The Journal of Veterinary Medical Science

NOTE
Clinical Pathology

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

Moeko KOHYAMA[1,2], Masato KITAGAWA[3], Hiroaki KAMISHINA[4,5], Yui KOBATAKE[4,5], Akira YABUKI[1,2], Mariko SAWA[1,2], Shusaku KAKITA[1] and Osamu YAMATO[1,2]*

[1]Laboratory of Clinical Pathology, Joint Faculty of Veterinary Medicine, Kagoshima University, 1-21-24 Kohrimoto, Kagoshima 890-0065, Japan
[2]The United Graduate School of Veterinary Science, Yamaguchi University, 1677-1 Yoshida, Yamaguchi 753-8511, Japan
[3]Laboratory of Veterinary Neurology, College of Bioresource Sciences, Nihon University, 1866 Kameino, Fujisawa, Kanagawa 252-0880, Japan
[4]Department of Veterinary Medicine, Faculty of Applied Biological Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan
[5]The United Graduate School of Veterinary Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan

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 as 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, 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 were 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.

KEY WORDS: canine *SOD1* gene, Collie, degenerative myelopathy, immunohistochemistry, mutant allele frequency

J. Vet. Med. Sci.
79(2): 375–379, 2017
doi: 10.1292/jvms.16-0391

Received: 27 July 2016
Accepted: 1 December 2016
Published online in J-STAGE: 12 December 2016

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) [4, 15]. 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 [4, 11]. 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 [4, 8].

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

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

©2017 The Japanese Society of Veterinary Science

This is an open-access article distributed under the terms of the Creative Commons Attribution Non-Commercial No Derivatives (by-nc-nd) License <http://creativecommons.org/licenses/by-nc-nd/4.0/>.

[2, 7, 15]. 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) [2, 14]. These mutations are likely to cause formation of misfolded proteins that accumulate into insoluble aggregates [7, 15]. According to the information from the Online Mendelian Inheritance in Animals (OMIA) [10], 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 [2, 12, 14, 15]. The Collie breed also has an allele of c.118G>A [15]; 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 [9]. 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 [2]. 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.

The caudal thoracic spinal cord from the Rough Collie with DM [9] 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 histopathological and immunohistochemical analyses, part of the spinal cord was embedded in paraffin using a standard method and cut into thin sections. As a 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 a 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.

Hematoxylin and eosin (HE) and Luxol fast blue (LFB) staining was performed using a standard method. 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. Epitope retrieval was performed with an autoclave in Target retrieval solution (pH 6.0, Dako, Glostrup, Denmark). Endogenous peroxidase activity was quenched using 0.3% H_2O_2 hydrogen peroxide in methanol for 20 min, and tissue sections were incubated in 10% normal goat serum (Dako) 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, and the mixture was incubated at room temperature for 30 min. 3,3′-diaminobenzidine tetrahydrochloride (Dako) was used as a chromogenic substrate. 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 [3]. 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, U.S.A.) until use. Preparation of DNA templates from the FTA cards and genotyping survey were performed as described previously [3]. 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).

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. 1). 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. 2A), 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. 2B). As expected, weak reactivity was observed in the cytoplasm of spinal cord neurons in the negative control dog (Fig. 2C). 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. 2). 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.

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 [9], was retrospectively investigated by histopathological analysis using LFB-HE staining and immunohistochemical analysis using an anti-SOD1 antibody. The histopathological and immunohistochemical 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 [2, 7, 15]. 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 [4]. 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

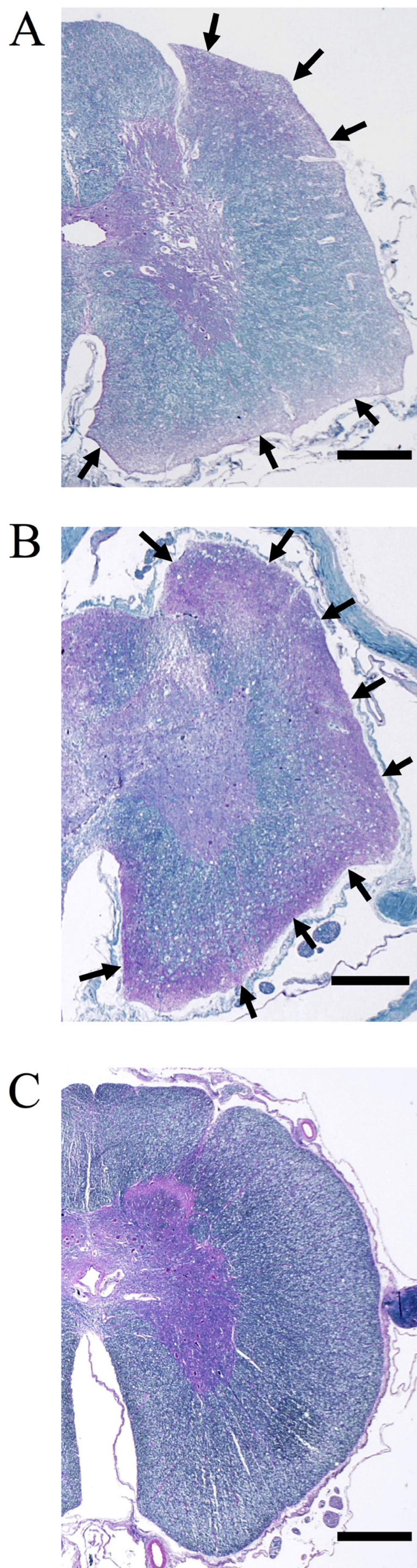

Fig. 1. Histopathological analysis of spinal cords from a Collie diagnosed clinically and histopathologically as having degenerative myelopathy (A), a Pembroke Welsh Corgi with degenerative myelopathy, which is homozygous for the *SOD1*:c.118G>A mutation (B), and a neurologically normal Labrador Retriever dog that is homozygous for a wild-type c.118G allele (C). Myelin loss in the white matter is depicted by loss of blue color (arrows, A and B). Hematoxylin and eosin and Luxol fast blue staining. Bar=400 μm.

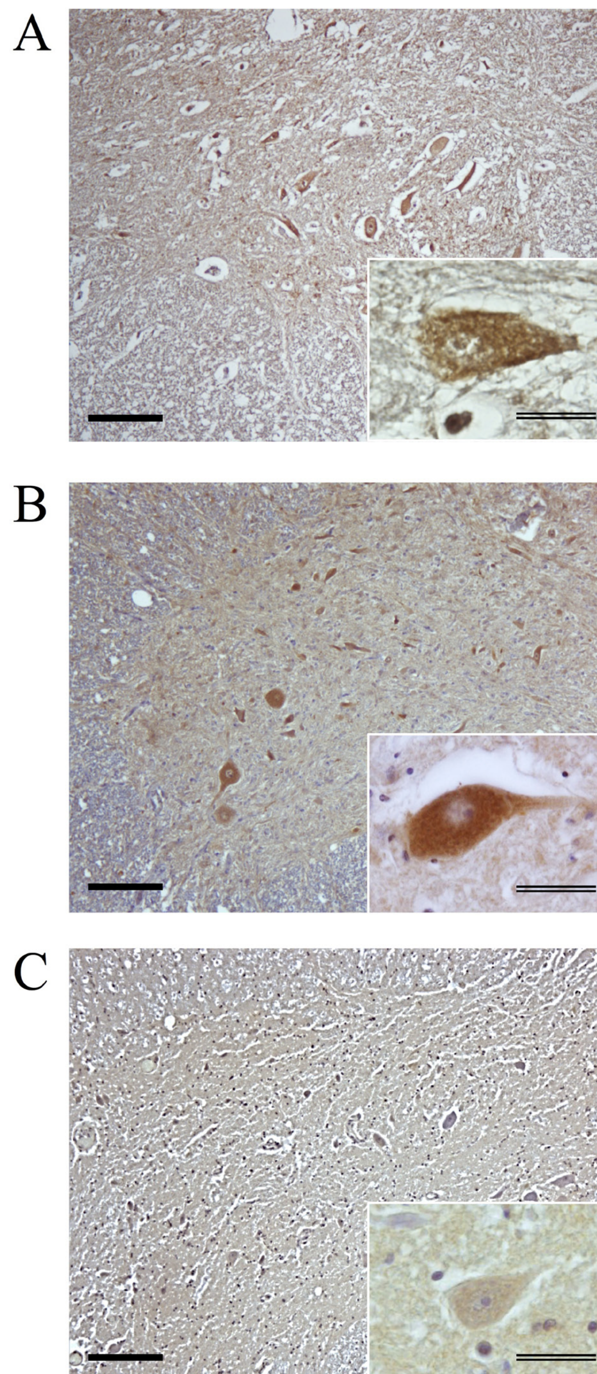

Fig. 2. Immunohistochemical analysis of ventral horn of spinal cords from a Collie diagnosed clinically and histopathologically as having degenerative myelopathy (A), a Pembroke Welsh Corgi with degenerative myelopathy, which is homozygous for the *SOD1*:c.118G>A mutation (B), and a neurologically normal 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) at a dilution of 1:500. The sections were counterstained with hematoxylin. Bar (single line)=150 μm, bar (double lines)=30 μm.

PCR [13]. 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 [5]. 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 [1], 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. [6], 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 [15]. 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 [3, 6]. Epidemiological information on the genetic disorders in each breed is essential for establishing prevention schemes [6]. Therefore, epidemiological surveys should be performed in each country and region.

In conclusion, the data in the present study suggest that the Collie breed is predisposed to DM, a disorder associated with the *SOD1*:c.118G>A mutation. Prevention of DM among Collies in Japan could be achieved with the help of strategies based on epidemiological and genetic testing highlighted by the findings in this study.

ACKNOWLEDGMENTS. The authors are grateful to all Collies' owners for providing DNA samples of their dogs for this study. We have already provided the genotyping data to the owners to facilitate efforts for prevention and control of DM in Collies in Japan. This study was supported by the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT KAKENHI Grant Numbers 25292181 and 26660242 to OY, and JSPS KAKENHI Grant Number JP16J06970 to MK).

REFERENCES

1. American Kennel Club http://www.akc.org Accessed Dec 2, 2016.
2. Awano, T., Johnson, G. S., Wade, C. M., Katz, M. L., Johnson, G. C., Taylor, J. F., Perloski, M., Biagi, T., Baranowska, I., Long, S., March, P. A., Olby, N. J., Shelton, G. D., Khan, S., O'Brien, D. P., Lindblad-Toh, K. and Coates, J. R. 2009. Genome-wide association analysis reveals a *SOD1* mutation in canine degenerative myelopathy that resembles amyotrophic lateral sclerosis. *Proc. Natl. Acad. Sci. U.S.A.* **106**: 2794–2799. [Medline] [CrossRef]
3. Chang, H. S., Kamishina, H., Mizukami, K., Momoi, Y., Katayama, M., Rahman, M. M., Uddin, M. M., Yabuki, A., Kohyama, M. and Yamato, O. 2013. 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. *J. Vet. Med. Sci.* **75**: 795–798. [Medline] [CrossRef]
4. Coates, J. R. and Wininger, F. A. 2010. Canine degenerative myelopathy. *Vet. Clin. North Am. Small Anim. Pract.* **40**: 929–950. [Medline] [CrossRef]
5. Japan Kennel Club http://www.jkc.or.jp Accessed Jan 11, 2017.
6. Mizukami, K., Yabuki, A., Kohyama, M., Kushida, K., Rahman, M. M., Uddin, M. M., Sawa, M. and Yamato, O. 2016. Molecular prevalence of multiple genetic disorders in Border collies in Japan and recommendations for genetic counselling. *Vet. J.* **214**: 21–23. [Medline] [CrossRef]
7. Nakamae, S., Kobatake, Y., Suzuki, R., Tsukui, T., Kato, S., Yamato, O., Sakai, H., Urushitani, M., Maeda, S. and Kamishina, H. 2015. Accumulation and aggregate formation of mutant superoxide dismutase 1 in canine degenerative myelopathy. *Neuroscience* **303**: 229–240. [Medline] [CrossRef]
8. Ogawa, M., Uchida, K., Park, E. S., Kamishina, H., Sasaki, J., Chang, H. S., Yamato, O. and Nakayama, H. 2011. Immunohistochemical observation of canine degenerative myelopathy in two Pembroke Welsh Corgi dogs. *J. Vet. Med. Sci.* **73**: 1275–1279. [Medline] [CrossRef]
9. Okada, M., Kitagawa, M., Kanayama, K., Yamamura, H. and Sakai, T. 2009. Negative MRI findings in a case of degenerative myelopathy in a dog. *J. S. Afr. Vet. Assoc.* **80**: 254–256. [Medline] [CrossRef]
10. Online Mendelian Inheritance in Animals http://omia.angis.org.au/home/ Accessed Jan 11, 2017.
11. Oyake, K., Kobatake, Y., Shibata, S., Sakai, H., Saito, M., Yamato, O., Kushida, K., Maeda, S. and Kamishina, H. 2016. Changes in respiratory function in Pembroke Welsh Corgi dogs with degenerative myelopathy. *J. Vet. Med. Sci.* **78**: 1323–1327. [Medline] [CrossRef]
12. Pfahler, S., Bachmann, N., Fechler, C., Lempp, C., Baumgärtner, W. and Distl, O. 2014. Degenerative myelopathy in a SOD1 compound heterozygous Bernese mountain dog. *Anim. Genet.* **45**: 309–310. [Medline] [CrossRef]
13. Schander, C. and Halanych, K. M. 2003. DNA, PCR and formalinized animal tissue −a short review and protocols. *Org. Divers. Evol.* **3**: 195–205. [CrossRef]
14. Wininger, F. A., Zeng, R., Johnson, G. S., Katz, M. L., Johnson, G. C., Bush, W. W., Jarboe, J. M. and Coates, J. R. 2011. Degenerative myelopathy in a Bernese Mountain Dog with a novel *SOD1* missense mutation. *J. Vet. Intern. Med.* **25**: 1166–1170. [Medline] [CrossRef]
15. Zeng, R., Coates, J. R., Johnson, G. C., Hansen, L., Awano, T., Kolicheski, A., Ivansson, E., Perloski, M., Lindblad-Toh, K., O'Brien, D. P., Guo, J., Katz, M. L. and Johnson, G. S. 2014. Breed distribution of *SOD1* alleles previously associated with canine degenerative myelopathy. *J. Vet. Intern. Med.* **28**: 515–521. [Medline] [CrossRef]