



NOTE

Internal Medicine

The intratumor heterogeneity of *TP53* gene mutations in canine histiocytic sarcoma

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ABSTRACT. The mutations of *TP53* gene are frequently observed in canine histiocytic sarcoma (HS). The objective of this study was to examine the distribution of tumor cells with *TP53* gene mutations. Tumor tissues were divided into three or four regions and *TP53* gene mutations were examined. *TP53* gene mutations were detected only in parts of the HS tissues from six of the eight dogs, and the frequency of the mutant allele varied (0–65%) among the tumor regions. This study suggests that canine HS can exhibit intratumor heterogeneity. Further studies are needed to examine the clinical significance of the intratumor heterogeneity of *TP53* gene mutations.

KEY WORDS: histiocytic sarcoma, intratumor heterogeneity, mutation, *TP53* gene

Canine histiocytic sarcoma (HS) is a tumor type originating from histiocytic cell lineages including dendritic cells and macrophages and characterized by aggressive biological behavior and poor prognosis [1, 7]. Our recent study revealed that 12 of 26 (46%) dogs with HS had mutations in *TP53*, a tumor suppressor gene, and 10 of 12 (83%) dogs with mutations had the same mutation, a two-base (AT) insertion mutation in exon 5 (c.446_447insAT) [2].

In human medicine, genetic heterogeneity in individual tumor tissues, known as intratumor heterogeneity (ITH), has been reported for many types of tumors [5, 8]. ITH is considered a key factor that can contribute to cancer progression, therapeutic failure due to drug resistance, and prognosis of patients [6]. However, there have been no studies on ITH in canine tumors. We hypothesized that canine HS might also exhibit ITH, and the aim of this study was to examine the distribution of the tumor cells that harbor the *TP53* gene mutations in the tumor tissues of dogs with HS.

Formalin-fixed paraffin-embedded (FFPE) tissue samples were obtained from eight dogs with HS that had been identified to have the c.446_447insAT mutation and/or point mutations in our previous study [2]. Written informed consent was obtained from all dog owners prior to study enrollment. The dogs were diagnosed with HS by histological examination of the surgically resected specimens or a post-mortem biopsy specimen based on a previous study [1]. These tissue samples were obtained from primary lesions before chemotherapy. The detailed information of dogs included in this study is shown in the Supplementary Table 1.

Each FFPE tissue sample was processed into five 8 μm -thick sections or one 6 μm -thick section as sequential sections. One 6 μm -thick section was stained with hematoxylin and eosin or toluidine blue, and the tumor region was divided into three or four regions. Five 8 μm -thick whole sections were subjected to mutation analysis without dividing the regions to increase the number of tumor cells for analysis. Before mutation analysis, microscopic examinations were conducted, and it was revealed that the divided regions were filled with tumor cells and there was no apparent difference in the morphological or histological structures within the tumor tissue regions. The normal marginal cell region was also isolated in three dogs.

Genomic DNA was extracted from five 8 μm -thick whole sections and the tumor tissue regions divided from one 6 μm -thick section of each dog, and it was amplified by PCR and subjected to mutation analysis. Primers for the detection of *TP53* gene mutations are listed in Table 1. Point mutations were analyzed by direct sequencing, and GeneScan analysis [3, 4] was performed to detect the c.446_447insAT mutation. The detection limit and accuracy of quantification of c.446_447insAT allele frequency were evaluated and the frequencies of mutant alleles were evaluated as described in Supplementary Materials and Methods.

The representative divisions of the tumor tissues are shown in Supplementary Fig. 1 and the representative results of mutation

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Table 1. Primer sequences for detection of *TP53* gene mutations

Mutation	Forward primer	Reverse primer	Exon	Amplicon size (bp)
c.446_447insAT	5'-FAM-ACCCCCACCCAATACCTG-3' (761–778) ^{a)}	5'-GCCTTGTCCTATCTGTAG-3' (889–906) ^{a)}	5	146
c.386C>T	5'-GACCTGTCCATCTGTCT-3' (659–676) ^{a)}	5'-ATAGATGGCCATAGCGCG-3' (782–800) ^{a)}	5	142
c.485G>A	5'-ACCCCCACCCAATACCTG-3' (761–778) ^{a)}	5'-GCCTTGTCCTATCTGTAG-3' (889–906) ^{a)}	5	146
c.926A>G	5'-GCTCAAAAACATACTCTTCTCT-3' (2,437–2,457) ^{a)}	5'-TGCCTTATCTGTTCTCCC-3' (2,568–2,586) ^{a)}	9	150
c.859C>T	5'-GCTTCTCTTCTCACCTG-3' (2,036–2,054) ^{a)}	5'-CTCCTTCACCTCCTTGT-3' (2,210–2,228) ^{a)}	8	193
c.1,019A>G				
c.1,021G>A				
c.1,030G>A	5'-AATGGTACTGTGGCTTCC-3' (2,878–2,895) ^{a)}	5'-CAAGCCGCCCCAGGTCA-3' (3,047–3,063) ^{a)}	10	92
c.1,033A>G				
c.1,048A>G				

a) The number in the parentheses indicates the nucleotide numbers registered in GenBank (NC_006587).

analysis by direct sequencing or GeneScan are shown in Fig. 1. The frequencies of mutant alleles are shown in Table 2.

Mutation analysis using five 8 μm -thick whole sections detected two of nine point mutations, c.485G>A in Dog 2 and c.386C>T in Dog 4. The c.485G>A mutation was detected in all divided regions for Dog 2, and the frequency of the allele with the c.485G>A mutation was 55% in the whole sections and 40–55% in the regions. Thus, it was suggested that c.485G>A may be a somatic heterozygous mutation harbored by all the tumor cells in Dog 2. It was also possible that the mutation may be homozygous in 50% of the tumor cells. On the other hand, the c.386C>T mutation was detected in two of three regions for Dog 4, and the frequency of the allele with the c.386C>T mutation varied among the regions (0–65%). Therefore, it was suggested that c.386C>T may be a passenger mutation, a type of mutation that occurs during tumor progression and is observed only in parts of the tumor. In addition, it was suggested that tumor cells with c.386C>T might not exist homogeneously but located in some regions of tumor tissues. The other seven point mutations that had been detected by the PCR-SSCP method in our previous study were not detected in the present study. This may be attributed to the lower sensitivity of direct sequencing compared with the PCR-SSCP method. It was suggested that these mutations may be passenger mutations observed only in a few tumor cells and these results also indicated the intratumor heterogeneity.

The c.446_447insAT mutation was detected in seven dogs that had been identified to have the mutation in our previous study [2]. In the mutation analysis using the regions divided from one 6 μm -thick section, the c.446_447insAT mutation was detected in all divided regions for Dog 1, whereas it was detected in one of four regions for Dog 3 and not detected in any regions for the other five dogs. The frequency of the allele with the c.446_447insAT mutation was approximately 50% in the whole sections and the divided regions in Dog 1, but it was 4–18% among the other dogs. Therefore, it was suggested that the frequency of the allele

Table 2. Mutation analysis by direct sequencing and GeneScan

Dog	<i>TP53</i> gene mutation	Whole sections (allele frequency; %)	Divided regions (allele frequency; %)				Normal cell regions
			Region 1	Region 2	Region 3	Region 4	
1	c.446_447insAT	Detected (47)	Detected (47)	Detected (43)	Detected (43)	-	ND
2	c.446_447insAT	Detected (7)	ND (0)	ND (0)	ND (0)	ND (0)	ND
	c.485G>A	Detected (55)	Detected (55)	Detected (40)	Detected (45)	Detected (50)	ND
3	c.446_447insAT	Detected (18)	Detected (39)	ND (0)	ND (0)	ND (0)	ND
4	c.386C>T	Detected (30)	Detected (15)	Detected (65)	ND (0)	-	-
	c.446_447insAT	Detected (7)	ND (0)	ND (0)	ND (0)		
	c.926A>G	ND	ND	ND	ND		
5	c.446_447insAT	Detected (4)	ND (0)	ND (0)	ND (0)	ND (0)	-
6	c.446_447insAT	Detected (12)	ND (0)	ND (0)	ND (0)	ND (0)	-
	c.859C>T	ND	ND	ND	ND	ND	
	c.1,019A>G	ND	ND	ND	ND	ND	
7	c.1,021G>A	ND	ND	ND	ND	ND	-
	c.1,030G>A	ND	ND	ND	ND	ND	
	c.1,033A>G	ND	ND	ND	ND	ND	
	c.1,048A>G	ND	ND	ND	ND	ND	
8	c.446_447insAT	Detected (9)	ND (0)	ND (0)	ND (0)	ND (0)	-

ND; Not detected, -; Not examined.

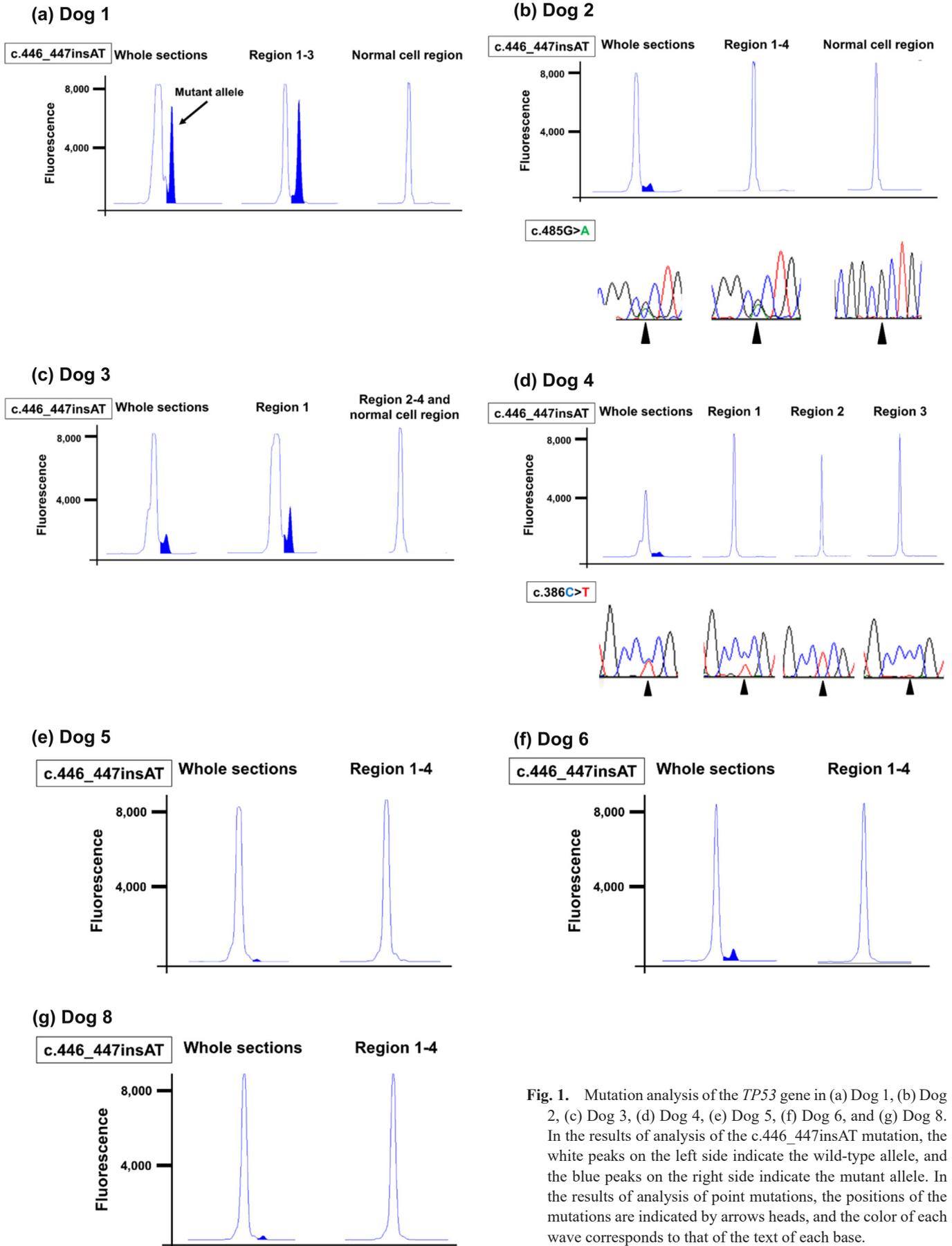


Fig. 1. Mutation analysis of the *TP53* gene in (a) Dog 1, (b) Dog 2, (c) Dog 3, (d) Dog 4, (e) Dog 5, (f) Dog 6, and (g) Dog 8. In the results of analysis of the c.446_447insAT mutation, the white peaks on the left side indicate the wild-type allele, and the blue peaks on the right side indicate the mutant allele. In the results of analysis of point mutations, the positions of the mutations are indicated by arrows heads, and the color of each wave corresponds to that of the text of each base.

with the c.446_447insAT mutation might be different among dogs with HS and the mutation might be a passenger mutation in some cases with HS. In the mutation analysis for the divided regions, one 6 μm -thick section was subjected to the analysis. On the other hand, five 8 μm -thick whole sections were subjected to the analysis in the mutation analysis for the whole sections to increase the number of tumor cells for analysis. Thus, it is reasonable to think that c.446_447insAT mutation was detected only in whole sections in Dogs 2, 4–6 and 8 because the number of analyzed tumor cells was larger in whole sections compared to divided regions even though the divided regions were collected. It is a limitation of the present study that the frequency of the mutant allele might not be accurately evaluated due to the small number of analyzed tumor cells in the divided regions. In addition, it was suggested that tumor cells with the c.446_447insAT mutation might not homogeneously exist but located in some regions of tumor tissues in some cases. However, factors such as copy number aberrations of the *TP53* gene locus in the tumor cells could affect the mutant allele frequencies. Therefore, further studies are needed to elucidate the role of *TP53* gene mutations in the tumorigenesis of canine HS.

Although the present study revealed that *TP53* gene mutations can exhibit ITH in canine HS tissues, there was no apparent relationship between the frequencies of mutant alleles and tumor size, lesion location or subtypes of HS in the present study. In addition, the association of the frequencies of mutant alleles with sensitivity to treatment or prognosis could not be also investigated. Therefore, further studies using a sufficient number of dogs are needed to evaluate the associations of the ITH with tumor progression or subtypes of HS and the clinical significance of the ITH of the *TP53* gene mutations.

In conclusion, *TP53* gene mutations were heterogeneous in most of the canine HS tissues, suggesting that canine HS is a tumor that can exhibit ITH, and it was unclear if the mutations of the *TP53* gene mutations were important for the tumorigenesis of canine HS. Further studies are needed to investigate the clinical significance of the ITH of *TP53* gene mutations in canine HS.

CONFLICT OF INTEREST. The authors do not have any financial or personal relationships that could inappropriately influence or bias the content of the study.

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