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

Hajime ASADA¹, Masaya TSUBOI², James K. CHAMBERS², Kazuyuki UCHIDA², Hirotaka TOMIYASU¹, Yuko GOTO-KOSHINO¹, Koichi OHNO¹ and Hajime TSUJIMOTO¹*

¹Department of Veterinary Internal Medicine, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan
²Department of Veterinary Pathology, Graduate School of Agricultural and Life Science, The University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan

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.

*Correspondence to: Tsujimoto, H.; atsuji@mail.ecc.u-tokyo.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. (CC-BY-NC-ND 4.0: https://creativecommons.org/licenses/by-nc-nd/4.0/)
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'-ATGCAAGACCACAGTCAG-3' (6–24) | 5'-GAGCCTGCAGGCCCCTC-3' (87–103) | 2    | 98                | 15                              |
| B        | 5'-GCACTGAACCTCTTCGCTC-3' (204–222) | 5'-GACCTCCACACACCCAGGTC-3' (260–277) | 3    | 74                | 15                              |
| C1       | 5'-TTGCGCGGCTGGGCGCG-3' (318–335) | 5'-GGTGACCTTTGCGGAAATGG-3' (502–519) | 4    | 204               | 15                              |
| C2       | 5'-CCCATCTACATCTCTGC-3' (483–500) | 5'-GCCAGCCCCATGGAAACC-3' (604–621) | 4    | 140               | 15                              |
| D1       | 5'-GACCTGGCATCCACGCTT-3' (1,058–1,075) | 5'-ATAGATGGGATAGGCCTG-3' (1,181–1,199) | 5    | 143               | 15                              |
| D2       | 5'-ACCCACCACCCACATCGG-3' (1,160–1,177) | 5'-GCTTGGCCCACTGTCG-3' (1,288–1,305) | 5    | 167               | 20                              |
| E        | 5'-TGATGTTCTCCTCCTGAC-3' (1,330–1,347) | 5'-AGACCCCTCAAGGGCAG-3' (1,474–1,491) | 6    | 162               | 20                              |
| F        | 5'-ACCTGGGGGCTGCCACT-3' (1,664–1,682) | 5'-AGGTGCTAGGAGGTC-3' (1,804–1,820) | 7    | 144               | 15                              |
| G        | 5'-GCTCTGCTCTTCTACCT-3' (2,036–2,054) | 5'-CTCCTCTCCTCTCTCCTG-3' (2,210–2,228) | 8    | 193               | 15                              |
| H        | 5'-GTCGCAACATCGACTCTCT-3' (2,437–2,457) | 5'-TGCCCTTATCTGCTCCAT-3' (2,568–2,586) | 9    | 150               | 15                              |
| I        | 5'-AATGCTCTTCGGTCTCC-3' (2,878–2,895) | 5'-CAAGCGGGCCAGGTCG-3' (3,047–3,063) | 10   | 92                | 15                              |
| J        | 5'-CTCCCACTTGGCTATATCGT-3' (3,613–3,632) | 5'-TGAGGCTGTGCTGGTGGG-3' (3,768–3,785) | 11   | 167               | 15                              |

a) The number in the parenthesis 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 the TP53 (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 detected, 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 plasmids 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 sequence 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 \( P < 0.05 \) was considered significant.
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).
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.

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.1106G >A (p.Arg362His), 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 mutation with clinical variables

| Variable           | c.446_447insAT (+) | c.446_447insAT (−) | P value |
|--------------------|--------------------|--------------------|---------|
| Breed              |                    |                    | 0.32    |
| Pembroke welsh corgi | 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                  |         |
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.

ACKNOWLEDGMENT. This study was supported by the Japan Society for the Promotion of Science, KAKENHI [grant number 26292158, 17H05043 and 17H03921].

REFERENCES

1. Affolter, V. K. and Moore, P. F. 2002. Localized and disseminated histiocytic sarcoma of dendritic cell origin in dogs. Vet. Pathol. 39: 74–83. [Medline] [CrossRef]

2. Asada, H., Tomiyasu, H., Goto-Koshino, Y., Fujino, Y., Ohno, K. and Tsujimoto, H. 2015. Evaluation of the drug sensitivity and expression of 16 drug resistance-related genes in canine histiocytic sarcoma cell lines. J. Vet. Med. Sci. 77: 677–684. [Medline] [CrossRef]

3. Chu, L. L., Rutteman, G. R., Kong, J. M., Ghahremani, M., Schmeing, M., Misdorp, W., van Garderen, E. and Pelletier, J. 1998. Genomic organization of the canine p53 gene and its mutational status in canine mammary neoplasia. Breast Cancer Res. Treat. 50: 11–25. [Medline] [CrossRef]

4. Kirpensteijn, J., Kik, M., Teske, E. and Rutteman, G. R. 2008. TP53 gene mutations in canine osteosarcoma. Vet. Surg. 37: 454–460. [Medline] [CrossRef]

5. Koshino, A., Goto-Koshino, Y., Setoguchi, A., Ohno, K. and Tsujimoto, H. 2016. Mutation of p53 gene and its correlation with the clinical outcome in dogs with lymphoma. J. Vet. Intern. Med. 30: 223–229. [Medline] [CrossRef]

6. Levine, A. J. 1997. p53, the cellular gatekeeper for growth and division. Cell 88: 323–331. [CrossRef] [PubMed]

7. Moore, P. F., Affolter, V. K. and Vernau, W. 2006. Canine hemophagocytic histiocytic sarcoma: a proliferative disorder of CD11d+ macrophages. Vet. Pathol. 43: 632–645. [Medline] [CrossRef]

8. Oshika, Y., Nakamura, M., Tokunaga, T., Fukushima, Y., Abe, Y., Ozeki, Y., Yamazaki, H., Tamaoki, N. and Ueyama, Y. 1998. Multidrug resistance-associated protein and mutant p53 protein expression in non-small cell lung cancer. Mod. Pathol. 11: 1059–1063. [Medline]

9. Ozaki, T. and Nakagawara, A. 2011. p53: the attractive tumor suppressor in the cancer research field. J. Biomed. Biotechnol. 2011: 603925. [Medline] [CrossRef]

10. Padgett, G. A., Madewell, B. R., Keller, E. T., Jodar, L. and Packard, M. 1995. Inheritance of histiocytosis in Bernese mountain dogs. J. Small Anim. Pract. 36: 93–98. [Medline] [CrossRef]

11. Rassnick, K. M., Moore, A. S., Russell, D. S., Northrup, N. C., Kristal, O., Bailey, D. B., Flory, A. B., Kiselow, M. A. and Intile, J. L. 2010. Phase II, open-label trial of single-agent CCNU in dogs with previously untreated histiocytic sarcoma. J. Vet. Intern. Med. 24: 1528–1531. [Medline] [CrossRef]

12. Skorupski, K. A., Clifford, C. A., Paoloni, M. C., Lara-Garcia, A., Barber, L., Kent, M. S., LeBlanc, A. K., Sabhlok, A., Mauldin, E. A., Shofer, F. S., Couto, C. G. and Sarenno, K. U. 2007. CCNU for the treatment of dogs with histiocytic sarcoma. J. Vet. Intern. Med. 21: 121–126. [Medline] [CrossRef]

13. Takahashi, M., Tomiyasu, H., Hotta, E., Asada, H., Fukushima, K., Kanemoto, H., Fujino, Y., Ohno, K., Uchida, K., Nakayama, H. and Tsujimoto, H. 2014. Clinical characteristics and prognostic factors in dogs with histiocytic sarcomas in Japan. J. Vet. Med. Sci. 76: 661–666. [Medline] [CrossRef]

14. Vail, D. M., Kravis, L. D., Cooley, A. J., Chun, R. and MacEwen, E. G. 1997. Preclinical trial of doxorubicin entrapped in sterically stabilized liposomes in dogs with spontaneously arising malignant tumors. Cancer Chemother. Pharmacol. 39: 410–416. [Medline] [CrossRef]

15. Van Leeuwen, I. S., Hellmén, E., Cornelisse, C. J., Van den Burgh, B. and Rutteman, G. R. 1996. P53 mutations in mammary tumor cell lines and corresponding tumor tissues in the dog. Anticancer Res. 16: 3737–3744. [Medline]

16. Wattel, E., Proudhomme, C., Hecqvet, B., Vanrumbeke, M., Quensel, B., Derivite, I., Morel, P. and Fenaux, P. 1994. p53 mutations are associated with resistance to chemotherapy and short survival in hematologic malignancies. Blood 84: 3148–3157. [Medline]

17. York, D., Higgins, R. J., LeCouture, R. A., Wolfe, A. N., Grab, R., Olby, N., Campbell, M. and Dickinson, P. J. 2012. TP53 mutations in canine brain tumors. Vet. Pathol. 49: 796–801. [Medline] [CrossRef]