NOTE *Internal Medicine*

Genotyping Assays for the Canine Degenerative Myelopathy-Associated c.118G>A (p.E40K) Mutation of the *SOD1* Gene Using Conventional and Real-Time PCR Methods: A High Prevalence in the Pembroke Welsh Corgi Breed in Japan

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ABSTRACT. Canine degenerative myelopathy is an adult-onset, progressive neurodegenerative disease that occurs in multiple dog breeds, particularly Pembroke Welsh Corgis. Recently, a degenerative myelopathy-associated mutation of the canine *SOD1* gene was identified as c.118G>A (p.E40K). In the present study, genotyping assays using conventional and real-time PCR methods were developed, and a preliminary genotyping survey was performed on 122 randomly selected Pembroke Welsh Corgis without any degenerative myelopathy-related clinical signs to determine the current allele frequency in Japan. Both of the assays provided clear-cut genotyping. The survey demonstrated the frequencies of the G/G wild-type, G/A heterozygote and A/A homozygote to be 9.0, 42.6 and 48.4%, respectively, indicating that the prevalence of the mutant A allele (69.7%) in Pembroke Welsh Corgis is extremely high in Japan.

KEY WORDS: canine *SOD1* gene, degenerative myelopathy, genotyping assay, Pembroke Welsh Corgi dog, prevalence.

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Canine degenerative myelopathy (DM) is an adult-onset, progressive neurodegenerative disease that occurs in multiple dog breeds including Pembroke Welsh Corgis (PWCs), Boxers and German Shepherds [3]. Most affected dogs are at least 8 years of age at the onset of clinical signs, which include progressive, asymmetric upper motor neuron paraparesis, pelvic limb to general proprioceptive ataxia and lack of paraspinal hyperesthesia, ultimately leading to paraplegia and necessitating euthanasia. A single nucleotide substitution from guanine to adenine in exon 2 at nucleotide position 118 (c.118G>A) in the coding region of the canine gene encoding the superoxide dismutase 1 protein (*SOD1*), which causes an amino acid substitution from glutamate to lysine (p.E40K), has been identified to be associated with canine DM [1]. As in the United States [2, 5], dogs with DM have frequently been found, especially in PWCs in Japan in recent years [8].

The present study developed and evaluated rapid and simple genotyping assays using conventional and real-time PCR methods for the canine DM-associated mutation. Furthermore, a preliminary genotyping survey was performed using the real-time genotyping assay on 122 PWCs to determine the current allele frequency in Japan.

Control samples for each genotype, which were confirmed by direct sequencing, were used to evaluate genotyping assays. DNA templates were prepared using whole blood spotted onto Flinders Technology Associates filter paper (FTA card; Whatman International Ltd., Piscataway, NJ, U.S.A.). For the PCR-restriction fragment length polymorphism (RFLP) assay, a disc 1.2 mm in diameter punched out of the FTA card was used as a template after quick washing as reported previously [6]. Each PCR test was carried out targeting a sequence around c.118G>A of the canine *SOD1* gene with forward and reverse primers shown in Table 1 using a general protocol. The PCR product was digested with the restriction endonuclease, *Acu*I (New England Biolabs Inc., Ipswich, MA, U.S.A.). Both the unprocessed and digested PCR products were subjected to electrophoresis in 3% agarose (Agarose 21, Nippon Gene Co., Ltd., Tokyo, Japan). The presence of the mutation was reflected by a single 270-base pair (bp) band that remained undigested due to the mutant sequence [CTAAAG$(N)_{16}$], whereas the absence of the mutation was reflected by the presence of 2 fragments, 181 and 89 bp, due to the restriction site [CTGAAG$(N)_{16}$↓] in the wild-type sequence.

DNA templates for the real-time PCR assay were also pre-

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Table 1. Characteristics of the primers and TaqMan probes used

Assay	Primer/probe	Reporter	Sequence* 5'→ 3' (mer)	Position	Tm (°C)	Concentration (nM)
PCR-RFLP	Forward primer	–	TTTTGTGCTTTTCTTTGAGTGAAG (24)	c.73–24_1	53.6	625
PCR-RFLP	Reverse primer	–	GATCATTTCCCTAAGGCTGAC (21)	c.166+132_152	56.5	625
Real-time PCR	Forward primer	–	TGGGCCTGTTGTGGTATCAG (20)	c.78_97	58.3	450
Real-time PCR	Reverse primer	–	CAAACTGATGGACGTGGAATCC (22)	c.130_151	58.6	450
Real-time PCR	Wild-type probe	VIC	CTCGCCTTCAGTCAGC (16)	c.111_126	–	100
Real-time PCR	Mutant-type probe	FAM	CTCGCCTTTAGTCAGC (16)	c.111_126	–	100

RFLP, restriction fragment length polymorphism; VIC, 6-carboxyrhodamine; FAM, 6-carboxyfluorescein. *Primers and probes were designed based on the GenBank data (NW_003726114 and AF346417). An underlined letter in the sequence of a primer is mismatched to that of the canine *SOD1* gene to alter the unrelated recognition sequence (CTGAAG) of *Acu*I by the primer.

pared using a disc 1.2 mm in diameter punched from the FTA card as reported previously [6]. Real-time PCR amplification was conducted with the primers and probes listed in Table 1 using the same protocol and reagents described previously [6], except for the annealing temperature (58°C).

A preliminary genotyping survey was performed on 122 randomly selected PWCs without any DM-related clinical signs in Japan. The blood samples were obtained from client-owned dogs (age range: 2 months to 15 years) with the consent of owners, which were presented for reasons other than DM at local veterinary clinics and veterinary teaching hospitals. Genotyping was carried out using the real-time PCR method developed in the present study. The 122 PWCs were categorized into 4 age-groups, i.e., 38 dogs aged less than 5 years, 41 dogs aged 5 to less than 10 years, 33 dogs aged 10 years and over and 10 dogs of uncertain age. The allele frequency was calculated and analyzed for the total and each group.

As shown in Fig. 1, a 270-bp fragment was amplified by PCR and digested with *Acu*I, producing 181- and 89-bp bands in wild-type (G/G) dogs. However, the 270-bp fragment could not be digested with *Acu*I in homozygous mutant-type (A/A) dogs, resulting in a band left on the gel after the digestion. Heterozygous (G/A) dogs showed 1 undigested and 2 digested fragments on the gel. The real-time PCR assay amplified a 74-bp fragment and clearly determined all the genotypes without any nonspecific allelic amplification (Fig. 2). An allelic discrimination plot constructed based on the 3 genotypes of amplification plots is shown in Fig. 3. The genotypes of all 15 dogs examined were consistent with the results of the direct sequencing and the PCR-RFLP assay. In the survey on 122 PWCs, 11 (9.0%) were G/G homozygous, 52 (42.6%) were G/A heterozygous and 59 (48.4%) were A/A homozygous (Table 2). The prevalence of the mutant A allele was 69.7% in total. There were no marked differences in the prevalence among groups categorized by age.

In the present study, both PCR-RFLP and real-time PCR assays provided clear-cut genotyping of the c.118G>A mutation in the canine *SOD1* gene. The genotyping survey demonstrated that the prevalence of the mutant allele (approximately 70%) in PWCs is extremely high in Japan, compared with that for other fatal canine inherited diseases, such as neuronal ceroid lipofuscinosis (4.1%) [6] and trapped neutrophil syndrome (5.6%) [7] in Border Collies in Japan.

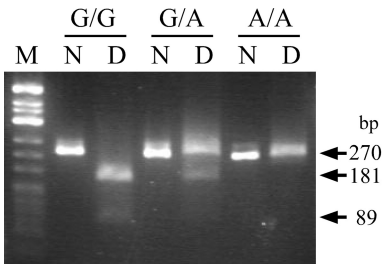

Fig. 1. Canine degenerative myelopathy genotyping of wild-type (G/G), heterozygous (G/A) and homozygous mutant (A/A) dogs by a polymerase chain reaction-restriction fragment length polymorphism assay using agarose gel electrophoresis. The amplified DNA without digestion (N) and DNA digested with the restriction endonuclease *Acu*I (D) were analyzed simultaneously. Lane M shows the molecular size markers and base pairs (bp).

That seems to be because canine DM has a later onset in adulthood and even homozygous mutant dogs are likely to be as fertile as dogs with other genotypes, suggesting DM to be one of the most serious canine inherited diseases. Therefore, the genotyping assays developed in the present study will contribute to prevention and control of the mutation in the population of PWCs and other breeds with a high prevalence.

A definitive diagnosis of DM is determined post-mortem by histopathologic examination of the spinal cord [3]. A differential diagnosis is also difficult, because PWC is a chondrodystrophic breed and prone to Hansen type I intervertebral disc disease, which often mimics and coexists with DM. Furthermore, at present, there exists no prophylactic or curative treatment for canine DM, but intensive physiotherapy may prolong survival times of DM-affected dogs [3, 4]. There is a need for the development of effective therapeutic and prophylactic strategies. The genotyping assays may be useful not only to support the diagnosis of individual dogs, but also to evaluate the risk of DM and early start therapies developed in the future.

Currently, homozygosity for the c.118G>A allele is

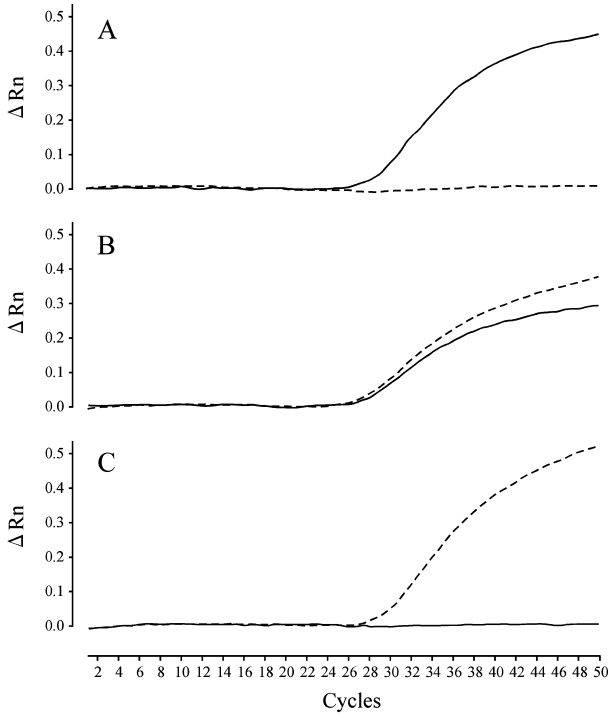

Fig. 2. Real-time polymerase chain reaction amplification plots of wild-type and mutant alleles in canine degenerative myelopathy. Amplification was plotted as fluorescence intensity (ΔRn value) against cycle number. The ΔRn value is the reporter dye signal normalized to an internal reference dye and corrected for the baseline signal established in the first few cycles of the reaction. Each of 3 amplification plots showed the G/G homozygote (A), A/G heterozygote (B) and A/A homozygote (C). Solid and dotted lines indicate amplification in the presence of wild-type and mutant probes, respectively.

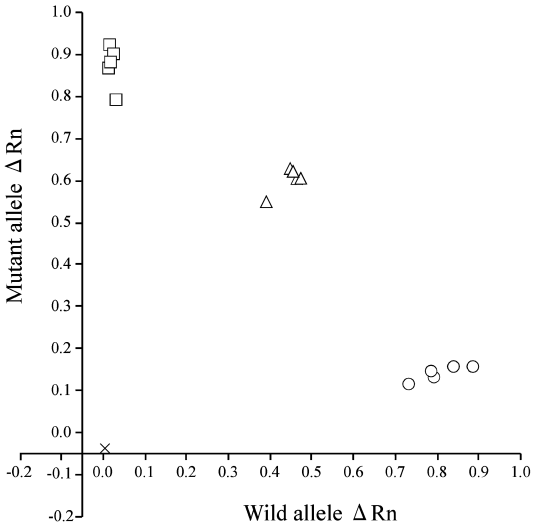

Fig. 3. Allelic discrimination plot of end-point fluorescence real-time polymerase chain reaction data showing the 3 genotypes of canine degenerative myelopathy. The allelic discrimination plot was depicted using 15 representative DNA samples in Pembroke Welsh Corgis that had already been genotyped by direct sequencing. The plot is expressed as the fluorescence intensity (ΔRn value) for each allele along the X- and Y-axes. The ΔRn value in this figure is the end-point reporter dye signal normalized to an internal reference dye and corrected for the baseline signal established in the first few cycles of the reaction. ×, no template control; ○, wild-type (G/G); △, heterozygous (G/A); □, homozygous mutant (A/A) genotypes.

demonstrated to be a major risk factor for canine DM [1, 3]. However, it is also true that many dogs homozygous for this mutation do not develop clinical signs, suggesting an age-related incomplete penetrance [3] and/or involvement of other mutations and genes. In the present study, there were no marked differences in the prevalence among the age-groups, and even the group aged 10 years and over included a number of A/A homozygous dogs without DM-related clinical signs. These findings also suggest that a small percentage of A/A homozygous dogs may become clinically affected. Therefore, it is important to clarify the incidence of DM and identify other factors involved in the onset of DM. More recently, another novel DM-associated mutation was identified as c.52A>T (p.T18S) in a Bernese Mountain Dog [9], which is also known as a breed predisposed to DM [3, 4]. Based on these observations, veterinarians should know that the genotyping assays for the c.118G>A mutation have application limits. In the near future, the accumulated screening data from the genotyping assays should help provide a better overall picture of canine DM.

Table 2. Prevalence of the mutant A allele in 122 randomly selected Pembroke Welsh Corgis without any degenerative myelopathy-related clinical signs

Age-group	Number				Prevalence (%) of A allele
	Total	G/G (%)	G/A (%)	A/A (%)	
Less than 5 years	38	3 (8)	22 (58)	13 (34)	63.2
5 to less than 10 years	41	7 (17)	13 (32)	21 (51)	67.1
10 years and over	33	1 (3)	10 (30)	22 (67)	81.8
Uncertain age	10	0 (0)	7 (70)	3 (30)	65.0
Total	122	11 (9)	52 (43)	59 (48)	69.7

G/G: wild-type, G/A: heterozygous, and A/A: homozygous mutant-type dogs.

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