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A 2-base insertion in exon 5 is a common mutation of the *TP53* gene in dogs with histiocytic sarcoma

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ABSTRACT. Canine histiocytic sarcoma (HS) is a malignancy originating from the histiocytic cell lineage and characterized by poor response to chemotherapy and short survival time. Mutation of the *TP53* gene and its association with poor prognosis has been reported in several canine tumors. However, the mutation of this gene has not been investigated in canine HS. The aim of this study was to examine a *TP53* gene mutation in dogs with HS. Aberrations of the *TP53* gene were examined by polymerase chain reaction-single strand conformational polymorphism analysis and DNA sequence analysis, revealing mutations of the *TP53* gene in 12 (46%) of 26 dogs affected by HS. The incidence of the *TP53* gene mutation was relatively high in canine HS compared with other canine tumors. Among these mutations, 10 of 12 dogs (83%) with a *TP53* gene mutation harbored the same mutation: a 2-base (AT) insertion in exon 5, resulting in the introduction of a stop codon (c.446_447insAT, p.Tyr150SerfsX8). Further studies are needed to examine the functional change due to the mutation and its association with the pathogenesis of canine HS.

KEY WORDS: dog, histiocytic sarcoma, mutation, PCR-SSCP, *TP53* gene

Canine histiocytic sarcoma (HS) is a rare neoplasm originating from histiocytic cell lineages, including dendritic cells (DCs) and macrophages [1, 7]. HS arises from interstitial DCs and is subdivided into two categories: localized and disseminated HS. In addition, a third subtype, hemophagocytic HS, that arises from macrophages, was recently described [7]. HS is characterized by aggressive biological behavior and dogs with HS have poor prognosis. Although there have been several reports describing its response to doxorubicin [14], liposomal doxorubicin [14] and paclitaxel [10], treatment with lomustine (CCNU) is selected in most cases based on the reported response rates [11–13]; however, HS often acquires chemotherapy resistance within a short time, which leads to a median survival time of less than 100 days in these study. Therefore, further study is needed to understand the pathogenesis to develop more effective therapeutic strategy for canine HS.

p53, encoded by a tumor suppressor gene, *TP53* [6], functions as a transcription factor that regulates gene expression that promotes apoptosis, cell cycle arrest and DNA repair. Mutation of the *TP53* gene is known to result in tumorigenesis and drug resistance [8, 16]. In humans, *TP53* is the most frequently altered gene in tumor cells and more than 50% of all patients with tumors carry *TP53* gene mutations [9].

As is the case with human tumors, mutations of the *TP53* gene have been reported in several canine tumors, including osteosarcoma [4], lymphoma [5], mammary tumors [15] and brain tumors [17], and the proportions of patients with *TP53* mutation in each tumor were 40.7, 16, 15 and 3.4%, respectively. Recently, we reported that three of four canine HS cell lines had aberrations of the *TP53* gene, and it was suggested that the *TP53* gene might be frequently mutated in canine HS [2]. However, there have been no reports of *TP53* gene mutations in primary canine HS tissues. The objectives of this study were to examine a *TP53* gene mutation in canine HS for the elucidation of its pathogenesis.

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Table 1. Primer sequences used for PCR-SSCP analysis of the *TP53* gene

Fragment	Forward primer	Reverse primer	Exon	Amplicon size (bp)	Electrophoresis temperature (°C)
A	5′-ATGCAAGAGCCACAGTCAG-3′ (6–24)[a)]	5′-GAGCCTGGCCTGCCCTC-3′ (87–103)[a)]	2	98	15
B	5′-GCACTGACTTTCTGCTCTC-3′ (204–222)[a)]	5′-GACCTCCCCACACCCAGT-3′ (260–277)[a)]	3	74	15
C1	5′-CTTGACTCTGGTCTCGCC-3′ (318–335)[a)]	5′-GGGTAGGTCTTCGGGGAA-3′ (502–519)[a)]	4	204	10[b)]
C2	5′-CCCTATCATCCTCTGTCC-3′ (483–500)[a)]	5′-GCCAGCCCCATGGAAACC-3′ (604–621)[a)]	4	140	15[b)]
D1	5′-GACCTGTCCATCTGTCCT-3′ (1,058–1,075)[a)]	5′-ATAGATGGCCATAGCGCGG-3′ (1,181–1,199)[a)]	5	143	15[b)]
D2	5′-ACCCCCACCCAATACCTG-3′ (1,160–1,177)[a)]	5′-GCCTTGTCCCATCTGTAG-3′ (1,288–1,305)[a)]	5	167	20[b)]
E	5′-TGATTCCTCCCCGATGGC-3′ (1,330–1,347)[a)]	5′-AGACCCCTCAGATGCCAA-3′ (1,474–1,491)[a)]	6	162	20[b)]
F	5′-ACCCTGGGCCTACCTTCTA-3′ (1,664–1,682)[a)]	5′AGGGTGGCAGGCAGGTC-3′ (1,804–1,820)[a)]	7	144	15[b)]
G	5′-GCTTCTCTCTTCTCACCTG-3′ (2,036–2,054)[a)]	5′-CTCCTTCACCTCCTCTTGT-3′ (2,210–2,228)[a)]	8	193	15[b)]
H	5′-GCTCAAAACATACTCTTCTCT-3′ (2,437–2,457)[a)]	5′-TGCCTTATCTGTTCCTCCC-3′ (2,568–2,586)[a)]	9	150	10
I	5′-AATGGTACTGTGGCTTCC-3′ (2,878–2,895)[a)]	5′-CAAGCCGGCCCAGGTCA-3′ (3,047–3,063)[a)]	10	92	15
J	5′-CTCCCACTTGCTAATATCGT-3′ (3,613–3,632)[a)]	5′-TGAGGGTGTGCGTGTTGG-3′ (3,768–3,785)[a)]	11	167	15

a) The number in the parenthese indicates the nucleotide numbers registered in GenBank (NC_006587). b) Electrophoresis temperatures for these primers were determined in a previous report [5].

MATERIALS AND METHODS

Patients

Twenty-six dogs with HS referred to the Veterinary Medical Center of the University of Tokyo from 2009 to 2016 were included in this study. Written informed consent was obtained from all dog owners prior to study enrollment. The patients were diagnosed with HS by histological evaluation of surgically resected specimens for treatment or biopsy (n=24) or cytologic evaluation of fine-needle aspirates (FNA) of tumor tissues (n=2). Each case was diagnosed with HS based on the morphological or histopathological features described in a previous report [1]. Reactivities to the antibodies directed to human leukocyte antigen (HLA)-DR alpha-chain (2 dogs), ionized calcium-binding adaptor molecule 1 (Iba-1) (4 dogs), or CD204 (1 dog) were examined by immunohistochemical staining for confirmation of the diagnosis. The cytochemical staining for alpha-naphthyl butyrate esterase (α-NBE) and inhibition of the enzyme by sodium fluoride were performed as markers of monocyte/macrophage lineage in the two dogs diagnosed based on the cytologic evaluation. Tumor cell samples were collected from formalin-fixed paraffin-embedded tissues (n=19) or freshly frozen tumor tissues (n=5) or FNA samples (n=2). Information extracted from the medical records included signalment, lesion locations and subtype of HS (localized, disseminated or hemophagocytic histiocytic sarcomas).

Detection of TP53 gene mutations

Genomic DNA samples were extracted from each tumor cell sample using a QIAmp DNA Mini Kit (QIAGEN, Limburg, Netherlands). Genomic DNA was also obtained from the peripheral blood of a healthy beagle. The sequence of *TP53* gene of the DNA sample of the beagle was confirmed to be the same as that registered in GenBank (NC_006587) in our preliminary experiment. The Veterinary Medicine Institutional Animal Care and Use Committee of the University of Tokyo approved the study (Approval Number, P16-172). Twelve primer pairs were synthesized to amplify overlapping genomic DNA fragments spanning the coding region (exons 2–11) of the *TP53* gene as previously reported [3] (Table 1), and the positions of these primers are shown in Fig. 1. Mutations of the regions were screened by polymerase chain reaction-single strand conformational polymorphism (PCR-SSCP) analysis, as described previously [5], using a GeneGel Exel 12.5/24 Kit (GE Healthcare, Amersham Place, U.K.). Electrophoresis was performed using a GenePhor DNA Separation System (GE Healthcare). The optimal electrophoresis temperature for each primer pair that had not been determined previously (exon 2, 3, 9, 10 and 11) was examined and determined in preliminary experiments as shown in Table 1.

Following the PCR-SSCP analysis, direct sequencing was conducted for the DNA samples extracted from abnormal bands demonstrating a mobility shift. These extracted DNA samples were amplified by PCR with the same primers used in the PCR-SSCP analysis. The products were directly sequenced using the BigDye terminator v3.1/1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, U.S.A.) and genetic analyzer (3130XL, Applied Biosystems). When the sequence could not be directly analyzed, PCR products were inserted into a T/A cloning vector (pGEM-T Easy, Promega Corp. Leiden, The Netherlands). Thereafter, the vectors were transfected into competent cells (DH5α, TOYOBO, Osaka, Japan), and the plasmides extracted from the DH5α cells were subjected to sequence analysis as described above. The nucleotide sequence of each fragment was compared with the reference sequence of the canine *TP53* gene (GenBank accession number NC_006587), and mutations of the *TP53* gene that resulted in changes of the amino acid sequences were extracted.

Statistical analysis

The chi-square test was used to compare clinical variables between dogs with a *TP53* gene mutation and those without. A value of P less than 0.05 was considered significant.

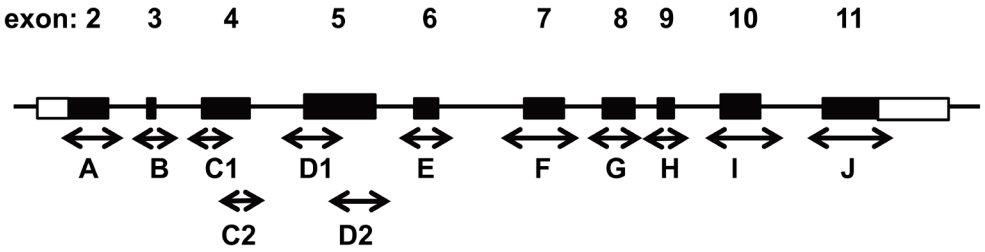

Fig. 1. Schematic diagram of the positions of the DNA fragments examined in this study. Each black arrow indicates the position of the fragment that was amplified with each primer pair.

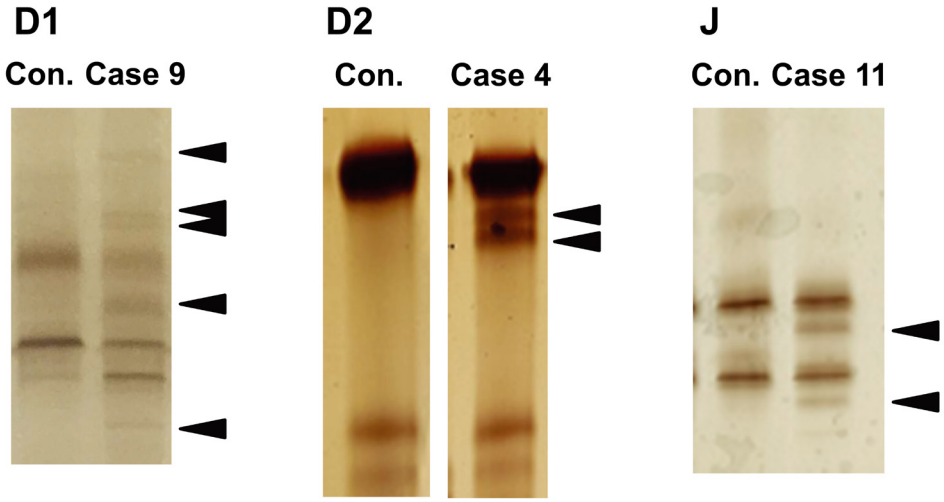

Fig. 2. Results of PCR-SSCP analysis of Fragments D1, D2 (exon 5) and J (exon 11) from the representative cases containing abnormal bands. The sample of a healthy beagle was used as normal control, and the case number above each lane indicates a specific dog with a *TP53* gene mutation. Arrowheads indicate abnormal bands that demonstrate a mobility shift.

RESULTS

Cases

Twenty-six dogs with HS were examined in this study, and breeds included seven Pembroke Welsh Corgis, five Flat-coated Retrievers, three Bernese Mountain dogs, two Golden Retrievers and one each of Rottweiler, Labrador Retriever, Pointer, Beagle, Shiba, Yorkshire Terrier, Norfolk Terrier, Pomeranian and mixed breed dog. The mean age was 9.2 years (range 1.7–15.1 years), and the mean body weight was 22.1 kg (range 2.8–40.5 kg). Five dogs were intact males, 10 were castrated males, two were intact females and nine were spayed females. Fourteen dogs had disseminated HS and 12 had localized HS. Six dogs were considered to have hemophagocytic HS based on the histological findings and clinicopathologic features. However, it was difficult to confirm the diagnosis of this type of HS, as immunohistochemical staining for macrophage markers, such as MHC class II and the leuko-integrin CD11d/CD18, could not be examined in these cases using fresh or frozen tissue samples. Ten of the 26 dogs had lesions in the spleen (38%), 8 (31%) had lesions in the lung, 8 (31%) had lesions in skin/soft tissue, 5 (19%) had lesions in the liver, 3 (12%) had lesions in lymph nodes, 2 (8%) had lesions in bones and 1 each (4%) had lesions in the gastrointestinal tract and the central nervous system.

Mutation of TP53 gene

In PCR-SSCP, the bands that were observed in healthy control samples were detected in HS tissue samples of all dogs. Results of the PCR-SSCP analysis of the representative cases with abnormal bands are shown in Fig. 2. These abnormal bands showing a mobility shift were predicted to have mutations, and DNA samples extracted from them were subjected to DNA sequence analysis. The positions of detected mutations are shown in Fig. 3a. Of the 26 dogs with HS, 12 dogs (46%) had a mutation in the *TP53* gene, and these mutations were distributed in the DNA binding domain, the tetramerization domain or the regulatory domain. The details of the mutations are listed in Table 2. Ten of the 26 dogs (38%) with a *TP53* gene mutation had the same mutation: a 2-base (AT) insertion in exon 5, resulting in the introduction of a stop codon (c.446_447insAT, p.Tyr150SerfsX8) (Fig. 3b and 3c). The other mutations detected were point mutations, and none of these mutations was common among the cases (Table 2). Four mutations were detected in one dog, three dogs had three mutations, two dogs had two mutations and six dogs had only one mutation.

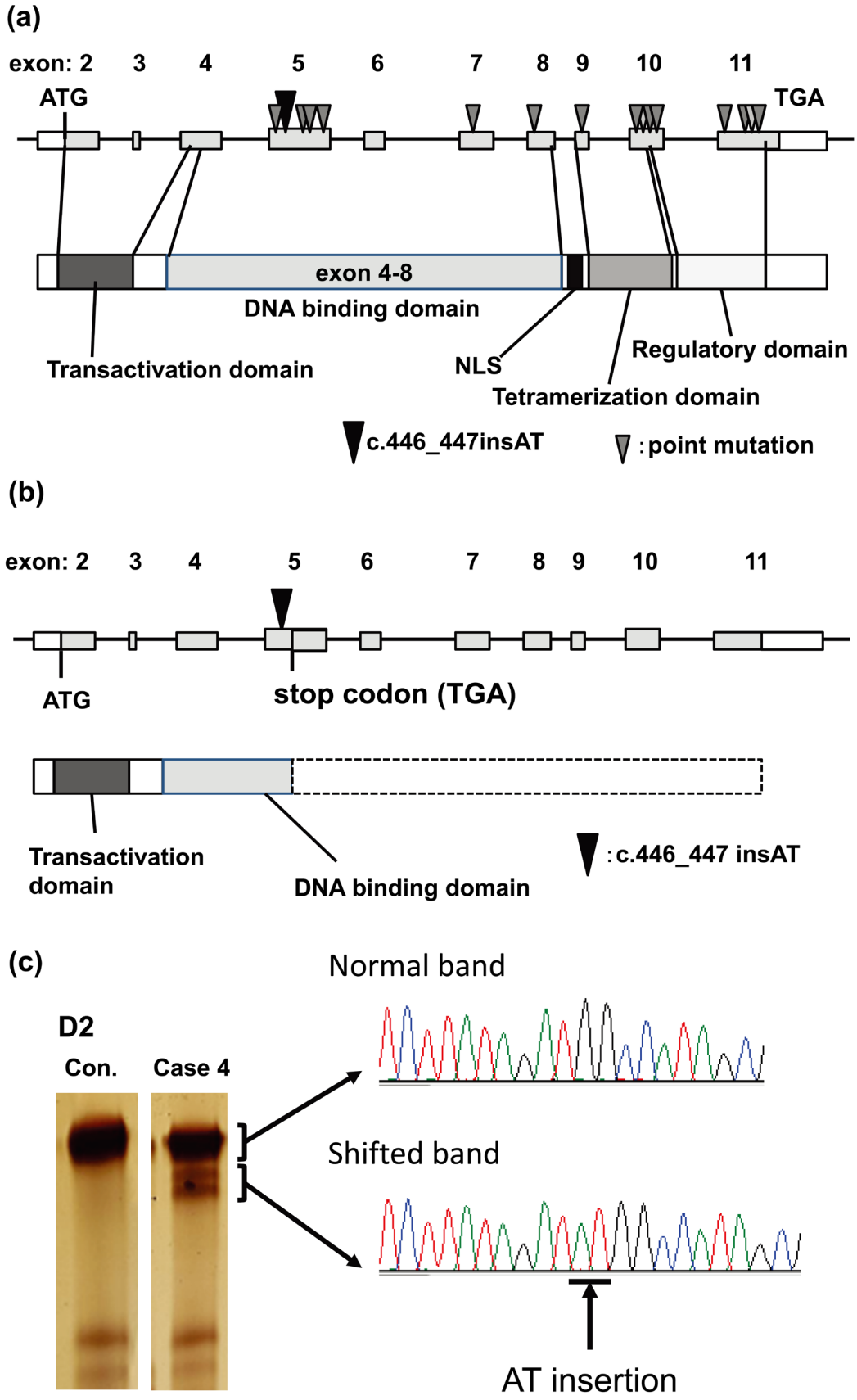

Fig. 3. Schematic diagram of the locations of all mutations of the *TP53* gene observed in the present study (a) and a schematic diagram of the c.446_447insAT mutation (b). An arrowhead represents each mutation. Black arrow-heads indicate the c.446_447insAT mutation and gray arrowheads indicate various point mutations. Gray and white boxes represent coding and non-coding regions, respectively. The result of sequence analysis of c.446_447insAT mutation are presented in (c).

Table 2. Mutation of the *TP53* gene in dogs with HS

Case No.	Breed	Subtype of HS	Exon	c.446_447insAT (p.Tyr150SerfsX8)	Other mutations
4	Shiba	Disseminated	5	(+)	
5	Yorkshire terrier	Localized	5	(−)	c.442G >A (p.Ala148Thr)
8	Norfolk terrier	Disseminated	5	(+)	
9	Pembroke welsh corgi	Localized	5, 9	(+)	c.386C >A (p.Pro129His), c.926A >G (p.Lys309Arg)
10	Beagle	Disseminated	5, 8, 10	(+)	c.859C >T (p.Pro287Ser), c.1019A >G (p.Asp340Gly)
11	Pembroke welsh corgi	Localized	5, 11	(+)	c.1100G >A (p.Arg162His), c.1116G >A (p.Met372Ile)
12	Flat-coated retriever	Disseminated	5, 8	(+)	c.802G >A (Gly268Arg)
13	Pembroke welsh corgi	Disseminated	5, 7	(+)	c.682A >G (Ser228Gly)
17	Flat-coated retriever	Disseminated	10	(−)	c.1021G >A (Ala341Thr), c.1030G >A (Gly344Arg), c.1033A >G (Lys345Glu), c.1048A >G (Ser350Gly)
19	Golden retriever	Localized	5	(+)	
25	Flat-coated retriever	Disseminated	5	(+)	
26	Pembroke welsh corgi	Localized	5	(+)	

Table 3. Associations of the c.446_447insAT mtation with clinical variables

Variable	c.446_447insAT (+)	c.446_447insAT (−)	*P* value
Breed			0.32
Pembroke welsh corgis	3	3	
Flat-coated retrievers	2	3	
Bernese mountain dogs	0	3	
Age			0.77
<10	7	9	
>10	3	7	
Sex			0.55
Male	7	8	
Female	3	8	
Subtype			0.92
Localized	4	8	
Disseminated	6	8	
Affected organ			0.98
Spleen	4	6	
Lung	3	4	
Skin/soft tissue	2	4	
Liver	1	2	

Association with clinical variables

The clinical variables were compared between the dogs with the c.446_447insAT mutation and those without the insertion (Table 3). There were no significant associations between this insertion and breed, age, sex, subtype of HS or affected organ. Furthermore, there was no association between the insertion and specific subtype of HS when HS was divided into 3 subtypes; localized, disseminated and hemophagocytic HS (data not shown).

DISCUSSION

In this study, *TP53* gene mutations were examined in canine HS and detected in 12 of 26 dogs (46%). The proportion of canine HS patients with a mutation of the *TP53* gene was relatively high compared with other canine tumors [4, 5, 15, 17], and it was suggested that *TP53* gene mutations might be common in canine HS and may play a role in the tumorigenesis of the disease. Interestingly, 10 (38%) of 26 dogs affected with HS harbored a common AT insertion in exon 5, resulting in the introduction of a stop codon. This insertion mutation has not been reported in canine tumors, and it was suggested that the mutation might be characteristic to canine HS. This mutated gene is predicted to produce a protein that does not contain the nuclear localization signal, the tetramerization domain and the regulatory domain. p53 functions in the nucleus as a tetramer, and its regulatory domain interacts with MDM2 to result in its own down-regulation. This suggests that the mutant p53 protein no longer has normal function as a transcription factor. It is also possible that the nonsense mutation detected in this study leads to loss of protein translation by degrading mRNA. Further studies are needed to examine the function of the mutant p53 protein and its association with the pathogenesis of HS.

In the statistical analyses, there were no significant associations between the presence of the c.446_447insAT mutation and breed, age, sex, subtype of HS or affected organ, suggesting that this insertion is not a genetic abnormality in a specific subgroup of canine HS. In this study, the number of dogs with HS was small and the treatment differed among cases. Therefore, the difference of survival time and response to treatment could not be evaluated between dogs with c.446_447insAT mutation and those without the insertion. Association of the *TP53* gene mutation with prognosis and response to treatment should be examined in the future studies using a sufficient number of dogs with HS.

In PCR-SSCP analysis, the bands derived from wild-type *TP53* gene were detected in HS tissue samples of all dogs with *TP53* gene mutations. In this study, each sample contained non-neoplastic cells as well as neoplastic cells, and it was unclear whether the band of wild-type *TP53* gene in PCR-SSCP analysis was derived from neoplastic cells or non-neoplastic cells. Therefore, it could not be confirmed if the mutations observed in this study were homozygous or heterozygous within tumor cells. Fluorescent *in situ* hybridization or separation of tumor cells from non-neoplastic cells by microdissection are needed for further elucidation of the status of the *TP53* gene mutations in canine HS.

Incidence of mutation of the *TP53* gene was relatively high in comparison to those in other canine tumors previously reported. Moreover, 10 of the 26 dogs with HS harbored the same 2-base (AT) insertion in the exon 5 of *TP53* gene. Further studies are needed to know the significance of this mutation on the pathogenesis of canine HS.

CONFLICT OF INTEREST. None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the report.

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