NOTE *Internal Medicine*

Real-Time PCR Genotyping Assay for GM2 Gangliosidosis Variant 0 in Toy Poodles and the Mutant Allele Frequency in Japan

Mohammad Mahbubur RAHMAN[1,2)], Akira YABUKI[1)], Moeko KOHYAMA[1)], Sawane MITANI[1)], Keijiro MIZUKAMI[1)], Mohammad Mejbah UDDIN[1,2)], Hye-Sook CHANG[1)], Kazuya KUSHIDA[1)], Miori KISHIMOTO[3)], Remi YAMABE[4)] and Osamu YAMATO[1)]*

[1)]*Laboratory of Clinical Pathology, Department of Veterinary Medicine, Joint Faculty of Veterinary Medicine, Kagoshima University, 1–21–24 Kohrimoto, Kagoshima 890–0065, Japan*
[2)]*Chittagong Veterinary and Animal Sciences University, Chittagong 4202, Bangladesh*
[3)]*Tokyo University of Agriculture and Technology, 3–5–8 Saiwai-cho, Fuchu, Tokyo 183–8509, Japan*
[4)] *Monolis, Inc., 8–31–6 Higashi-machi, Shindaiji, Chohu, Tokyo 182–0012, Japan*

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ABSTRACT. GM2 gangliosidosis variant 0 (Sandhoff disease, SD) is a fatal, progressive neurodegenerative lysosomal storage disease caused by mutations of the *HEXB* gene. In canine SD, a pathogenic mutation (c.283delG) of the canine *HEXB* gene has been identified in toy poodles. In the present study, a TaqMan probe-based real-time PCR genotyping assay was developed and evaluated for rapid and large-scale genotyping and screening for this mutation. Furthermore, a genotyping survey was carried out in a population of toy poodles in Japan to determine the current mutant allele frequency. The real-time PCR assay clearly showed all genotypes of canine SD. The assay was suitable for large-scale survey as well as diagnosis, because of its high throughput and rapidity. The genotyping survey demonstrated a carrier frequency of 0.2%, suggesting that the current mutant allele frequency is low in Japan. However, there may be population stratification in different places, because of the founder effect by some carriers. Therefore, this new assay will be useful for the prevention and control of SD in toy poodles.

KEY WORDS: canine GM2 gangliosidosis variant 0, carrier frequency, real-time PCR genotyping, Sandhoff disease, toy poodle dog.

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GM2 gangliosidoses are a group of progressive neurodegenerative lysosomal storage diseases resulting from excessive accumulation of the GM2 ganglioside and related glycoconjugates in lysosomes, especially in lysosomes of the neurons [3]. Three main variant forms of GM2 gangliosidoses (0, B and AB) are present caused by defects of different genes. The diseases are inherited in an autosomal recessive manner, and affected individuals die prematurely due to brain damage, with progressive neurological signs, including motor and psychointellectual dysfunction and visual defects. In veterinary species, naturally occurring GM2 gangliosidoses have been shown in dogs, cats, sheep, pigs, deer and flamingos [4, 11].

GM2 gangliosidosis variant 0, called Sandhoff disease (SD), is a form of GM2 gangliosidosis caused by deleterious mutations of the *HEXB* gene encoding the β-subunit protein of acid β-hexosaminidase A and B [1]. SD has been shown in dogs [12, 15] and cats [1, 2, 9, 16]. Canine SD has been shown in two breeds: golden retriever in 2004 [15] and toy poodle in 2010 [12]. The clinical signs of SD in toy poodles include motor disorders and tremor starting between 9 and 12 months of age and death resulting from progressive neurological deterioration between 18 and 23 months of age. The causative mutation of SD in toy poodles has been identified as a homozygous single base pair (bp) deletion of guanine in exon 3 at nucleotide position 283 of the putative open reading frame of the canine *HEXB* gene (c.283delG), allowing antemortem molecular diagnosis by using a conventional PCR-based DNA test [11].

The toy poodle is one of the most popular breeds in the world. Especially in Japan, the annual number of toy poodles registered with the Japan Kennel Club has been the largest among all the canine breeds since 2008 (http://www.jkc.or.jp/), indicating toy poodles to be the most common dog in Japan. The presence of a fatal inherited disease in such a popular breed is a serious problem. Thus, it is necessary to control and decrease the prevalence of SD in order to maintain the quality of toy poodles and supply healthy dogs to prospective breeders and owners. Control and prevention require the establishment of simple, rapid and reliable genotyping assays for large-scale surveys and clarification of the current mutant allele frequency to plan preventive measures. Therefore, in the present study, a real-time PCR genotyping assay was developed and evaluated for rapid and large-scale genotyping and screening for this mutation. Furthermore, a genotyping survey was carried out in a population of toy poodles in Japan to determine the current mutant allele frequency.

All experimental procedures by using animals and their

*CORRESPONDENCE TO: YAMATO, O., Laboratory of Clinical Pathology, Department of Veterinary Medicine, Joint Faculty of Veterinary Medicine, Kagoshima University, 1–21–24 Kohrimoto, Kagoshima 890–0065, Japan. e-mail: osam@vet.kagoshima-u.ac.jp

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samples were performed in accordance with the guidelines regulating animal use at Kagoshima University.

DNA templates for the real-time PCR assay were prepared in different ways, as follows. A post-mortem liver specimen was collected from a toy poodle with SD, and the genomic DNA was isolated using a commercial kit (QIAamp DNA Micro Kit, Qiagen, Valencia, CA, U.S.A.), in accordance with the manufacturer's protocol. Whole blood or saliva samples spotted onto the Flinders Technology Associates filter paper (FTA card, Whatman International, Piscataway, NJ, U.S.A.) were collected from two affected, four heterozygous carrier and five wild-type toy poodles, genotypes of which were determined previously using direct sequencing analysis [11]. In addition, whole blood or saliva samples were collected from 496 toy poodles for an epidemiological survey. A disc punched out of the FTA cards was used for DNA extraction in accordance with the procedure reported previously [7].

Amplifications were performed using real-time PCR system (Applied Biosystems, Foster City, CA, U.S.A.) by a specific primer pair and TaqMan minor groove binder (MGB) probes bound with each fluorescent reporter dye (6-carboxyrhodamine or 6-carboxyfluorescein) at the 5'-end and a nonfluorescent quencher dye at the 3'-end (Table 1), which were synthesized by a commercial company (Applied Biosystems). The primers and probes were designed based on the sequence of the canine *HEXB* gene in a wild-type dog and a dog with SD (GenBank accession no. AB674550), yielding a 73-bp amplicon in the wild-type allele and a 72-bp amplicon in the mutant allele. The real-time PCR amplifications were carried out in a final volume of 10 μl consisting of a 2× PCR master mix (TaqMan GTXpress Master Mix, Applied Biosystems), an 80× genotyping assay mix (TaqMan SNP Genotyping Assays, Applied Biosystems) containing specific primers and TaqMan MGB probes, and an appropriate volume of template DNA. The holding stage before PCR was performed at 25°C for 30 sec. The cycling conditions were 20 sec at 95°C followed by 50 cycles of 3 sec at 95°C and 20 sec at 60°C. The holding stage after PCR was performed at 25°C for 30 sec. In addition, an allelic discrimination plot was constructed based on the three types of amplification plot. The obtained data were analyzed using a software (StepOne software, version 2.1, Applied Biosystems) with the calculations based on results obtained using DNA samples from 12 dogs (three affected, four heterozygous carrier and five wild-type dogs).

The genotyping survey was carried out by using whole blood or saliva samples spotted onto FTA cards collected randomly from 496 client-owned toy poodles with the cooperation on their owners and veterinarians from different animal hospitals and a training school for veterinary technicians in Japan. Sample collection was performed between 2009 and 2012. Genotyping was carried out by using the real-time PCR assay developed in the present study.

Pedigree analysis was performed to elucidate the genetic relationships of affected and carrier dogs identified in Japan and clarify the pathway of transmission and distribution of the mutant allele. The genetic relationships and records of champion dogs were analyzed using the pedigree papers and studbooks issued by the Japan Kennel Club (http://www.jkc.or.jp/). The ancestry in North America was traced using pedigree information provided by the Poodle Pedigree Database (http://www.poodlepedigree.com). The pedigree data of toy poodles with SD were updated based on the previous data [11, 12] and the newly found information in the present study.

The real-time PCR assay with TaqMan MGB probes determined all SD genotypes in toy poodles without nonspecific allelic amplification after 50 cycles of amplification (Fig. 1). The amplification curve of the mutant allele showed a higher plateau signal than that of the wild-type allele, but the difference in the signal intensity between wild-type and mutant alleles did not interfere in the discrimination of the three genotypes. An allelic discrimination plot was constructed based on the three genotypes of amplification plots obtained using DNA samples from 12 dogs, including five wild-type, four heterozygous carrier and three affected genotypes (Fig. 2). Three genotypes of the 12 dogs were clearly determined by this allelic discrimination plot, and the results are consistent with those of the direct DNA sequencing. In the genotyping survey of 496 toy poodles in Japan, one heterozygous carrier was identified, resulting in a carrier frequency of 0.2%. Pedigree information on this carrier was not available, because the owner could not be contacted. No affected dogs were identified in this survey.

The pedigree analysis was performed mainly by using the information of ancestral dogs in North America, which was provided by the Poodle Pedigree Database. The pedigree data obtained by this analysis were added to the previous pedigree data of toy poodles with SD, revealing that all dogs carrying the mutant allele in Japan share a blood relationship and common ancestral pedigree line in North America (Fig. 3). This pedigree line included American champion dogs (Dogs a, b and d in Fig. 3) and a Canadian champion dog (Dog c). Two of them (Dogs c and d) were imported to and used for breeding in Japan. One of the dog's offspring (Dog e) was a Japanese champion and used for breeding in Japan. The pedigree with SD in Japan was mainly formed using the above-mentioned three breeding dogs (Dogs c, d and e).

Recently, real-time quantitative PCR approaches have been developed to detect mutations in genes causing inherited diseases even in veterinary species including dogs [6, 7], cats [5, 14] and cattle [17]. Real-time PCR is generally considered more sensitive, rapid and less time-consuming than that by conventional PCR for the analysis of genetic variability. Large numbers of samples can be analyzed by real-time PCR in a single experiment within a short time, which is particularly applicable for preventive screening programs. In the present study, a real-time PCR assay by using TaqMan MGB probes was developed, and this assay aided in clearly determining all SD genotypes (Fig. 1). In addition, allelic discrimination plot analysis yielded multiple accurate results simultaneously in a single plot (Fig. 2). These results showed that the assay is a simple, rapid and reliable tool for large-scale epidemiological survey as well as a method to diagnose SD in toy poodles.

Table 1. Characteristics of primers and probes used for the real-time PCR assay in the present study

Primer/probe	Sequence 5'→3' (mer)	Reporter (5')	Quencher (3')	Concentration (nM)
Forward primer	GATTAACCACCTTCTCCCCTCATG (24)	NA	NA	450
Reverse primer	CTGTTGGCCTTGAGGAAAGC (20)	NA	NA	450
Probe for wild-type allele	CAGATAGTTTGGTTGTGAAAG (21)	VIC	NFQ	100
Probe for mutant allele	CAGATAGTTTGGTTTGAAAG (20)	FAM	NFQ	100

NA=not applicable; VIC=6-carboxyrhodamine; FAM=6-carboxyfluorescein; NFQ=nonfluorescent quencher. The underlined letter in the sequence of the probe is guanine corresponding to a deletion mutation (c.283delG) in dogs with GM2 gangliosidosis variant 0.

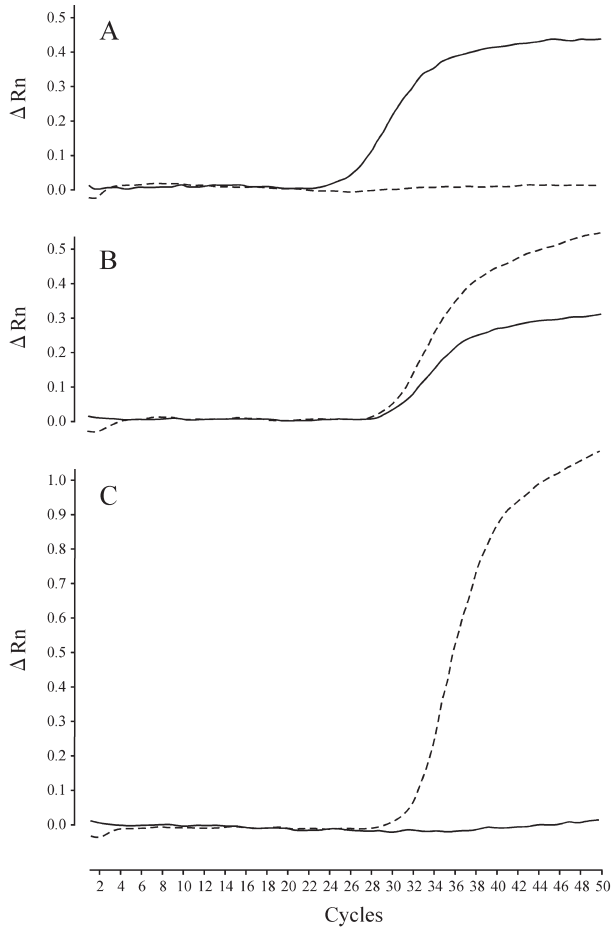

Fig. 1. Real-time PCR amplification plots of wild-type and mutant alleles in GM2 gangliosidosis variant 0 in toy poodles. Amplification was plotted as fluorescence intensity (ΔRn value) against cycle number. The ΔRn value is the reporter dye signal normalized to the internal reference dye and corrected for the baseline signal established in the first few cycles of PCR. Each of the three amplification plots showed the wild-type (A), heterozygous carrier (B) and mutant genotypes (C). Solid and dotted lines indicate wild-type and mutant alleles, respectively.

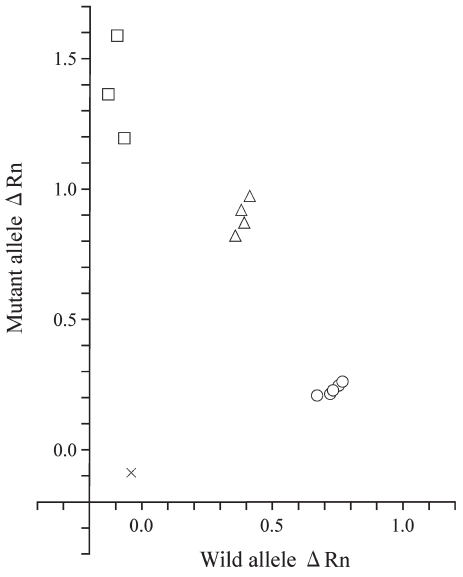

Fig. 2. An allelic discrimination plot of end point fluorescence real-time PCR data showing the three genotypes of GM2 gangliosidosis variant 0 in toy poodles. The allelic discrimination plot shows representative DNA samples of the three genotypes. The plot is expressed as fluorescence intensities (ΔRn 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 PCR. ×=no template control; ○=wild-type (five samples); △=heterozygous carrier (four samples); □=mutant (three samples) genotypes.

The genotyping survey in the present study demonstrated the current carrier frequency in Japan to be 0.2% (1/496 dogs). This carrier frequency seems low compared to that in other fatal, inherited canine diseases, for example, neuronal ceroid lipofuscinosis (8.1%) [7] and trapped neutrophil syndrome (11.1%) [6] in border collies and GM1 gangliosidosis in shiba inus (1.0%) [13]. Therefore, the carrier frequency of SD in toy poodles is not currently high enough for control and prevent measures to be warranted in Japan. The low carrier frequency in Japan may be because a pedigree related to this disease was limited to certain kennels, and subsequently, the mutant allele was not distributed throughout Japan. Indeed, several years ago, many breeding dogs including the Dog e shown in Fig. 3, which belonged to a kennel, died in an accident involving air conditioning failure on a midsummer's day (Yamato's personal communication).

The pedigree analysis indicated that dogs carrying the mutant allele in Japan have a blood relationship with a pedigree line in North America, which produced several champion dogs in the United States and Canada (Fig. 3). If these champion dogs are carriers of SD and frequently used for breeding

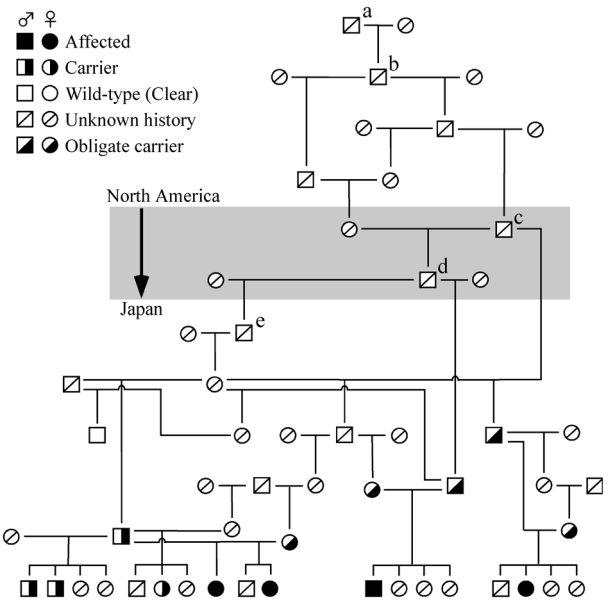

Fig. 3. Genetic relationship of affected and carrier dogs identified in Japan. The analysis was carried out using pedigree papers and studbooks issued by the Japan Kennel Club. The ancestry in North America was traced using pedigree information provided by the Poodle Pedigree Database. The gray area indicates a border between North America and Japan. Dog a: born in 1966, American champion; Dog b: unknown birth year, American champion; Dog c: born in 1986, Canadian champion; Dog d: born in 1988, American champion; Dog e: born in 1991, Japanese champion.

in North America, population stratification might occur, that is, the carrier frequency might be higher in North America than in Japan. A study regarding the canine population structure by using genotyping analysis of 722 SNPs demonstrated that there is significant intra-breed genetic diversity resulting from the difference of geographic locations (i.e., stratification) [10]. Furthermore, in inherited canine diseases, such as neuronal ceroid lipofuscinosis [8] and GM1 gangliosidosis [13], population stratification can easily occur unknowingly in a geographic region by using carriers for breeding (i.e., founder effect), resulting in an elevation of the carrier frequency and disease prevalence in that region. Therefore, measures to control and prevent SD in toy poodles would be warranted in places where the carrier frequency is high or when it increases. The real-time PCR assay developed in this study will be useful in such situations.

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