to be membranous cytoplasmic bodies by electron microscopy (Figs. 1B, C). Immunohistochemical analysis demonstrated the accumulation of GM2 ganglioside (Fig. 1D) and led to the definitive diagnosis of SD. This is the third report of canine SD and the first time the disease has been recognized in a mixed breed.

Few findings differed in the present case from those reported in a golden retriever and

toy poodles in earlier reports (Yamato et al., 2002; Matsuki et al., 2005; Tamura et al., 2010). Miosis and loss of palpebral and pupillary light reflexes were observed in the golden retriever; the toy poodles showed decreased or absent corneal reflexes causing corneal ulcer and panophthalmitis, with frequent vomiting starting early in the course of the disease (Yamato et al., 2002; Matsuki et al., 2005; Tamura et al., 2010). However, none of these signs were observed in the dog in this report. Abnormal cytoplasmic vacuoles were seen in approximately 10% of peripheral lymphocytes in the toy poodles, but not in the golden retriever or in the dog in this report, suggesting that lymphocyte vacuolation is not a very frequent finding in canine SD (Tamura et al., 2010). The excessive cytoplasmic vacuolation of monocytes observed in the dog in this report may be specific to canine SD; therefore, this finding may be a useful contribution to the clinical diagnosis of canine SD, particularly when the lymphocyte vacuolation is not observed. Among MR findings, bilateral T2 hyperintensity and T1 hypointensity in the caudate nucleus, along with mild atrophy of the cerebral cortex, were observed in the affected golden retriever, while diffuse T2 hyperintensity of the subcortical white matter in the cerebrum and bilateral T2 hyperintensity and T1 hypointensity in the caudate nucleus were observed in the toy poodles (Matsuki et al., 2005; Tamura et al., 2010). However, there was no abnormality in the MR images of the dog in this report. The different findings in the same disease may be attributable to different mutations of the canine *HEXB* gene. The dog in this report did not have the same mutation as the toy poodles;

however, whether it had the same mutation as the golden retriever remains to be seen, because the latter's mutation has not yet been identified. Further studies are needed to identify all pathogenic mutation(s) and elucidate the phenotype–genotype correlation.

In the present report, pedigree analysis was performed and the activities of Hex A and B were measured using leukocytes from other pedigree members: the dam, a littermate, and a relative of the proband (Table 1). The littermate showed about half of the normal Hex A and Hex B activity, while the dam and relative showed slightly lower-than-normal activities. Therefore, these three pedigree members were suspected to be heterozygous carriers, but the carrier status could not be determined using only the Hex activity. Indeed, it has been shown that Hex activity cannot be used for genotyping carriers with feline SD owing to the wide overlap in enzyme activity between normal and carrier animals, although the mean value of leukocyte Hex activity in carriers is almost half the normal value (Yamato et al., 2008). It is therefore necessary to identify the pathogenic mutation(s) and develop specific DNA assay(s) for detection of carriers and diagnosis.

Most canine heritable diseases have been reported in purebred dogs and are very rare in mixed-breed dogs (OMIA – Online Mendelian Inheritance in Animals <http://omia.angis.org.au/home/> Accessed Sep 10, 2015). If mixed-breed dogs have a purebred ancestor, they may be susceptible to inherited diseases present in that purebreed. The physical size and hair coat of the dog in this report were similar to those of

European-bred dogs such as setters and border collies. There is a possibility that the pathogenic mutation(s) for this disease might have been transmitted from purebreeds, although the mutation might also have occurred de novo in this closed, mixed-breed pedigree.

Further studies are needed to elucidate this issue.

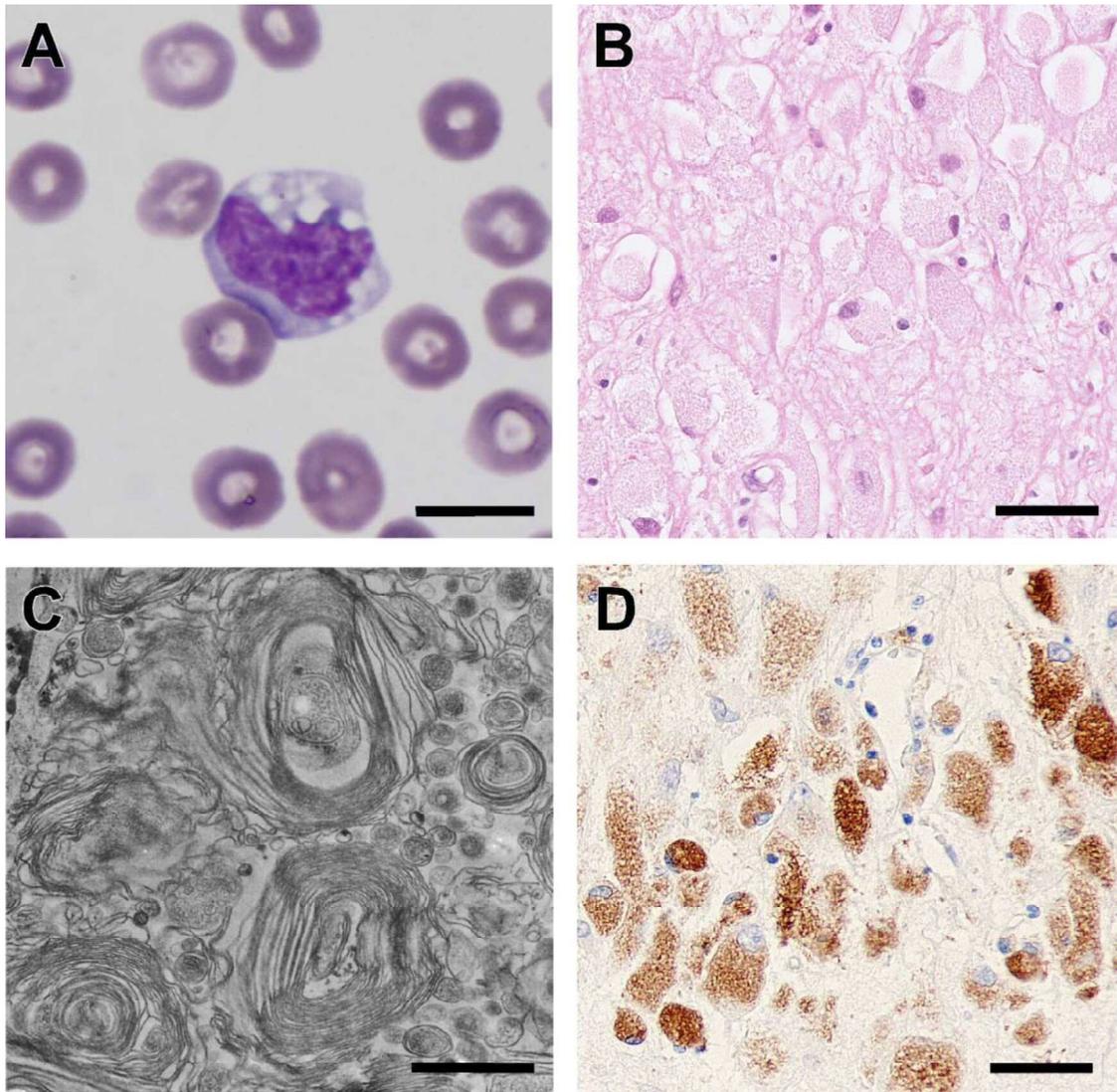
**Table 1.** Activities of lysosomal acid  $\beta$ -hexosaminidases A and B and  $\beta$ -galactosidase in the peripheral leukocytes of a mixed-breed dog with GM2 gangliosidosis variant 0 (Sandhoff disease), its pedigree members, and a control dog, with reference values.

Dog	$\beta$ -hexosaminidase A <sup>a</sup>	$\beta$ -hexosaminidase B <sup>a</sup>	$\beta$ -galactosidase <sup>a</sup>
Affected dog (male) <sup>b</sup>	25	0.2	135
Dam (female) <sup>b</sup>	758	172	114
Littermate (female) <sup>b</sup>	596	86	126
Relative (female) <sup>b</sup>	875	171	57
Control (male) <sup>b</sup>	1115	216	139
Reference value (n = 22) <sup>c</sup>	1454 $\pm$ 401	143 $\pm$ 85	145 $\pm$ 29

<sup>a</sup> The activities are expressed as nmol/h/mg of protein.

<sup>b</sup> Blood samples were collected and treated in the same way as in an adult control dog that was healthy, male, and of mixed breed.

<sup>c</sup> Mean  $\pm$  standard deviation calculated from the data obtained from 22 clinically healthy dogs.



**Figure 1.** Hematological, histopathological, ultrastructural, and immunohistochemical findings in a mixed-breed dog with GM2 gangliosidosis variant 0 (Sandhoff disease). (A) Vacuolated cytoplasm of a circulating monocyte. Giemsa stain. Bar = 10  $\mu\text{m}$ . (B) Cerebral cortex, hematoxylin and eosin stain. Bar = 50  $\mu\text{m}$ . (C) Cytoplasm of a cerebral neuron showing membranous cytoplasmic bodies. Bar = 2  $\mu\text{m}$ . (D) Cerebral cortex, stained using a combination of biotin-conjugated monoclonal antibody against *N*-acetyl GM2 ganglioside, peroxidase-labeled streptavidin, and 3,3'-diaminobenzidine as a peroxidase substrate, with the background counterstained with Mayer's hematoxylin stain. Bar = 50  $\mu\text{m}$ .

## CHAPTER 1.2

### ***In situ* detection of GM1 and GM2 gangliosides using immunohistochemical and immunofluorescent techniques for auxiliary diagnosis of canine and feline gangliosidoses**

The above-titled work originally appeared in “*BMC Veterinary Research* (Kohyama et al., 2016)” as: ***In situ* detection of GM1 and GM2 gangliosides using immunohistochemical and immunofluorescent techniques for auxiliary diagnosis of canine and feline gangliosidoses** authored by:

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### **1.2.1. ABSTRACT**

GM1 and GM2 gangliosidoses are progressive neurodegenerative lysosomal storage diseases resulting from the excessive accumulation of GM1 and GM2 gangliosides in the lysosomes, respectively. The diagnosis of gangliosidosis is carried out based on comprehensive findings using various types of specimens for histological, ultrastructural, biochemical and genetic analyses. Therefore, the partial absence or lack of specimens might have resulted in many undiagnosed cases. The aim of the present study was to establish immunohistochemical and immunofluorescent techniques for the auxiliary diagnosis of canine and feline gangliosidoses, using paraffin-embedded brain specimens stored for a long period. Using hematoxylin and eosin staining, cytoplasmic accumulation of pale to eosinophilic granular materials in swollen neurons was observed in animals previously diagnosed with GM1 or GM2 gangliosidosis. The immunohistochemical and immunofluorescent techniques developed in this study clearly demonstrated the accumulated material to be either GM1 or GM2 ganglioside. Immunohistochemical and immunofluorescent techniques using stored paraffin-embedded brain specimens are useful for the retrospective diagnosis of GM1 and GM2 gangliosidoses in dogs and cats.

### 1.2.2. INTRODUCTION

GM1 and GM2 gangliosidoses are progressive neurodegenerative lysosomal storage diseases resulting mainly from the excessive accumulation of GM1 and GM2 gangliosides in the lysosomes, respectively (Haskins et al., 2008). These diseases are inherited in an autosomal recessive manner and result in the premature death of affected individuals due to brain damage with progressive neurological signs. In GM1 gangliosidosis, the accumulation of GM1 ganglioside is caused by an inherited deficiency of the lysosomal acid  $\beta$ -galactosidase (Suzuki et al., 2001). In GM2 gangliosidosis, the accumulation of GM2 ganglioside is caused by an inherited deficiency of the lysosomal acid  $\beta$ -hexosaminidase A or GM2 activator protein in GM2 gangliosidosis, and the disease is accordingly categorized into three variants: Tay-Sachs disease (B variant), Sandhoff disease (O variant), and GM2 activator protein deficiency (AB variant) (Gravel et al., 2001).

Gangliosidosis is more likely to occur in many animal species and breeds compared to other lysosomal diseases. Naturally occurring GM1 gangliosidosis has been reported in dogs, including mixed Beagles (Read et al., 1976), English Springer Spaniels (Alroy et al., 1985), Portuguese Water dogs (Saunders et al., 1988), Alaskan Huskies (Müller et al., 1998), Shiba Inus (Yamato et al., 2000), and a mixed-breed dog (Whitfield et al., 2000), and in cats, including Siamese (Baker et al., 1971; Uddin et al., 2012), Korat (De Maria et al., 1998), and several families of domestic cats (Blakemore et al., 1972; Barnes et al., 1981; Baker et al.,

1986; Uddin et al., 2013; Ueno et al., 2016). In addition, GM1 gangliosidosis has been reported in ruminants such as Friesian calves (Donnelly et al., 1973), Suffolk sheep (Ahern-Rindell et al., 1988), Coopworth Romney-cross sheep (Skelly et al., 1995), and Romney sheep (Ryder et al., 2001), and in wild species such as American black bears (Muthupalani et al., 2014) and emus (Bermudez et al., 1995). Naturally occurring GM2 gangliosidosis has been reported in dogs, including German Shorthair Pointers (Karbe, 1973), Japanese Spaniels (Chins) (Cummings et al., 1985; Sanders et al., 2013), a Golden Retriever (Yamato et al., 2002), Toy Poodles (Tamura et al., 2010), and mixed-breed dogs (Rotmistrovsky et al., 1991; Kohyama et al., 2015), and in cats, including Korat (Neuwelt et al., 1985), European Burmese (Bradbury et al., 2009), and several families of domestic cats (Cork et al., 1977; Yamato et al., 2004c; Martin et al., 2005). In addition, GM2 gangliosidosis has been reported in Yorkshire pigs (Kosanke et al., 1978), Jacob sheep (Torres et al., 2010), a rabbit (Rickmeyer et al., 2013), Muntjak deer (Fox et al., 1999), and American flamingos (Zeng et al., 2008).

The diagnosis of GM1 and GM2 gangliosidoses is carried out based on comprehensive findings, which include clinical, biochemical, histopathological, and genetic findings using various types of specimens (Gravel et al., 2001; Suzuki et al., 2001). The clinical findings are progressive neurological, motor, and visual dysfunctions, but they are not specific to these diseases (Hasegawa et al., 2013). The biochemical findings include the cerebral accumulation

of specific glycoconjugates and deficiency of specific enzyme activities, which are determined by specialized techniques such as thin-layer chromatography (TLC) and fluorometric enzymatic assays, respectively, using fresh or frozen tissues (Yamato et al., 2004a; Yamato et al., 2004c). The histopathological and ultrastructural findings demonstrate swollen neurons filled with periodic acid-Schiff stain-positive storage materials and osmiophilic membranous cytoplasmic bodies in the lysosomes of neurons, respectively, but these characteristics are not completely specific to these diseases (Yamato et al., 2000; Yamato et al., 2004b; Tamura et al., 2010; Kohyama et al., 2015). Genetic tests can be used to directly diagnose the diseases, but they are limited to diseases for which specific mutations have been identified (Chang et al., 2010; Rahman et al., 2011; Rahman et al., 2014). Therefore, it is possible that a correct diagnosis has not been established in many animal cases, as a result of the partial absence or lack of specimens for biochemical, histological, ultrastructural, or genetic examination.

The aim of the present study was to establish immunohistochemical and immunofluorescent techniques for the auxiliary diagnosis of canine and feline gangliosidoses using paraffin-embedded brain specimens, which are often stored for a long time in veterinary diagnostic laboratories worldwide.

### 1.2.3. METHODS

#### *Specimens*

Stored paraffin-embedded cerebral cortex samples of dogs and cats with GM1 or GM2 gangliosidosis were used in this study. These cases occurred in different parts of Japan and the original diagnosis was made using specific genetic tests and biochemical analyses at the Laboratory of Clinical Pathology, Joint Faculty of Veterinary Medicine, Kagoshima University, which has been exclusively supporting the diagnosis of inherited metabolic diseases in animals in Japan. These animals included a 14-month-old Shiba Inu with GM1 gangliosidosis diagnosed in 2009, an 11-month-old domestic shorthair cat with GM1 gangliosidosis diagnosed in 2004, a 20-month-old Toy Poodle with GM2 gangliosidosis diagnosed in 2006, and a 20-month-old domestic shorthair cat with GM2 gangliosidosis diagnosed in 2010. The diagnosis of these animals was established using genetic and/or biochemical tests reported previously (Yamato et al., 2004c; Chang et al., 2010; Uddin et al., 2012; Rahman et al., 2014). Stored paraffin-embedded cerebral cortex samples of a dog and a cat without any brain disease were also used as controls. Thin sections at 4  $\mu\text{m}$  were prepared from these paraffin-embedded tissue blocks by standard method. These sections were stained with hematoxylin and eosin (HE) and subjected to the immunohistochemical and

immunofluorescent techniques described below. All experimental procedures involving animals and their samples were performed in accordance with the guidelines regulating the use of animals and their samples at Kagoshima University with the approval number VM15041.

### *Immunohistochemical study*

Each section was deparaffinized with xylene and rehydrated through a graded ethanol series. Antigen retrieval was conducted by heating the sample in a 10 mM citrate buffer (pH 6.0) in a microwave oven. Thereafter, the samples were washed in deionized water, treated with 3% hydrogen peroxide, and washed in 0.01 M phosphate-buffered saline (PBS; pH 7.4). Blocking was performed with 0.25% casein in 0.01 M PBS and incubated overnight at 4°C with the respective reagents.

For the detection of GM1 ganglioside, biotinylated cholera toxin B subunit (1:1000; List Biological Laboratories, Inc., Campbell, CA, USA) was used. For the detection of GM2 ganglioside, mouse anti-GM2 ganglioside monoclonal IgM antibody (1:1000; Tokyo Chemical Industry, Co., Ltd., Tokyo, Japan) was used as a primary antibody, and biotinylated goat anti-mouse IgM antibody (1:200; Vector Laboratories, Inc., Burlingame, CA, USA) was used as a secondary antibody. Subsequently, these sections were incubated with

peroxidase-labeled streptavidin (KPL, Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, USA). The immunoreactivity was detected by a 3,3'-diaminobenzidine (DAB) system using DAB Tablet (Merck KGaA, Darmstadt, Germany) as a peroxidase substrate. The sections were counterstained with hematoxylin.

### *Immunofluorescent study*

Each section was pretreated in the same way as described above for immunohistochemistry. For the detection of GM1 ganglioside, biotinylated cholera toxin B subunit (1:500; List Biological Laboratories, Inc.) and Alexa Fluor 488-conjugated streptavidin (1:1000; Life Technologies, Inc., Gaithersburg, MD, USA) were used. For the detection of GM2 ganglioside, mouse anti-GM2 monoclonal IgM antibody (1:500; Vector Laboratories, Inc.) was used as a primary antibody, and Alexa Fluor 488-conjugated goat anti-mouse IgM antibody (1:1000; Life Technologies, Inc.) was used as a secondary antibody. Subsequently, these sections were incubated with a 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) solution (1:1000; Dojindo Laboratories, Inc., Kumamoto, Japan) for nuclear staining. The fluorescence was observed using a fluorescence microscopy (BX53-33-FL2; Olympus, Corp., Tokyo, Japan).

#### 1.2.4. RESULTS

Using the HE stain, cytoplasmic accumulation of pale to eosinophilic granular materials in balloon-swollen neurons was observed in the cerebral cortex samples of dogs and cats previously diagnosed with GM1 or GM2 gangliosidosis (Fig. 2, A–D), whereas there was no such abnormal change observed in the samples of the control animals (Fig. 2, E and F).

Using the immunohistochemical technique for the detection of GM1 ganglioside, the accumulated cytoplasmic materials were positively stained and mainly identified as GM1 ganglioside in cells of animals with confirmed GM1 gangliosidosis (Fig. 3, A and B). In animals with GM2 gangliosidosis, the accumulated cytoplasmic materials were very weakly positively stained in a portion of the cells of the affected cat (Fig. 3, C and D). In the control animals, the cytoplasm in some normal-shaped cells was also positively stained to indicate the presence of GM1 ganglioside (Fig. 3, E and F). The nuclei of several cells were positively stained in a portion of the samples such as in the case of feline GM2 gangliosidosis and in both control animals (Fig. 3, D–F).

Using the immunohistochemical technique for the detection of GM2 ganglioside, the accumulated cytoplasmic materials were positively stained and mainly identified as GM2 ganglioside in the cells of animals with GM2 gangliosidosis (Fig. 4, C and D), whereas these materials were not strongly stained in animals with GM1 gangliosidosis (Fig. 4, A and B). In the control animals, the cytoplasm in some normal-shaped cells was also weakly stained

using this method (Fig. 4, E and F). The nuclei of several cells were weakly positively stained in a portion of the samples such as in the case of canine GM1 gangliosidosis and in both control animals (Fig. 4 A, E and F).

The results of the immunofluorescent technique were almost identical to those of the immunohistochemical technique. The accumulated materials in the swollen neurons of animals with gangliosidoses were positively stained and clearly identified as either GM1 or GM2 ganglioside by using the respective detection techniques for each ganglioside (Figs. 5 and 6). The accumulated materials in the neurons of animals with GM2 gangliosidosis were very weakly stained using the technique for GM1 ganglioside (Fig. 5, C and D), and vice versa (Fig. 6, A and B). In the control animals, some cells showed cytoplasm that was positively stained for GM1 and GM2 gangliosides (Figs. 5 and 6, E and F). In addition, some of the cells of a cat with GM2 gangliosidosis and both control animals showed positive staining of nuclei using the technique for the detection of GM1 ganglioside (Fig. 5, D–F). Some of the cells of a control cat showed weakly positive staining of nuclei using the technique for the detection of GM2 ganglioside (Fig. 6F).

### 1.2.5. DISCUSSION

Gangliosides are glycosphingolipids consisting of a hydrophobic ceramide (*N*-acylsphingosine) and a hydrophilic oligosaccharide chain bearing one or more *N*-acetylneuraminic acid (sialic acid) residues, and are typical components of the outer leaflet of the plasma membranes of animal cells (Gravel et al., 2001; Suzuki et al., 2001). GM1 and GM2 gangliosides are present as the main glycolipids in neurons and are likely to be involved in cell differentiation and cell–cell interactions, but their specific physiological functions remain obscure. Therefore, developing techniques for the detection of GM1 and GM2 gangliosides is important not only for advancement in brain science but also for the correct diagnosis of gangliosidoses, because the intralysosomal accumulation of each ganglioside in neurons is characteristic to either GM1 or GM2 gangliosidosis. Therefore, in the past few decades, various determination methods for the profiling, quantification, or evaluation of gangliosides, including GM1 and GM2 gangliosides, in tissues, cultured cells, or extracellular fluids have been reported. These methods include TLC coupled with densitometric or immunochemical detection (Sato et al., 2004; Yamato et al., 2004c), high-performance liquid chromatography coupled with tandem mass spectrometric detection (Huang et al., 2014), enzyme-linked immunosorbent assay (Dawson, 2005), and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (Sato et al., 2011).

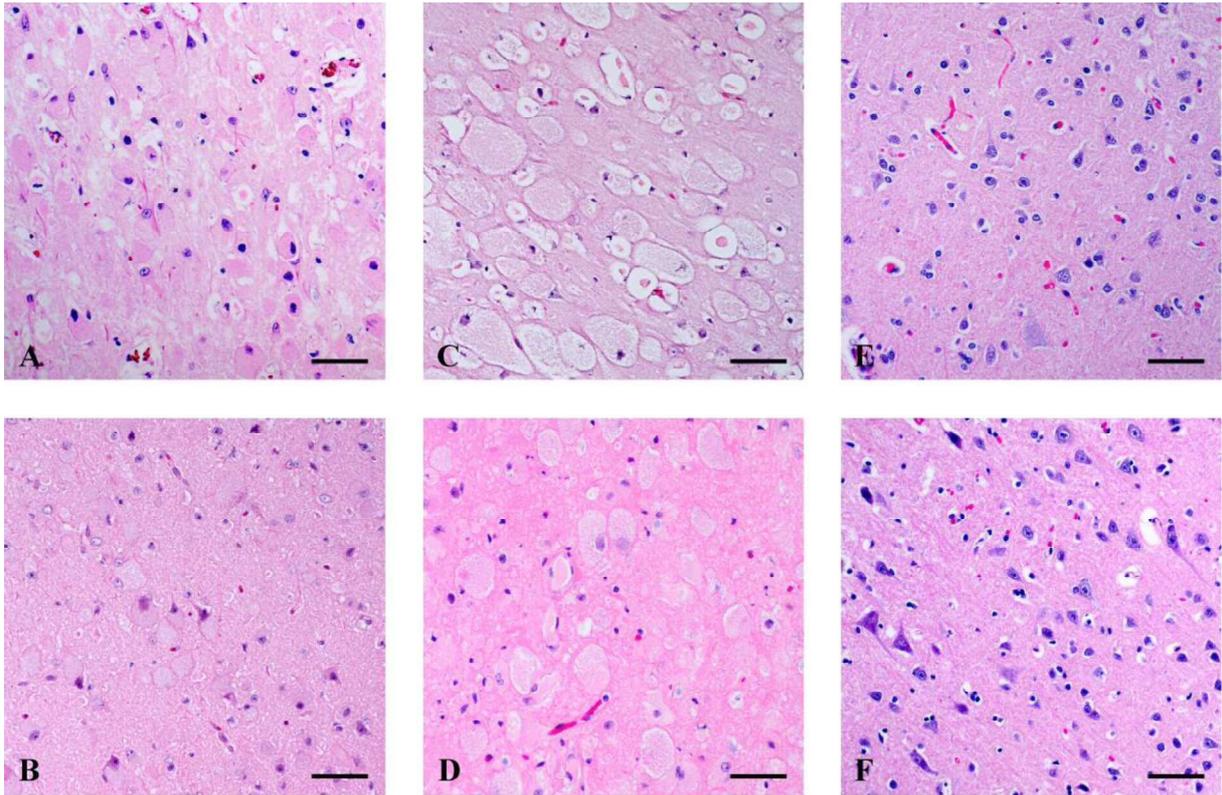
The *in situ* detection of gangliosides in tissue sections is also very important not only for diagnosis of the diseases but also to obtain reliable information on their tissue, cellular, and subcellular distributions (Petr et al., 2010). Furthermore, confirming that the histological detection of GM1 and GM2 gangliosides is applicable to paraffin-embedded specimens stored for a long period would also be useful for the retrospective diagnosis of the diseases, but very few studies have evaluated such *in situ* detection methods using long-term stored paraffin-embedded specimens from canine and feline gangliosidoses. In the present study, immunohistochemical and immunofluorescent techniques for the detection of GM1 and GM2 gangliosides were developed, and their application was evaluated using canine and feline paraffin-embedded specimens stored for 5 to 11 years. As a result, these two techniques could clearly detect the presence of both GM1 and GM2 gangliosides in neurons of the control animals (Figs. 3, 4, 5 and 6E and F) as well as the accumulation of either GM1 or GM2 ganglioside in neurons of animals with diagnosed GM1 (Figs. 3, 4, 5 and 6A and B) and GM2 gangliosidoses (Figs. 3, 4, 5 and 6C and D). These data demonstrate that the two techniques are applicable to the retrospective *in situ* detection of GM1 and GM2 gangliosides, and consequently to the auxiliary diagnosis of gangliosidoses in dogs and cats. However, gangliosides can be accumulated as the secondary products without direct link to the primary protein defect in some lysosomal and a few non-lysosomal diseases (Suzuki et al., 2001; Walkley et al., 2009). Therefore, in cases in which the abnormal accumulation of each

ganglioside is found in swollen neurons, a definitive diagnosis should ultimately be made using DNA extracted from the same paraffin-embedded specimen via the identification of pathogenic mutation(s) in the responsible genes: the *GLB1* gene for GM1 gangliosidosis and the *HEXA*, *HEXB*, and *GM2A* genes for GM2 gangliosidosis.

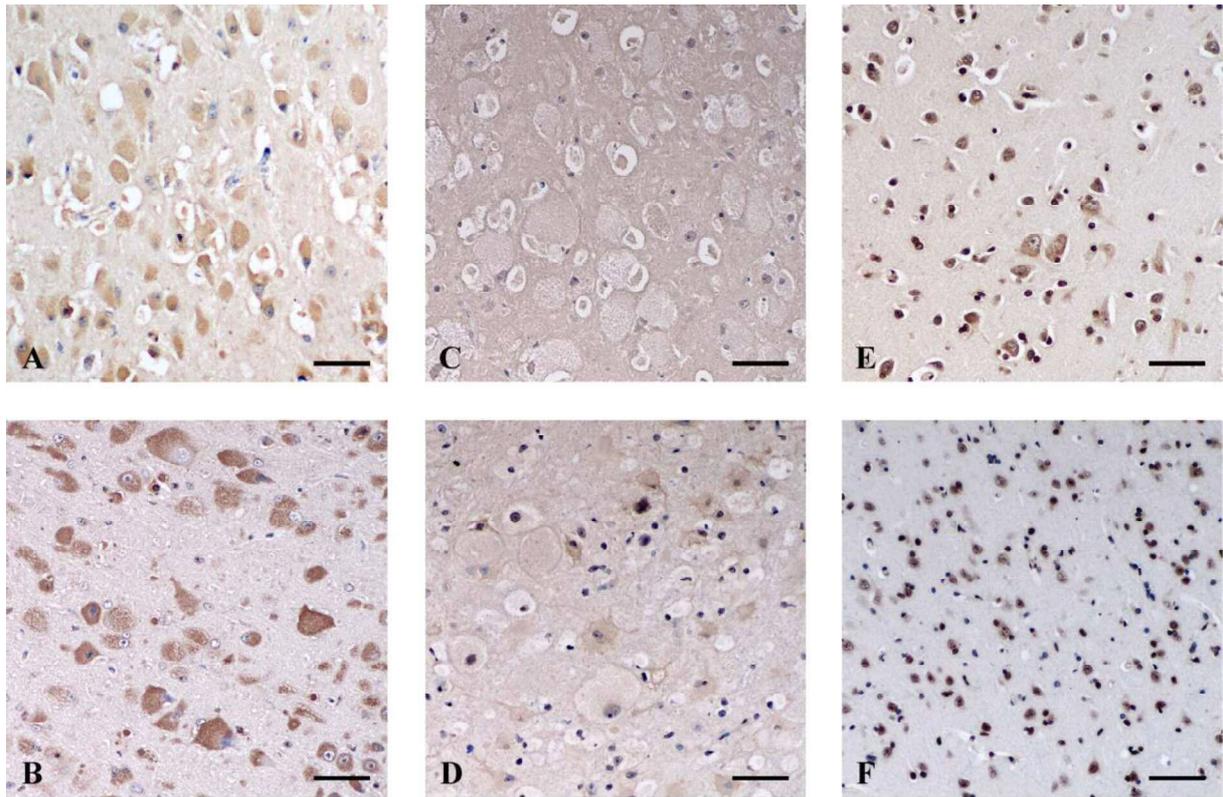
Comparing the two techniques developed in the present study, the immunofluorescent technique provided relatively less histopathological information than the immunohistochemical technique, due to the dark background when using immunofluorescence. Therefore, the tissue, cellular, and subcellular distributions of stained materials could not be easily determined in the immunofluorescent technique; however, this technique does have the advantage of requiring a lower amount of reagents (nearly half) because of its higher detection sensitivity compared to the immunohistochemical technique.

In addition, in the experiments conducted to detect GM1 and GM2 gangliosides, the nuclei were stained in some specimens using both techniques. The positive staining of the nuclei in some cells from affected and control animals may result from the natural components of GM1 and GM2 gangliosides because the nuclei of neuronal cells in rat brain contain these gangliosides (Saito et al., 2002). However, this stain of the nucleus was easily differentiated from the specific stain of cytoplasmic GM1 and GM2 gangliosides when using the immunohistochemical technique but not when using the immunofluorescent technique, owing to the reduced morphological visibility. Therefore, the simultaneous observation of

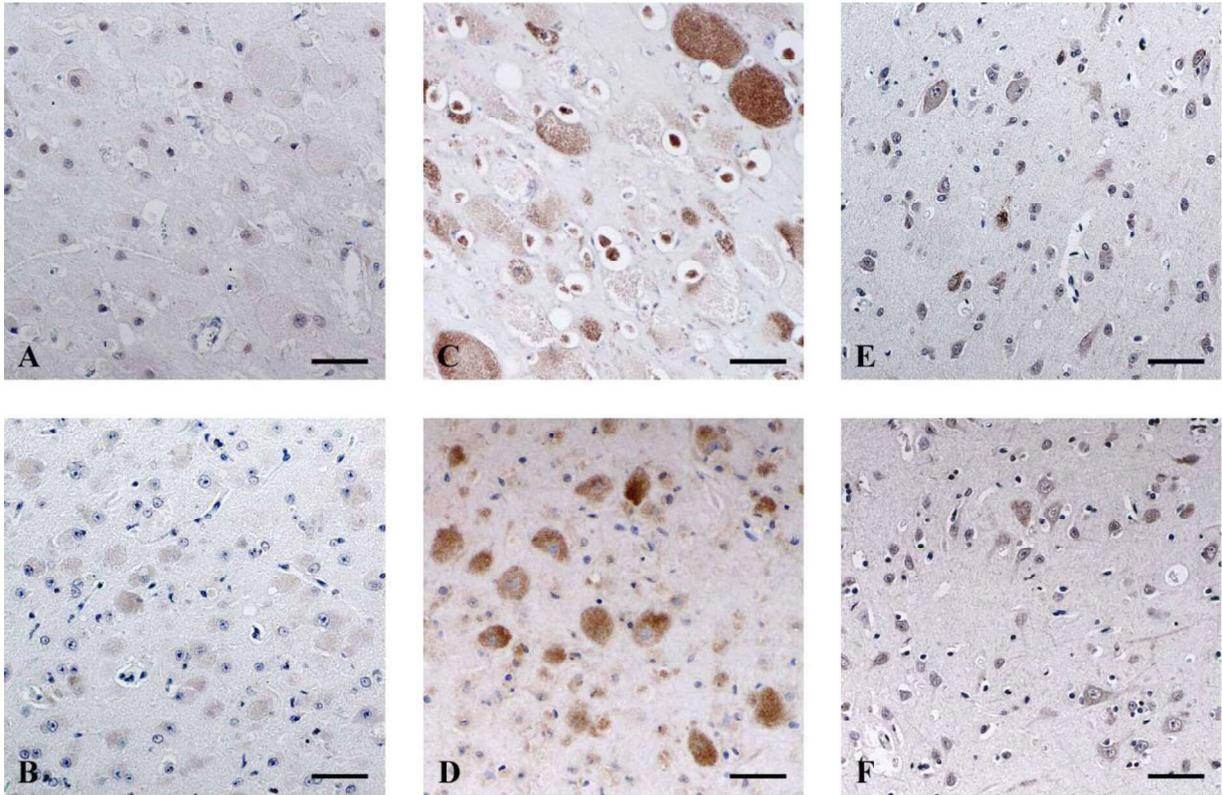
HE-stained cerebral tissues (Fig. 2) is necessary for accurate judgment of the results, especially when using an immunofluorescent technique.



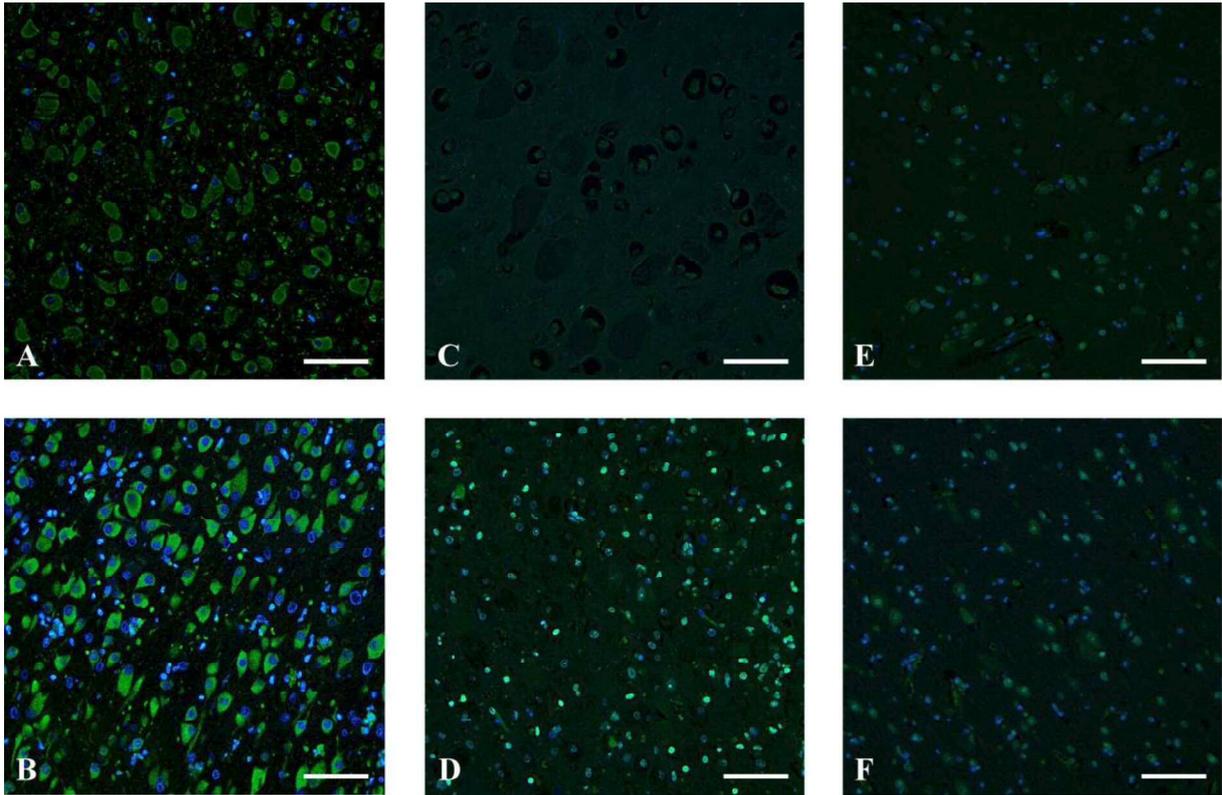
**Figure 2.** Histopathological findings in animals affected and unaffected with gangliosidoses. Hematoxylin and eosin staining was performed on paraffin-embedded sections of the cerebral cortex from the following animals: a dog (A) and a cat (B) affected with GM1 gangliosidosis; a dog (C) and a cat (D) affected with GM2 gangliosidosis; an unaffected control dog (E) and cat (F). Bar = 50  $\mu$ m.



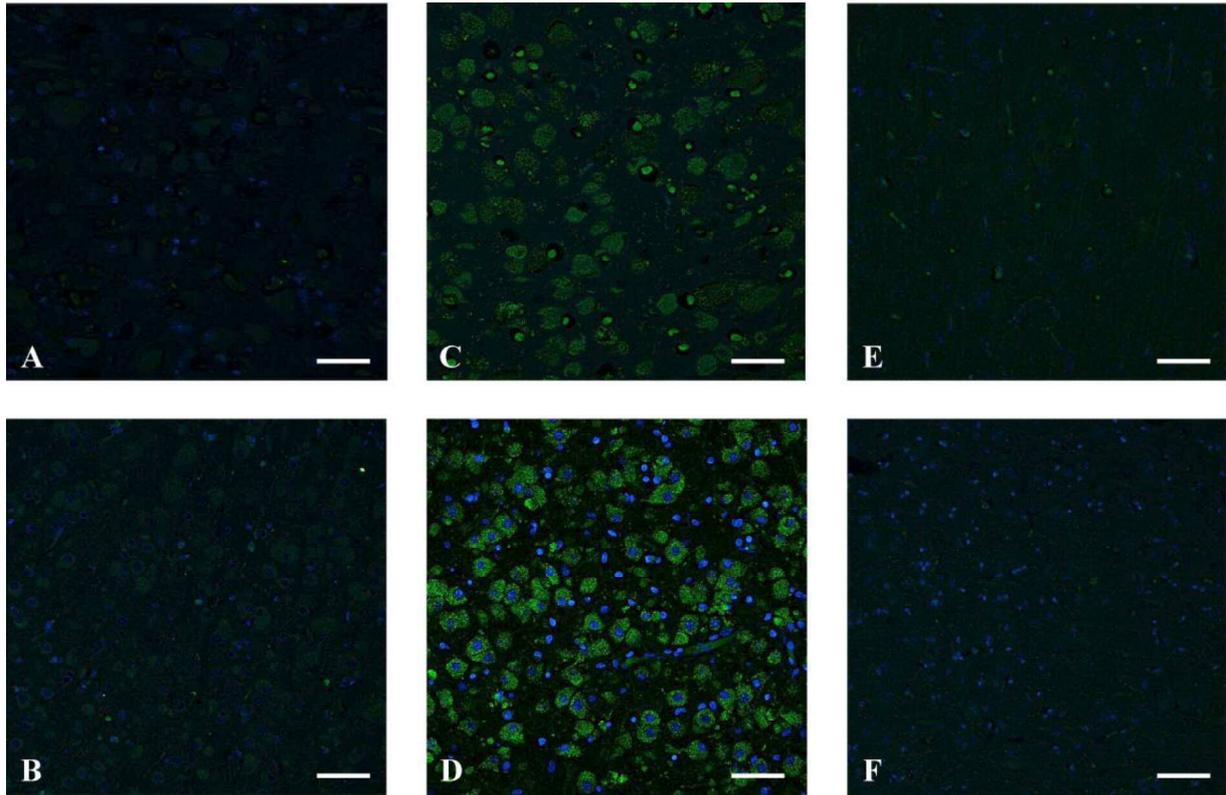
**Figure 3.** Immunohistochemical findings for the detection of GM1 ganglioside in animals affected and unaffected with gangliosidoses. The immunohistochemical technique for the detection of GM1 ganglioside was performed on paraffin-embedded sections of the cerebral cortex from the following animals: a dog (A) and a cat (B) affected with GM1 gangliosidosis; a dog (C) and a cat (D) affected with GM2 gangliosidosis; an unaffected control dog (E) and cat (F). For the detection of GM1 ganglioside, biotinylated cholera toxin B subunit and peroxidase-labeled streptavidin were used. The immunoreactivity was detected by 3,3'-diaminobenzidine as a peroxidase substrate. The sections were counterstained with hematoxylin. Bar = 50  $\mu\text{m}$ .



**Figure 4.** Immunohistochemical findings for the detection of GM2 ganglioside in animals affected and unaffected with gangliosidoses. The immunohistochemical technique for the detection of GM2 ganglioside was performed on paraffin-embedded sections of the cerebral cortex from the following animals: a dog (A) and a cat (B) affected with GM1 gangliosidosis; a dog (C) and a cat (D) affected with GM2 gangliosidosis; an unaffected control dog (E) and cat (F). For the detection of GM2 ganglioside, mouse anti-GM2 ganglioside monoclonal IgM antibody was used as a primary antibody, and biotinylated goat anti-mouse IgM antibody was used as a secondary antibody. Subsequently, these sections were incubated with peroxidase-labeled streptavidin. The immunoreactivity was detected by 3,3'-diaminobenzidine as a peroxidase substrate. The sections were counterstained with hematoxylin. Bar = 50  $\mu$ m.



**Figure 5.** Immunofluorescent findings for the detection of GM1 ganglioside in animals affected and unaffected with gangliosidoses. The immunofluorescent technique for the detection of GM1 ganglioside was performed on paraffin-embedded sections of the cerebral cortex from the following animals: a dog (A) and a cat (B) affected with GM1 gangliosidosis; a dog (C) and a cat (D) affected with GM2 gangliosidosis; an unaffected control dog (E) and cat (F). For the detection of GM1 ganglioside, biotinylated cholera toxin B subunit and Alexa Fluor 488-conjugated streptavidin were used. Subsequently, these sections were incubated with 4',6-diamidino-2-phenylindole dihydrochloride for nuclear staining. Bar = 30  $\mu$ m.



**Figure 6.** Immunofluorescent findings for the detection of GM2 ganglioside in animals affected and unaffected with gangliosidoses. The immunofluorescent technique for the detection of GM2 ganglioside was performed on paraffin-embedded sections of the cerebral cortex from the following animals: a dog (A) and a cat (B) affected with GM1 gangliosidosis; a dog (C) and a cat (D) affected with GM2 gangliosidosis; an unaffected control dog (E) and cat (F). For the detection of GM2 ganglioside, mouse anti-GM2 monoclonal IgM antibody was used as a primary antibody, and Alexa Fluor 488-conjugated goat anti-mouse IgM antibody was used as a secondary antibody. Subsequently, these sections were incubated with 4',6-diamidino-2-phenylindole dihydrochloride for nuclear staining. Bar = 30  $\mu$ m.

## CHAPTER 2.1

### **A retrospective immunohistochemical analysis and a molecular epidemiological survey of degenerative myelopathy in the Collie breed**

The above-titled work originally appeared in “*The Journal of Veterinary Medical Science* (Kohyama et al., 2017)” as: **Degenerative myelopathy in the Collie breed: a retrospective immunohistochemical analysis of superoxide dismutase 1 in an affected Rough Collie, and a molecular epidemiological survey of the *SOD1*: c.118G>A mutation in Japan** authored by:

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### 2.1.1. ABSTRACT

Canine degenerative myelopathy (DM) is an adult-onset, progressive neurodegenerative disease that occurs in multiple dog breeds. A DM-associated mutation of the canine superoxide dismutase 1 (*SOD1*) gene, designated c.118G>A (p.E40K), has been implicated as one of pathogenic determinants of the disease in many breeds, but it remains to be determined whether the c.118G>A mutation is responsible for development or progression of DM in Collies. Previously, a Rough Collie was diagnosed clinically and histopathologically as having DM in Japan, suggesting the possibility that the Collie breed may be predisposed to DM due to the high frequency of c.118G>A in Japan. In this study, accumulation and aggregate formation of SOD1 protein was retrospectively demonstrated in the spinal cord of the DM-affected dog by immunohistochemical analysis. Furthermore, a molecular epidemiological survey revealed a high carrier rate (27.6%) and mutant allele frequency (0.138) of c.118G>A in a population of Collies in Japan, suggesting that the Collie breed may be predisposed to DM associated with c.118G>A, and the prevention of DM in Collies in Japan should be addressed through epidemiological and genetic testing strategies.

### 2.1.2. INTRODUCTION

Canine degenerative myelopathy (DM) is an adult-onset, progressive neurodegenerative disease that occurs in multiple dog breeds, including Pembroke Welsh Corgis (PWCs), German Shepherd Dogs, Boxers and Bernese Mountain Dogs (BMDs) (Coates et al., 2010; Zeng et al., 2014). Most of the affected dogs are at least 8 years of age at the onset of clinical signs, which may include progressive, asymmetric upper motor neuron paraparesis, pelvic limb to generalized proprioceptive ataxia and lack of paraspinal hyperesthesia; these signs ultimately leading to paraplegia and dyspnea that may necessitate euthanasia (Coates et al., 2010; Oyake et al., 2016). In addition to identification of these typical clinical signs, diagnosis of DM is made by post-mortem histopathological analysis of the spinal cord for markers of disease, which typically include demyelination, axonal loss or degeneration and astrocytosis (Coates et al., 2010; Ogawa et al., 2011).

Cytoplasmic accumulation and aggregate formation of a mutant form of the superoxide dismutase 1 (SOD1) protein in the spinal cord of DM-affected dogs, which can be detected by anti-SOD1 antibodies, are closely associated with the pathogenesis of DM (Awano et al., 2009; Zeng et al., 2014; Nakamae et al., 2015). To date, two DM-associated mutations have been identified in the canine *SOD1* gene: c.118G>A (p.E40K) and c.52A>T (p.S18T) (Awano et al., 2009; Wininger et al., 2011). These mutations are likely to cause formation of misfolded proteins that accumulate into insoluble aggregates (Zeng et al., 2014; Nakamae et

al., 2015). According to the information from the Online Mendelian Inheritance in Animals (OMIA) (Online Mendelian Inheritance in Animals <http://omia.angis.org.au/home/> Accessed July 21, 2016), canine DM (OMIA 000263-9615) has been reported in 16 breeds: American Eskimo dog, BMD, Boxer, Cardigan Welsh Corgi, Chesapeake Bay Retriever, German Shepherd, Golden Retriever, Kerry Blue Terrier, Miniature Poodle, PWC, Pug, Rhodesian Ridgeback, Siberian Husky, Soft Coated Wheaten Terrier, Standard Poodle and Wirehaired Fox Terrier. At present, c.118G>A is widely distributed in 124 canine breeds, and c.52A>T is limited to BMDs (Awano et al., 2009; Wininger et al., 2011; Pfahler et al., 2014; Zeng et al., 2014). The Collie breed also has an allele of c.118G>A (Zeng et al., 2014); however, to the best of our knowledge, the predisposition of the Collie breed to DM has not been considered till date.

In 2009, DM was reported in an 11-year-old male Rough Collie in Japan, which was diagnosed on the basis of clinical and histopathological findings (Okada et al., 2009). Analysis of the molecular basis for DM in this dog was not performed, because the dog had been examined between 1997 and 1998; that was before c.118G>A was first identified in 2009 as a DM-associated mutation (Awano et al., 2009). In the present study, formalin-fixed spinal cord from this dog was further investigated by immunohistochemical analysis using an anti-SOD1 antibody to detect accumulation and aggregate formation of SOD1 in the spinal cord. Furthermore, a molecular epidemiological survey was performed to determine the

c.118G>A mutant allele frequency in a population of Collies in Japan, and to assess the relationship between the incidence of this disease-associated mutation and prevalence of DM in Collies.

### **2.1.3. MATERIALS AND METHODS**

The caudal thoracic spinal cord from the Rough Collie with DM (Okada et al., 2009) had been immersed in formalin for approximately 18 years. This was the only sample of the Rough Collie with DM for analysis in the present study. For immunohistochemical and histopathological analysis, part of the spinal cord was embedded in paraffin using a standard method and cut into thin sections. As positive control, spinal cord samples at twelfth thoracic vertebra from a 12-year-old female PWC with DM, which was homozygous for c.118G>A, were analyzed. As negative control, spinal cord samples at thirteenth thoracic vertebra were analyzed from a 12-year-old, neurologically normal, male Labrador Retriever dog that was homozygous for wild-type c.118G, and had died of hemangiosarcoma.

Immunohistochemistry was performed using a rabbit polyclonal anti-SOD1 antibody (SOD-1 FL-154, 1:500; Santa Cruz Biotechnology, Dallas, TX, U.S.A.) whose epitope corresponds to full length of human SOD1 protein. Hematoxylin and eosin (HE) and Luxol fast blue (LFB) staining was performed using a standard method. Formalin-fixed paraffin-embedded tissue sections were deparaffinized in xylene and ethanol. Epitope retrieval was performed with an autoclave (121°C for 15 min) in citrate buffer solution (Target retrieval solution, pH 6.0; Dako, Glostrup, Denmark). Endogenous peroxidase activity was quenched using 0.3% hydrogen peroxide in methanol for 20 min, and tissue sections were incubated in 10% normal goat serum (Dako) in phosphate-buffered saline at

room temperature for 60 min. Sections were incubated with the anti-SOD1 antibody at 4°C overnight. A horse radish peroxidase-conjugated goat anti-rabbit immunoglobulin (Dako) was added as a secondary antibody, and the mixture was incubated at room temperature for 30 min. 3,3'-diaminobenzidine tetrahydrochloride (Dako) was used as a chromogenic substrate. After the chromogenic reaction, sections were counterstained with hematoxylin.

DNA extraction from the formalin-immersed spinal cord of the DM-affected Collie was performed using QIAamp DNA micro kit (Qiagen, Hilden, Germany). DNA amplification was performed using a real-time PCR method described previously (Chang et al., 2013). For a molecular epidemiological survey, blood and saliva specimens were collected from 29 Collies in Japan with their owners' informed consent, and stored by spotting them onto the Flinders Technology Associates filter papers (FTA card; Whatman International Ltd., Piscataway, NJ, USA) until use. Preparation of DNA templates from the FTA cards and genotyping survey were performed as described previously (Chang et al., 2013). All experimental procedures and ethical issues involving animals and their samples were approved by the Animal Research Committee at Kagoshima University with the approval number VM15041 (29 September, 2015).

#### 2.1.4. RESULTS

Immunohistochemical analysis revealed strong reactivity of anti-SOD1 antibody with specific target sites in the cytoplasm of spinal cord neurons in the gray matter of the affected Collie (Fig. 7A), which was similar to the reactivity of the same antibody in the positive control spinal cord of PWC that was homozygous for c.118G>A (Fig. 7B). As expected, weak reactivity was observed in the cytoplasm of spinal cord neurons in the negative control dog (Fig. 7C). Aggregates of SOD1 protein were observed in part of spinal cord neurons of the DM-affected Collie and in the positive control PWC, but not in the negative control dog (Fig. 7). In the LFB-HE staining, axonal degeneration or loss, and demyelination were observed in the white matter of the affected Collie and PWC, while no abnormal finding was observed in the negative control dog (Fig. 8). Using DNA purified from the formalin-immersed spinal cord of the affected Collie, no specific sequence was amplified using the real-time PCR method (data not shown). The molecular epidemiological survey in 29 Collies in Japan revealed that 21 dogs (72.4%) were wild-type homozygotes and 8 dogs (27.6%) were heterozygous carriers, while no mutant homozygotes were observed. Based on the data from this survey, the mutant allele frequency was 0.138.

### 2.1.5. DISCUSSION

In the present study, the spinal cord from a Rough Collie, which had been diagnosed on the basis of clinical and histopathological findings as having DM in Japan (Okada et al., 2009), was retrospectively investigated by immunohistochemical analysis using an anti-SOD1 antibody and histopathological analysis using LFB-HE staining. The immunohistochemical and histopathological findings of the affected Collie are similar to those observed in the spinal cord neurons in several DM-affected dogs based on immunohistochemical analysis using other anti-SOD1 antibodies (Awano et al., 2009; Zeng et al., 2014; Nakamae et al., 2015). These results suggest that this Collie may have had the *SOD1* gene mutation associated with DM.

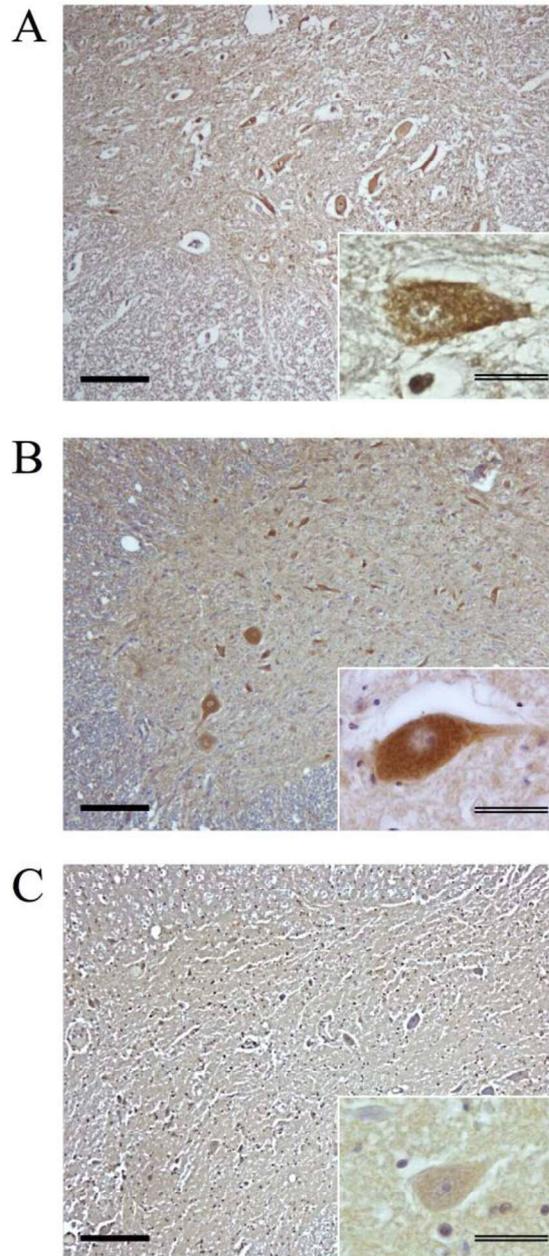
Amplification of DNA from the formalin-immersed spinal cord of the affected Collie was unsuccessful using the real-time PCR method, which can amplify a 74-base pair-specific band in exon 2 of the canine *SOD1* gene (Coates et al., 2010). Failure to amplify this fragment may be due to fragmentation of the genomic DNA into very short pieces that may not anneal to the specific oligonucleotide primers used, possibly as a result of long-term storage (approximately 18 years) in formalin. In general, prolonged storage in formalin makes formic acid, which causes the degradation of DNA that could make it difficult to amplify DNA by fragment target-specific PCR (Schander et al., 2003). However, the molecular epidemiological survey of c.118G>A in 29 Collies in Japan demonstrated the

heterozygous carrier rate to be 27.6%. This carrier rate is high enough to support accidental mating by carriers, which may produce offspring with a homozygous mutant genotype even by random breeding. Data from this epidemiological survey, coupled with the above immunohistochemical findings, support the conclusion that the DM-affected Collie was most probably homozygous for c.118G>A, which is associated with the onset of clinical and histopathological DM.

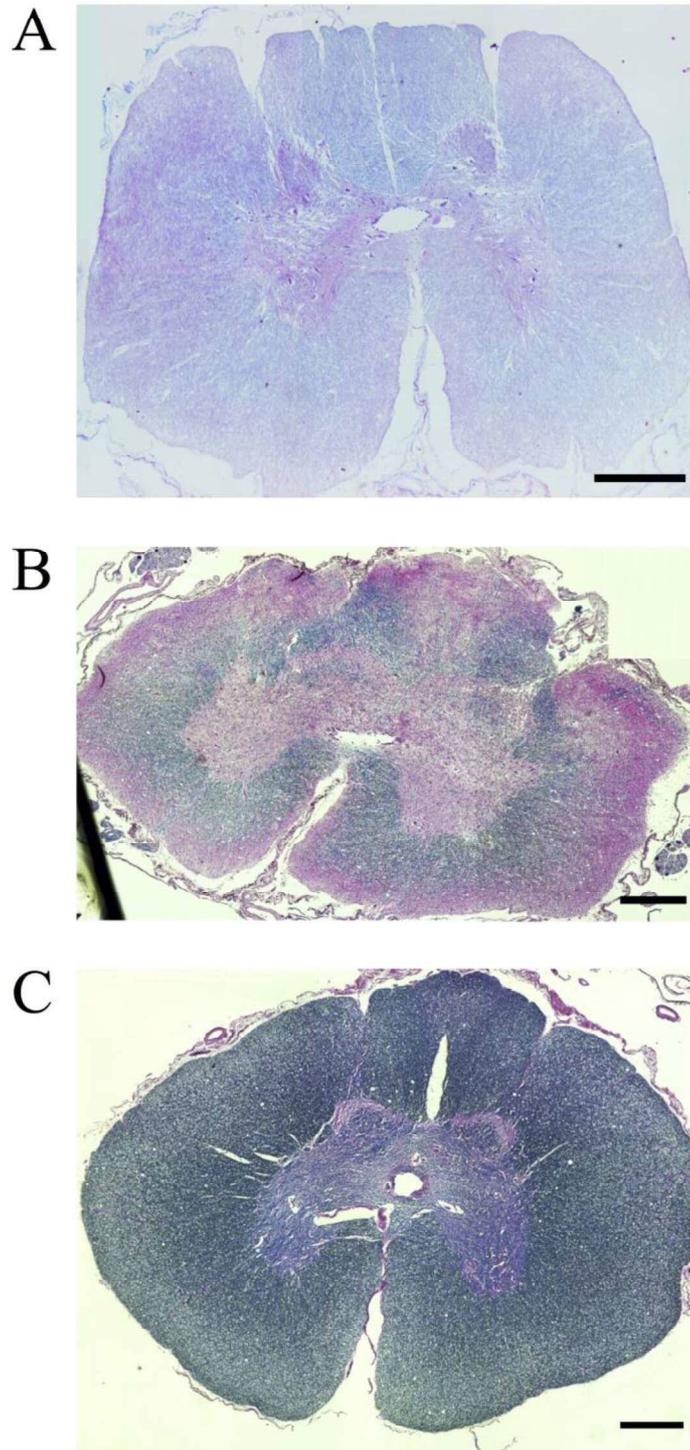
One of the limitations of our study is the small number of dogs examined. One reason is that the Collie breed is rare in Japan because of the small number of (less than 100) dogs that are registered annually into the Japan Kennel Club (Japan Kennel Club <http://www.jkc.or.jp> Accessed July 21, 2016). Therefore, there may be a high risk for inbreeding and production of DM-affected offspring. The genetic testing of Collies for breeding purposes is strongly recommended in an effort to reduce the mutant allele frequency, which may ultimately lead to the reduction and eventual eradication of DM in this breed.

The Collie breed is more popular in the U.S.A. than in Japan. According to data from the American Kennel Club (American Kennel Club <http://www.akc.org> Accessed July 21, 2016), the Collie ranked thirty-fifth or thirty-sixth among all the breeds in the U.S.A. between 2013 and 2015. Based on a report on DM in multiple dog breeds in the U.S.A. (Mizukami et al., 2016), the allele frequency of the c.118G>A mutation in Collies was calculated to be 0.387, which is higher than the frequency (0.138) in Japan, as revealed in the present study. These

data suggest that DM may occur more frequently than expected in Collies in the U.S.A. and other countries where the mutant allele frequency is very high. Indeed, SOD1 aggregate formation was demonstrated by immunohistochemical staining of specimen from a Rough Collie in the U.S.A, which was homozygous for c.118G>A and confirmed to be histopathologically affected with DM (Zeng et al., 2014). However, the data regarding the mutant allele frequency from that study might have been influenced by a bias in sample acquisition or population stratification. Interestingly, the mutant allele frequencies are higher in PWCs (0.792) and Border Collies (0.169) in the U.S.A. than in randomly collected samples from PWCs (0.697) and Border Collies (0.008) in Japan (Chang et al., 2013; Mizukami et al., 2016). Epidemiological information on the genetic disorders in each breed is essential for establishing prevention schemes (Mizukami et al., 2016). Therefore, epidemiological surveys should be performed in each country and region.



**Figure 7.** Immunohistochemical analysis of spinal cords from a Collie diagnosed clinically and histopathologically as having degenerative myelopathy (A), a Pembroke Welsh Corgi with degenerative myelopathy, which is homozygous for the *SOD1*:c.118G>A mutation (B), and a neurologically normal Labrador Retriever dog that is homozygous for a wild-type c.118G allele (C). Immunohistochemical staining was performed using a rabbit polyclonal anti-SOD1 antibody (SOD-1 clone FL-154, Santa Cruz Biotechnology, Dallas, TX, U.S.A.) at a dilution of 1:500. The sections were counterstained with hematoxylin. Bar (single line) = 150  $\mu\text{m}$ , bar (double lines) = 30  $\mu\text{m}$ .



**Figure 8.** Histopathological analysis of spinal cords from a Collie diagnosed clinically and histopathologically as having degenerative myelopathy (A), a Pembroke Welsh Corgi with degenerative myelopathy, which is homozygous for the *SOD1*:c.118G>A mutation (B), and a neurologically normal Labrador Retriever dog that is homozygous for a wild-type c.118G allele (C). Hematoxylin and eosin and Luxol fast blue staining. Bar = 400  $\mu$ m.

## CHAPTER 2.2

### **Real-time PCR genotyping assay for canine progressive rod-cone degeneration and mutant allele frequency in Toy Poodles, Chihuahuas and Miniature Dachshunds in Japan**

The above-titled work originally appeared in “*The Journal of Veterinary Medical Science* (Kohyama et al., 2016)” as: **Real-time PCR genotyping assay for canine progressive rod-cone degeneration and mutant allele frequency in Toy Poodles, Chihuahuas and Miniature Dachshunds in Japan** authored by:

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### 2.2.1. ABSTRACT

Canine progressive rod-cone degeneration (PRCD) is a middle- to late-onset, autosomal recessive, inherited retinal disorder caused by a substitution (c.5G>A) in the canine *PRCD* gene that has been identified in 29 or more purebred dogs. In the present study, a TaqMan probe-based real-time PCR assay was developed and evaluated for rapid genotyping and large-scale screening of the mutation. Furthermore, a genotyping survey was carried out in a population of the three most popular breeds in Japan (Toy Poodles, Chihuahuas and Miniature Dachshunds) to determine the current mutant allele frequency. The assay separated all the genotypes of canine PRCD rapidly, indicating its suitability for large-scale surveys. The results of the survey showed that the mutant allele frequency in Toy Poodles was high enough (approximately 0.09) to allow the establishment of measures for the prevention and control of this disorder in breeding kennels. The mutant allele was detected in Chihuahuas for the first time, but the frequency was lower (approximately 0.02) than that in Toy Poodles. The mutant allele was not detected in Miniature Dachshunds. This assay will allow the selective breeding of dogs from the two most popular breeds (Toy Poodle and Chihuahua) in Japan and effective prevention or control of the disorder.

### 2.2.2. INTRODUCTION

Canine progressive retinal atrophy (PRA) is a hereditary retinal dystrophy that includes a group of conditions with similar clinical presentation, although the age of onset and rate of progression varies considerably by breed (Petersen-Jones , 2014). PRA has been divided into various forms based on the age of onset, pathologic features and genetic mutations in certain genes (Mellersh, 2014; Petersen-Jones , 2014).

Progressive rod-cone degeneration (PRCD) is one of forms in canine PRA, which is a middle- to late-onset, autosomal recessive retinal disorder causing photoreceptor degeneration (Miyadera et al., 2012a; Petersen-Jones, 2014). It has been reported that a point mutation c.5G>A (p.Cys2Tyr) in the canine *PRCD* gene is associated with PRCD in 29 or more breeds: American Cocker Spaniel, American Eskimo Dog, Australian Cattle Dog, Australian Shepherd, Australian Shepherd (Miniature), Australian Stumpy Tail Cattle Dog, Chesapeake Bay Retriever, Chinese Crested, Cockapoos, Dwarf Poodle, English Cocker Spaniel, Entelbacher Mountain Dog, Finish Lapphund, Golden Retriever, Golden Doodle, Karelian Bear Dog, Kuvasz, Labradoodle, Labrador Retriever, Lapponian Herder, Markiesje, Moyen Poodle, Norwegian Elkhound, Nova Scotia Duck Tolling Retriever, Poodle (Miniature and Toy), Portuguese Water Dog, Spanish Water Dog, Swedish Lapphund and Yorkshire Terrier (Zangerl et al., 2006; Gentilini et al., 2009; Downs et al., 2014). However, to our knowledge, the carrier rate and/or mutant allele frequency in each breed have not been

clarified in Japan.

According to the Japan Kennel Club (<http://www.jkc.or.jp/>), a certified club by the Federation Cynologique Internationale (<http://www.fci.be/>), Poodles, Chihuahuas and Dachshunds have been the three most popular breeds in Japan. From 2003 to 2014, the annual number of these three breeds has accounted for 48 to 57% of the annual total number of all the registered purebred dogs. Therefore, it is very important to know the current carrier rate and/or mutant allele frequency of canine PRCD in these three popular breeds, in order to apply preventive measures for this ocular disorder. In addition, an easy and reliable genotyping method is required for screening the disorder.

In the present study, a TaqMan probe-based real-time PCR genotyping assay was developed and evaluated for rapid genotyping and large-scale screening for the mutation of canine PRCD. Furthermore, a genotyping survey was carried out in a randomly selected population of Toy Poodles, Chihuahuas and Miniature Dachshunds.

### 2.2.3. MATERIALS AND METHODS

All experimental procedures using animal samples were performed in accordance with the guidelines regulating the animal use at Kagoshima University. Whole blood samples of 200 Toy Poodles, 57 Chihuahuas and 100 Miniature Dachshunds were randomly collected from several animal hospitals in Japan with the cooperation of their owners and veterinarians. The whole blood samples were spotted onto Flinders Technology Associates filter papers (FTA card; Whatman International Ltd., Piscataway, NJ, USA) and stored until use. Control DNA samples from wild-type, carrier and mutant homozygous genotypes were prepared in the process of this study and used after confirming each genotype by direct DNA sequencing (Hokkaido Bio System Co. Ltd., Sapporo, Japan).

For the real-time PCR assay, primers and TaqMan minor groove binder (MGB) probes were designed based on the sequences of the canine *PRCD* gene in a wild-type dog (GenBank accession numbers NC\_006591.3 and NM\_001097560.1) and a dog possessing the *PRCD*-associated mutation (c.5G>A). These primers and probes (Table 2) were synthesized by a commercial company (Applied Biosystems, Foster City, CA, USA) and bound to a fluorescent reporter dye (6-carboxyrhodamine or 6-carboxyfluorescein) at the 5'-end and a nonfluorescent quencher dye at the 3'-end. DNA extraction was performed as previously described using a disc that punched out of each FTA card (Mizukami et al., 2011). The amplifications were carried out using the StepOne real-time PCR system (Applied

Biosystems) in a final volume of 10  $\mu$ l consisting of 2 $\times$  PCR master mix (TaqMan GTXpress Master Mix, Applied Biosystems), 80 $\times$  genotyping assay mix (TaqMan SNP Genotyping Assays, Applied Biosystems) containing the specific primers, TaqMan MGB probes and template DNA. Prior to PCR, the prepared mixtures were held at 25°C for 30 sec. The cycling conditions consisted of 20 sec at 95°C, followed by 60 cycles of 3 sec at 95°C and 20 sec at 63°C. The holding stage after PCR was carried out at 25°C for 30 sec. In the process of seeking the optimal annealing temperature (63°C), several annealing temperatures (60–64°C) were evaluated to differentiate the amplifications of wild-type alleles from those of mutant alleles. The obtained data were analyzed with StepOne version 2.3 (Applied Biosystems), and the calculations were based on the results obtained from DNA samples of control dogs.

#### 2.2.4. RESULTS

The real-time PCR assay clearly separated all three genotypes at the annealing temperature of 63°C (Fig. 9). The total required time for the 60-cycle amplification was less than one hour. At the annealing temperature of 60–62°C, nonspecific allelic amplification was observed in the wild-type allele, because false mutant-allelic amplification occurred together with the true wild-type-allelic amplification (Fig. 10). The false mutant-allelic amplification was inhibited moderately by an increase in the annealing temperature at 63°C and 64°C, but fluorescence intensity decreased markedly at 64°C compared to that at 60–63°C (Figs. 9 and 10). Consequently, the annealing temperature of 63°C was considered optimal for this method in which it was possible to clearly distinguish the allelic types, although a slight nonspecific allelic amplification still occurred.

The results of the survey among the three most popular purebred dogs in Japan are shown in Table 3. The survey demonstrated that the carrier rate in Toy Poodles was 16.5% and the mutant allele frequency was 0.088. We identified a dog that was homozygous for the mutation and had a risk for canine PRCD, but the dog could not be followed up clinically. The survey also demonstrated that the carrier rate in Chihuahuas was 3.7% and the mutant allele frequency was 0.019. The mutant allele was not detected in Miniature Dachshunds.

### 2.2.5. DISCUSSION

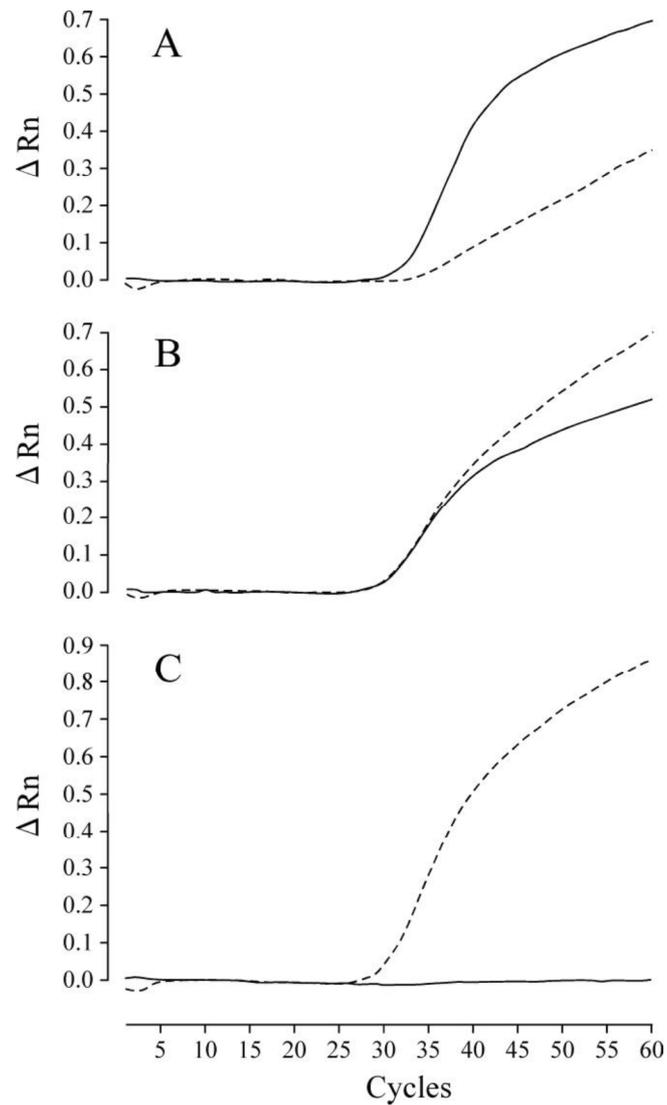
In the present study, a real-time PCR assay using TaqMan MGB probes was developed to provide clear-cut genotyping results for the wild-type and mutant alleles associated to canine PRCD (Fig. 9). The use of FTA cards for sampling that eliminated the need for traditional multi-step DNA extraction and purification procedures (Mizukami et al., 2011), combined with the relatively short amplification time (less than one hour), allowed rapid genotyping and screening of this mutation in less than two hours. However, there was a slight nonspecific false allelic amplification in this assay. The difference in the amplification plots of the wild-type and mutant alleles was probably caused by the difference in the melting temperature ( $T_m$ ) of each probe (Table 2) (Kutyavin et al., 2000). Although the  $T_m$  values of both probes seems to increase by the addition of an MGB moiety, false mutant-allelic amplification occurred at the standard annealing temperature of 60°C. In the present study, an increase in the annealing temperature at 63°C improved the accuracy of the assay. Overall, the data showed that the proposed assay is a simple, rapid and reliable tool for small- and large-scale surveys, and that this assay, along with the previous reported assay (Gentilini et al., 2009), will help prevention and control of canine PRCD.

The survey demonstrated that the mutant allele frequency in Toy Poodles, calculated from 33 carriers and one homozygous mutant genotype in a population of 200 randomly sampled dog, was approximately 0.09 (Table 3). This frequency is considered high enough to

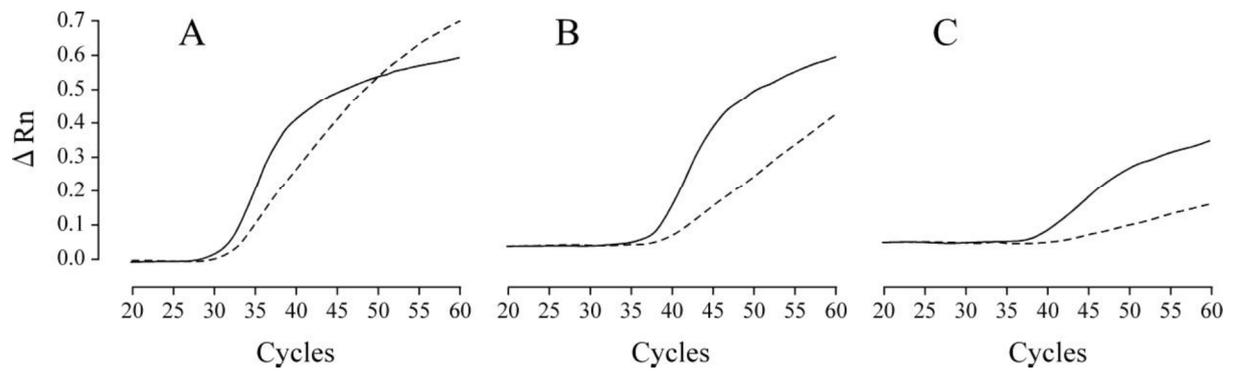
allow the establishment of measures for the control of the disorder in Toy Poodles in Japan. In addition, the mutant allele was detected for the first time in Chihuahuas and the frequency was approximately 0.02. Although the mutant allele frequency was lower than that in Toy Poodles, it is still high enough to allow the establishment of measures for the control of the disorder in Chihuahuas in Japan. The mutant allele frequency in Toy Poodles and Chihuahuas could be decreased and/or controlled, if the genotyping test was used at breeding kennels. Genotyping and selection could easily prevent the breeding of dogs that have a risk of PRCD or the breeding between carriers and thereby the production of dogs with risk for PRCD in the future, while preserving the gene pool in each breed. Genotyping should not be applied easily to healthy client-owned dogs, except for diagnostic purposes in dogs that show clinical signs of PRA, because it would increase owners' financial burden without offering any preventive benefits.

In the present study, the mutant allele was not detected in any of the 100 randomly collected Miniature Dachshunds (Table 3), suggesting that the mutant allele of canine PRCD is absent or almost absent in Miniature Dachshunds in Japan. Therefore, the genotyping does not have to be applied to this breed. However, canine cone-rod dystrophy is known to be prevalent in Miniature Dachshunds as another form of canine PRA, which is mainly associated with a certain mutation in the canine *RPGRIPI* gene (Miyadera et al., 2009; Miyadera et al., 2012b). The prevention and control of PRA in Miniature Dachshunds should

be considered based on this mutation. The total control of canine PRA should be planned based on specific data for each breed in each country.



**Figure 9.** Real-time PCR amplification plots of wild-type and mutant alleles in canine progressive rod-cone degeneration. Amplification was plotted as fluorescence intensity ( $\Delta Rn$  value) against cycle number. The  $\Delta Rn$  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 PCR. Each of the three amplification plots shows the homozygous wild-type (A), heterozygous carrier (B) and homozygous mutant genotypes (C) at the annealing temperature of 63°C. Solid and dotted lines indicate amplification of wild-type and mutant alleles, respectively.



**Figure 10.** Real-time PCR amplification plots of homozygous wild-type genotype in canine progressive rod-cone degeneration. Amplification plots were obtained at three different annealing temperatures of 60°C (A), 62°C (B) and 64°C (C). Solid and dotted lines indicate amplification of true wild-type and false mutant alleles, respectively.

**Table 2.** Primes and probes used in the real-time PCR assay for rapid genotyping and large-scale screening for the mutation of canine progressive rod-cone degeneration.

Primer/probe	Sequence 5'→3' (mer)	Reporter (5')	Quencher (3')	T <sub>m</sub> (°C)	Concentration (nM)
Forward primer	CCTTTCTCCTGCAGACTCTGT (21)	NA	NA	56.5	450
Reverse primer	CCAAGGTGCTGAGTAGGAAGAG (22)	NA	NA	57.0	450
Probe for wild-type allele	TGAGCCATGTGCACCAC (17)	VIC	NFQ	55.8	100
Probe for mutant allele	TGAGCCATGT <u>A</u> CACCAC (17)	FAM	NFQ	51.8	100

T<sub>m</sub> = melting temperature calculated by OligoAnalyzer 3.1 (<https://sg.idtdna.com/calc/analyzer>); NA = not applicable; VIC = 6-carboxyrhodamine; FAM = 6-carboxyfluorescein; NFQ = nonfluorescent quencher. Underlined letter in the sequence of the probe indicates the corresponding guanine to a substitution mutation (c.5G>A) in the canine *PRCD* gene.

**Table 3.** The rate of carriers and dogs with risk for progressive rod-cone degeneration and the allele frequency for canine PRCD-associated mutation in the three most popular breeds (Toy Poodle, Chihuahua and Miniature Dachshund) in Japan.

Dog breed	Number of examined dogs	Number of carriers (rate, %)	Number of risk* (rate, %)	Mutant allele frequency
Toy Poodle	200	33 (16.5)	1 (0.5)	0.088
Chihuahua	54	2 (3.7)	0 (0)	0.019
Miniature Dachshund	100	0 (0)	0 (0)	0
Total	354	35 (9.9)	1 (0.3)	0.052

\* Risk means the possibility of progressive rod-cone degeneration in a homozygous mutant genotype; however, the dog that had the risk could not be followed up clinically.

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