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Mutation of *p53* Gene and Its Correlation with the Clinical Outcome in Dogs with Lymphoma

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Background: *p53* plays a key role in the apoptotic event induced by chemotherapeutic agents. Mutation of *p53* gene has been observed in various spontaneous tumors in humans and is associated with a poor prognosis. *p53* abnormalities have been evaluated in several tumors in dogs; however, the association of *p53* gene mutation with clinical outcome in dogs with lymphoma has not been documented.

Hypothesis/Objectives: The aim of this study was to examine *p53* mutation in canine lymphoma cells and its association with the clinical outcome.

Animals: Forty-three dogs with previously untreated high-grade lymphoma referred to the University of Tokyo were included in this study.

Methods: Prospective cohort study. We examined *p53* gene (exon 4–8) mutation in the tumor tissues from 43 dogs with lymphoma using PCR-SSCP (polymerase chain reaction – single-strand conformational polymorphism) analysis, followed by nucleotide sequencing of the abnormal bands.

Results: Of the 43 dogs, 7 dogs (16%) had *p53* mutation, whereas 36 dogs (84%) were devoid of *p53* mutation. Overall response rate after remission induction was significantly lower (33% versus 88%, $P = .002$) in dogs with lymphomas having *p53* mutation than those with lymphomas devoid of *p53* mutation. Overall survival time was significantly shorter (67 days versus 264 days, $P = .004$) in dogs with lymphoma with *p53* mutation than those with lymphoma retaining wild-type *p53*.

Conclusion and Clinical Importance: Mutations of *p53* gene were detected in a proportion of canine lymphoma cells from untreated dogs and can be associated with a poor prognosis.

Key words: Chemotherapy; Multidrug resistance; P-glycoprotein (P-gp); Prognosis.

Lymphoma is defined as a neoplastic disease of lymphoid cells that primarily affects lymph nodes and a variety of organs except for bone marrow. It is the most common hematopoietic tumor in dogs, and its annual incidence was reported to be 13–107 per 100,000 dogs.[1–3]

Various chemotherapeutic protocols have been reported in the veterinary literatures for the treatment of lymphoma in dogs. Although overall response rates in dogs treated with multidrug combination chemotherapies were shown to be as high as 80–90%, most of the dogs eventually die or are euthanized from the recurrence of the disease in which multidrug resistant of the tumors is apparent.[4] To improve the treatment outcome after chemotherapy, better understanding of the mechanism of drug resistance is warranted.

Multidrug resistance is a cross-resistance to multiple structurally unrelated chemotherapeutics and is often recognized in canine lymphoma cells especially during or after chemotherapy. The mechanisms of multidrug resistance have been classified into decrease in the intracellular drug concentration by decreased expression of transporters or induction of drug efflux pumps,[5–7] alteration in metabolic or detoxification pathways,[8,9] modification of target molecules,[10,11] damage repair,[12,13] and resistance to apoptosis.[14–17]

Inhibition of apoptosis mediated by *p53* inactivation is associated with drug resistance of spontaneous tumors in humans and their xenografts in mice.[15,17] Fibroblasts obtained from *p53*-knockout mice showed apparent resistance to alkylating agents and topoisomerase-II inhibitors.[17] Moreover, transplanted tumor cells obtained from *p53*-knockout mice were shown to be more resistant to doxorubicin and radiation than those obtained from mice with normal *p53*.[15] In many tumors including non-Hodgkin's lymphoma,[18] there is a significant association of the *p53* mutation and poor prognosis in humans.[19–21] Furthermore, restoration of normal *p53* gene conferred the chemosensitivity[22] and radiosensitivity[23] in *p53*-null tumor cells.

Mutations of *p53* gene have been identified in various tumors in dogs including thyroid carcinoma,[24] oral papilloma,[25] osteosarcoma,[26] circumanal gland adenoma,[27] mammary tumor,[28,29] and lymphoma.[30–32] In dogs with mammary tumor,[28,29] mast cell tumor,[33] and lymphoma,[34] relation of *p53* abnormalities (mutation or overexpression) to their clinical outcome has been suggested.

To examine the mutation of *p53* gene in a large number of clinical specimens, PCR SSCP analysis was employed as a sensitive and accurate screening method in this study. PCR is used to amplify the region of interest and the resultant DNA is separated as single-strand molecules by electrophoresis. This method is

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based on the observation that under nondenaturing conditions, single-stranded DNA (ssDNA) fragments fall into unique conformations determined by their primary sequence. As a consequence, even a single base mutation can disrupt secondary structure of the ssDNA which leads to changes in mobility through the gel. Coupled with sequence analysis, it is an extremely useful method for identifying and characterizing genetic mutations and has been used widely for the detection of genetic polymorphisms and mutations in a variety of genes including *p53*.

The aim of this study was to examine *p53* mutation in previously untreated canine lymphoma cells and to investigate its association with the treatment outcome in the affected dogs.

Materials and Methods

Case Population

Forty-three dogs with high-grade lymphomas referred to the Veterinary Medical Center of the University of Tokyo in the period of 2000–2006 were included in this study. The dogs were diagnosed by the cytologic evaluation of fine-needle aspirates (FNA), the histologic evaluation of the surgically resected lesions when the cytology was inconclusive for diagnosis or both. Cytological classification of lymphoma to indicate the high-grade malignancy was performed according to the updated Kiel classification.[35] Histological diagnosis was based on the World Health Organization classification in 2002.[36]

Evaluation of Response to Chemotherapy and Survival Data

After sampling of the lymphoma cells by FNA or surgical resection of the lesions, all 43 dogs were treated with a CHOP-based combination chemotherapy protocol (L-VCA short protocol).[37] Dogs who received treatment with a different protocol or concurrent radiotherapy were excluded from this study. Response to chemotherapy was evaluated at 14 days after starting treatment and dogs who survived less than 14 days were excluded from this analysis. Dogs were considered to achieve complete response (CR) when they were clinically free of the disease, partial response (PR) when the tumor size reduced by more than 50%, stable disease (SD) when the reduction or increase was within 50% and progressive disease (PD) when the increase was more than 50%. Overall response rate was calculated from the number of dogs that achieved CR or PR of all dogs. Overall survival duration was defined as the time from the initiation of chemotherapy to death or the last follow-up evaluation, and duration of remission was defined as the time from the initiation of chemotherapy to the point of PD in dogs that responded to chemotherapy.

PCR-SSCP Analysis for p53 Gene

Mutations of *p53* gene at exons 4–8 which encodes its functional domains were screened using PCR-SSCP analysis followed by silver staining.[38] Tumor cells were obtained from lesions that were used for the diagnosis of lymphoma by FNA or surgical resection. A canine mammary gland tumor cell line (cIPm)[31] was used as a control to have wild-type *p53* gene. Cell lines with known *p53* mutation[38]; a canine osteosarcoma cell line (cHOS) and a canine mammary gland carcinoma cell line (cHMp) were used to verify the conditions of SSCP.

Genomic DNA samples were extracted with a method utilizing a silica-gel membrane.[a] Seven primer pairs to amplify overlapping genomic DNA fragments spanning exons 4–8 of canine *p53* gene were synthesized based on the sequence of canine *p53* gene previously reported (Table 1).[39] The genomic DNA samples (100 ng) were amplified by PCR using a pair of primers (15 pmol each), 1.25 units of *Taq* DNA polymerase,[b] and 0.2 μM of each of 4 deoxynucleotides in 50 μL of the reaction buffer supplied by the manufacturer.[b] After denaturation at 94°C for 2 minutes, 35 cycles of the reaction (94°C for 1 minute [denaturation], 58°C for 1 minute [annealing], and 72°C for 1 minute [polymerization]) were performed, followed by a final extension procedure at 72°C for 7 minutes.

After the PCR procedure, the reaction products were mixed with the same volume of denaturing solution (95% (v/v) formamide, 0.05% xylene cyanole FF, 0.05% bromophenol blue), denatured at 95°C for 5 minutes, and thereafter directly placed on ice. The samples (6 μL/lane) were loaded onto 12.5% polyacrylamide gels.[c] Electrophoresis was performed at 15 W for 80 minutes, temperature controlled by a peltier cooling system[d] at the optimally determined electrophoresis temperature for each primer pair (Table 1). Then, the gels were silver-stained[e] for visualization of the PCR products. PCR products showing mobility shifts were extracted from the gels and subjected to nucleotide sequence analysis.

Table 1. Primers used for PCR-SSCP analysis of canine *p53* genomic DNA

Primer	Primer Sequence[a]	Nucleotide Position of Primer[b]	Exon Scanned	Electrophoresis Temperature (°C)
C1S	5′-CTTGACTCTGGTCTCGCC-3’	nt -26 ~ nt -9	Exon 4	10
C1AS	5’-GGGTAGGTCTTCGGGGAA-3’	nt 176 ~ nt 159		
C2S	5’-CCCTATCATCCTCTGTCC-3’	nt 140 ~ nt 157	Exon 4	15
C2AS	5’-GCCAGCCCCATGGAAACC-3’	nt 278 ~ nt 261		
D1S	5’-GACCTGTCCATCTGTCCT-3’	nt 705 ~ nt 732	Exon 5	15
D1AS	5’-ATAGATGGCCATAGCGCGG-3’	nt 853 ~ nt 834		
D2S	5’-ACCCCCACCCAATACCTG-3’	nt 814 ~ nt 831	Exon 5	20
D2AS	5’-GCCTTGTCCCATCTGTAG-3’	nt 960 ~ nt 942		
ES	5’-TGATTCTCCCCGATGGC-3’	nt 983 ~ nt 1001	Exon 6	20
EAS	5’-AGACCCCTCAGATGCCAA-3’	nt 1145 ~ nt 1137		
FS	5’-ACCCTGGGCCTACCTTCTA-3’	nt 1317 ~ nt 1335	Exon 7	15
FAS	5’-AGGGTGGCAGGCAGGTC-3’	nt 1473 ~ nt 1457		
GS	5’-GCTCTCTCTTCTCACCTG-3’	nt 1690 ~ nt 1708	Exon 8	15
GAS	5’-CTCCTTCACCTCCTCTTGT-3’	nt 1880 ~ nt 1862		

[a] Sequence and [b] nucleotide positions of the primers were based on the reported sequence of canine *p53* gene.[39]

Sequence Analysis of Aberrant p53 Gene

After PCR-SSCP analysis, direct sequencing of the abnormal bands was performed. DNA samples extracted from the fragments with mobility shift were amplified by PCR method using the same primer pairs for PCR-SSCP. The PCR products were sequenced by dideoxy chain termination method.[f] Nucleotide sequences were determined on both DNA strands in opposite directions and were repeated four times for each PCR product.

Statistical Analysis

Comparisons of overall response rate and other clinical variables (age, gender, anatomic form) between dogs with *p53* mutation and dogs without *p53* mutation were analyzed with χ^2 test. Kaplan–Meier method was used to generate overall survival curves, and the difference between the pair of Kaplan–Meier curves was evaluated by log-rank test. Cases that were lost to follow-up were excluded from the study. *P* values <.05 were rated significant.

Results

Cases

Breeds of the 43 dogs with lymphomas analyzed in this study included Golden Retriever (7), mixed breed (6), Pembroke Welsh Corgie (5), Miniature Dachshund (4), Labrador Retriever (3), Shih Tzu (2), Beagle (2), Shiba Inu (2), Cavalier King Charles Spaniel (2), American Cocker Spaniel (1), Maltese (1), Flat-coated Retriever (1), French Bull Dog (1), Pug (1), Chihuahua (1), Standard Duchshund (1), Shetland Sheep Dog (1), Basset Hound (1), and Miniature Schnauzer (1). The median age at presentation was 8 years old (range, 10 months–13.9 years old). Twenty-two were male (neutered, 7) and 21 were female (neutered, 8). Of the 43 dogs diagnosed as lymphoma, the anatomic form was classified into multicentric form (32), alimentary form (6), thymic form (4), and cutaneous form (1).

Mutation of p53 Gene

Results of PCR-SSCP analysis for exons 4–8 of *p53* gene are shown in Figure 1. Two bands with mobility shift were detected in 4 dogs (case 2, 4, 6, 7) and 4 bands with mobility shift were detected in 3 dogs (case 1, 3, 5). Furthermore, in cases 2 and 4, normal bands derived from wild-type *p53* gene in the SSCP analysis were not visible. The mutations were detected at exons 4 (1 dog), 5 (1 dog), 6 (1 dog), 7 (2 dogs), and 8 (2 dogs). As a whole, 7 dogs (16%) had *p53* mutation, whereas p*53* mutation was not detected in 36 dogs (84%) in the sequence of exons 4–8.

According to direct sequencing of the abnormal bands, of the 7 dogs with *p53* mutation, 3 dogs had a single base insertion and 4 dogs had a single base substitution (Table 2). One dog (case 3) had a synonymous substitution; however, all other mutations were shown to cause changes of amino acid sequence (Table 2).

Mutation of *p53* gene was found in all anatomic forms (4 dogs with multicentric form, 1 dog with alimentary form, 1 dog with thymic form, and 1 dog with cutaneous form). There was no significant association between *p53* status and age, gender, or anatomic form.

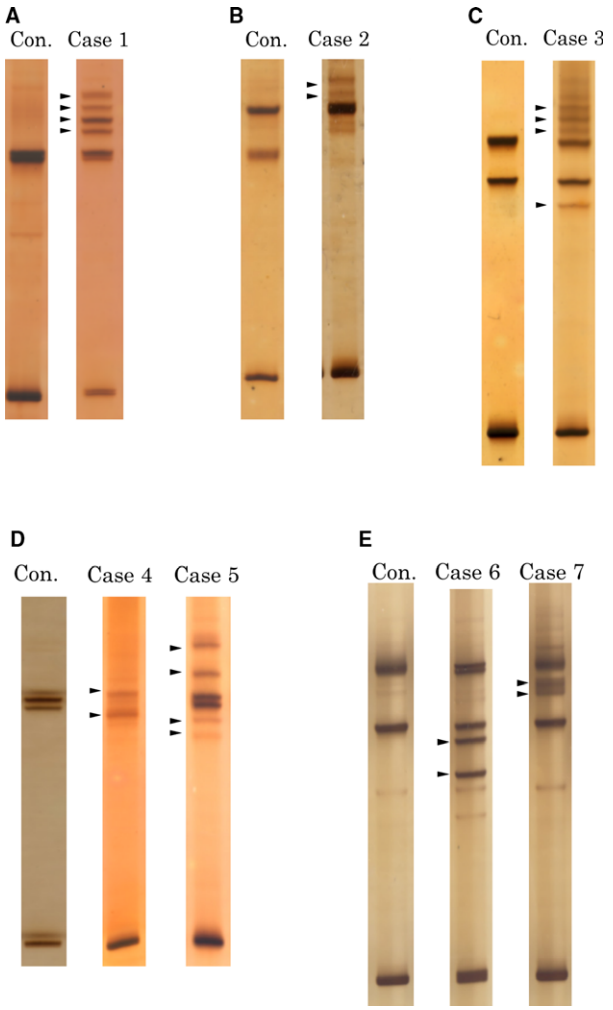

Fig 1. Results of PCR-SSCP analysis of *p53* gene in tumor cells obtained from dogs with lymphoma. **A**–**E** show the representative SSCP gels of each exon (A, exon4; B, exon5; C, exon6; D, exon7; E, exon8). The number above each lane indicating a specific dog with *p53* mutation corresponds with the case number in Table 2. Lane Con. is a dog mammary gland tumor cell line (cIPm) which was used as negative control. Tumor samples from case 1, 3, and 5 had 4 aberrant bands and, in other cases, 2 aberrant bands were detected. Lack of normal bands derived from the wild-type *p53* transcript was seen in samples from case 2 and 4. Abnormal bands that had a mobility shift are indicated (arrow heads).

Response to Treatment

Relation of *p53* status and response to chemotherapy is shown in Table 3. Thirty-nine dogs survived more than 14 days and were included in the analysis. In dogs without *p53* mutation, 29 of the 33 dogs (88%) responded to the chemotherapy. On the other hand, in dogs with *p53* mutation, only 2 of the 6 dogs (33%) responded to the chemotherapy. By χ^2 analysis, dogs

Table 2. *p53* mutations at exons 4–8 in 7 dogs with lymphoma

Case No.	Breed	Sex	Age	Diagnosis	Response	Exon	Mutation (Amino Acids)
1	Golden Retriever	SF	8Y0M	Alimentary Lymphoma	CR	4	c.287_288insT
2	Labrador Retriever	SF	11Y7M	Multicentric Lymphoma	PD	5	c.434C>T (p.Arg145His)
3	Shi Tzu	F	12Y0M	Multicentric Lymphoma	PR	6	c.603T>A (p.Arg201Arg)
4	American Cocker Spaniel	M	12Y7M	Multicentric Lymphoma	SD	7	c.679T>C (p.Asn227Asp)
5	Miniature Dachshund	M	0Y10M	Alimentary Lymphoma	SD	7	c.687_688insC
6	Shiba Inu	F	10Y0M	Thymic Lymphoma	SD	8	c.812C>T (p.Arg271Gln)
7	Mixed breed	SF	10Y10M	Cutaneous Lymphoma	SD	8	c. 796_797insA

Table 3. Relation of *p53* mutation and response to chemotherapy

Variable	Good Response Group (CR or PR)	Bad Response Group (SD or PD)	*P* Value
p53 mutation			
Positive	2	4	.002
Negative	29	4	

with *p53* mutation were significantly more likely to have a poorer response to chemotherapy ($P = .002$; Table 3).

Survival

Overall survival curves generated by Kaplan–Meier method are shown in Figure 2. In the present study, all cases could be followed to the time of death either by the dogs presenting to our Veterinary Medical Center or by communication with referring veterinarians. Median overall survival time was 67 days in dogs with *p53* mutation (7 dogs) and 264 days in dogs without *p53* mutation (36 dogs) (Fig 2A). Median duration of remission was 88 days in dogs with *p53* mutation (2 dogs) and 223 days in dogs without *p53* mutation (29 dogs). Analyzed by log-rank test, the median overall survival time was significantly shorter in dogs with lymphomas showing *p53* mutation than those with lymphomas devoid of *p53* mutation ($P = .004$).

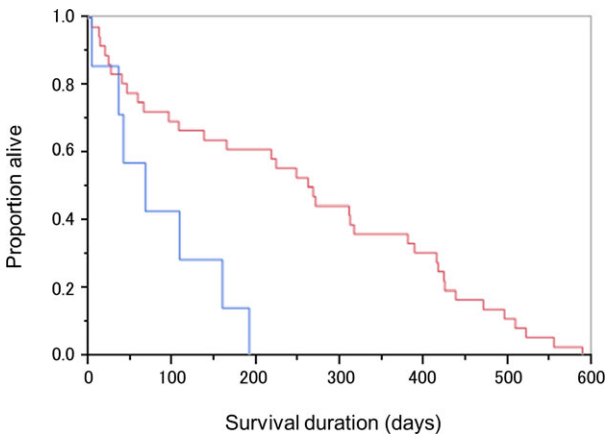

Fig 2. Overall survival curves of dogs with lymphomas with or without *p53* mutation. Red: no mutation (n = 36), Blue: mutation (n = 7). $P = .004$.

Discussion

In this study, mutation of *p53* gene was found using PCR-SSCP in 7 (16%) of the 43 dogs with previously untreated high-grade lymphoma. In previous studies evaluating *p53* mutation in lymphoma in dogs, *p53* mutation was detected in 14–26% of the dogs with lymphoma.[30–32] Based on these studies, *p53* mutation seems to be a relatively frequent event in lymphoma in dogs in comparison to that in lymphoma in cats with a very low frequency of *p53* mutation.[40] Since direct sequencing was only done in samples showing aberrant bands with PCR-SSCP, it is possible that some mutations were not detected in this study. However, mutation detection for PCR-SSCP is generally high and the sensitivity of SSCP increases with decreasing DNA fragment length. In one study examining genomic and cDNA sequences of the *p53* gene, sensitivity for PCR-SSCP analysis of more than 99% and 89% for 100–300 bp and 300–450 bp fragments were reported, respectively.[41] In our study, the primers used for PCR-SSCP were designed so that each DNA fragment will be less than 200 bp to optimize the condition of SSCP.

The locations and types of mutations in tumors in dogs are similar to those reported in tumors in human, and most of the mutations reported in tumors in dogs are point mutations located in the conserved domains of *p53* gene.[24–33] Sequence aberrations of *p53* gene detected in this study included point mutations and single nucleotide insertions. Several studies indicated that some type of tumors in human had *p53* mutations characteristic to the peculiar type of tumors: in smokers with lung cancer, many of the *p53* mutations were found at Arg^{157}, Arg^{248}, and Arg^{273}.[42] Mutation characteristic to each type of tumor has not been identified in tumors in dogs. Further studies examining *p53* mutation in tumors in dogs will elucidate the *p53* mutation specific to some type of tumors, leading to the understanding the molecular tumorigenesis.

In humans with colon cancer, 75–80% of the tumors examined were shown to have loss of both wild-type *p53* alleles.[42,43] In the present study, cases 1, 3, and 5 had 4 aberrant bands indicating aberrations in the *p53* gene on both alleles. Two other samples (cases 2 and 4) lacked normal bands derived from wild-type *p53* gene, indicating the loss of wild-type *p53* allele. These data suggest that mutation of *p53* gene exist not only on one allele but also on both alleles in lymphomas in dogs, leading to the loss of wild-type *p53* gene.

In addition to the mutations described in Table 2, lymphoma samples from 2 dogs showed single nucleotide deletions in intron 7. Intronic mutations in *p53* have been reported in several tumors in human including B-cell lymphomas.[44–46] Influence of these intronic changes could not be assessed in this study. However, *p53* mutation in intron 7 resulting in abnormal splicing of exon 7 and 8 has been reported in a human case of B-chronic lymphocytic leukemia,[45] indicating that intronic mutations may dysregulate P53 function.

In this study, overall response rate in dogs with *p53* mutation was 33%, whereas that in dogs without *p53* mutation was 88%. By statistical analysis, dogs with *p53* mutation were shown to have significantly poorer response to chemotherapy ($P = .002$; Table 3). Moreover, the dogs with *p53* mutation had significantly shorter overall survival duration than dogs without *p53* mutation (Fig 2). In the present study, we showed mutations of *p53* in lymphomas in dogs, but did not examine the function of the mutated P53, thereby we cannot conclude that *p53* is the direct cause of chemoresistance in these cases. However, it has been shown that loss of P53 function decreases the sensitivity of tumor cells to chemotherapy in a variety of tumors in humans,[15–17] suggesting the association between P53 inactivation and chemoresistance.

Relation between *p53* mutation and clinical outcome has been evaluated in many tumors in human, indicating the association of *p53* mutation with poor prognosis.[18–21] In a study evaluating 75 people with relapsed/refractory NHL, those with *p53* mutations were significantly more likely to be drug-resistant (56%) than patients without *p53* mutation (17%).[18] Moreover, it has been shown that NHL patients with *p53* mutation also have significantly shorter overall and progression-free survival time.[18,47] In veterinary medicine, Dhaliwal et al[34] evaluated the expression of P53 using immunohistochemistry in 31 dogs with lymphoma. They reported 7 dogs (22%) to be positive for P53 expression and the expression of P53 was statistically correlated with survival.

Although Nasir and Argyle reported germ-line *p53* mutation in two Bull Mastiffs,[48] many of the *p53* gene found in tumors in dogs are considered to be acquired during tumoregenesis or antineoplastic therapies. There have been several studies suggesting that *p53* mutation is a late event in the tumor development in humans,[49,50] which means that the patients with *p53* mutations are expected to have a shorter survival than patients without *p53* mutations because of the time lag. It will be important to carry out further studies to explore the timing of the occurrence of *p53* mutation in lymphomas in dogs, which may reveal the association of the *p53* mutations with the biological behavior of the disease.

Previously, several studies revealed the prognostic factors of lymphoma in dogs including the substage of World Health Organization (WHO) staging system,[51] immunophenotype of the tumor cells,[52] tumor location,[53] histologic subtype,[54] presence of anemia,[55,56] inactivation or overexpression of p16,[57,58] and phosphorylation of Rb.[57] In this study, dogs with lymphomas showing *p53* mutation were shown to have lower overall response rate and shorter survival duration than those with lymphomas retaining wild-type p53. These results indicate that *p53* mutation can be recognized as a new prognostic factor for lymphoma in dogs.

Dogs in this study had diverse types of lymphoma which included different anatomic forms. Also, immunophenotype and histologic subtype were not determined in majority of the cases. It has been well documented that these variables are associated with the biologic behavior and clinical outcome in lymphoma in dogs[52–54] and having different types of lymphoma could have biased the findings of this study. However, *p53* gene mutation was seen in all anatomic forms in the present study. The number of dogs in each group was too small to make any correlation between the anatomic form and the incidence of *p53* gene mutation. Additional study with large number of cases with the same subtype is needed to confirm the precise relationship of *p53* gene mutation and prognosis in lymphoma in dogs and to assess any correlation between the incidence of *p53* gene mutation and certain subtypes.

In conclusion, *p53* mutation can be found in a proportion of untreated dogs with lymphoma and dogs having *p53* mutation were shown to have poorer response to chemotherapy and shorter survival time than those with lymphomas devoid of *p53* mutation. These results indicate that *p53* mutation can be used as a prognostic factor of lymphoma in dogs, and suggest that the worse prognosis in dogs with lymphomas having *p53* mutation might be attributable to drug resistance mediated by *p53* mutation. To explicate this fact, further studies to show the relation between loss of P53 function and clinical drug resistance is needed.

Footnotes

[a] QIAamp DNA Mini Kit, Qiagen, Hilden, Germany
[b] Applied Biosystems, Foster City, CA
[c] GeneGel Excel 12.5/24 Kit, Amersham Pharmacia, Buckinghamshire, England
[d] GenePhor DNA Separation System, Amersham Pharmacia
[e] DNA Silver Staining Kit, Amersham Pharmacia
[f] BigDye Terminator v1.1 Cycle Sequencing Kit, Applied Biosystems

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Conflict of Interest Declaration: Authors disclose no conflict of interest.

Off-label Antimicrobial Declaration: Authors declare no off-label use of antimicrobials.

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