Topic

Simultaneous Inactivation of the *p16*, *p15*, and *p14* Genes Encoding Cyclin-Dependent Kinase Inhibitors in Canine T-lymphoid tumor cells

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Abstract

The *p16*, *p15*, and *p14* genes are widely known as tumor suppressor genes in human medicine. Although a large number of genetic and epigenetic aberrations in these genes have been reported in human malignancies, canine malignancies have not been well analyzed on the aberrations of these genes. In this study, the full-length complementary DNA (cDNA) of the canine *p16* gene was cloned using the 5′ and 3′ rapid amplification of cDNA ends methods. Based on the sequence data, primers specific for *p16*, *p15*, and *p14* were designed. Using these primers, the expression of *p16*, *p15*, and *p14* mRNAs could be individually evaluated by reverse transcriptase polymerase chain reaction. Genomic aberrations were also examined using genomic polymerase chain reaction. Two of the 6 canine lymphoid tumor cell lines did not express detectable levels of *p16*, *p15*, and *p14* mRNAs, and wide-ranging deletions in the *p15-p14-p16* genomic locus were suspected. Wide-ranging deletions were also speculated in 2 of 14 dogs with T-cell lymphoid tumors. On the other hand, similar failure of amplification suggesting wide-ranging deletions were not observed in any of the 14 dogs with B-cell lymphoma. Deletion of the *p15-p14-p16* genomic locus could be one of the molecular aberrations in canine lymphoid tumor cells.

Key words：canine, lymphoid tumor, *p16*

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INTRODUCTION

The *p16*, *p15*, and *p14* genes are widely known as tumor suppressor genes in human medicine[8,11]. They are clustered in a region on human chromosome 9 corresponding to mouse chromosome 4, and canine chromosome 11[9,41]. The P16 and P15 proteins belong to the inhibitor of cyclin-dependent kinase 4 (INK4) family, and are known as INK4a (cyclin-dependent kinase inhibitor 2A [CDKN2A]) and INK4b (CDKN2b), respectively. By binding directly to CDK4 and CDK6, P16 and P15 block the formation of cyclin D-CDK complexes, resulting in the phosphorylation of retinoblastoma (RB) proteins and G1-phase cell cycle arrest. The *p14* gene, which is also known as *alternative reading frame* (*ARF*), regulates the cell cycle via an alternative pathway. By binding to the murine double minute 2 (MDM2) protein, P14 inhibits the degradation of P53 by MDM2. Because P53 induces G1-phase cell cycle arrest in conjunction with P21, the outcome of this pathway is similar to that of the P16 and P15 pathways. The human *p16* and *p14* genes are composed of 3 exons each, and are known to share common exons 2 and 3.

A large number of genetic and epigenetic aberrations of the *p16*, *p15*, and *p14* genes have been reported in human malignancies. Of these 3 genes, inactivation of *p16* has been most frequently found in hematological malignancies, such as lymphoma[2,3,5,6,10,16,17,20,24,37,45], leukemia[13,18,19,22,30,33,39,43], and multiple myeloma[25,32] as well as other non-hematological malignancies of various types[25,32]. Inactivation of *p16* results from 4 types of alterations, namely, homozygous deletion, promoter hypermethylation, loss of heterozygosity, and point mutations. Inactivation of *p16*, occurring mainly due to deletions

and methylations[8], was observed in 44% of hematological tumor patients[40]. Furthermore, deletions of the 3 genes occur more frequently in patients with T-cell malignancies than in those with B-cell malignancies[8]. By contrast, patients with B-cell malignancies exhibit a higher incidence of inhibition of expression due to hypermethylations[8].

Partial or whole sequence data of the *p16*, *p15*, and *p14* open reading frames have been reported. Four reports on the canine *p16* gene have been published[7,9,21,27] since the identification of the partial sequence of its exon 2 (GenBank accession no. FJ542309)[21]. In another study[1], the entire coding sequence of the canine *p15* gene (GenBank accession no. NM_00114629) and partial sequence of the canine *p14* gene (GenBank accession no. FM883643) were reported. However, the genomic locus harboring *p16*, *p15*, and *p14* has not been thoroughly examined in dogs. The predicted canine *p16*, *p15*, and *p14* gene locus, deduced from the corresponding loci in other species, is shown in Fig. 1. Canine *p16* and *p14* genes are thought to share common exons 2 and 3, with a unique alternatively spliced first exon. Further, the previously reported partial sequence of canine *p16* gene exon 2 exhibited high homology to the sequence of *p15* gene exon 2. Therefore, the primers designed to amplify exon 2 of the canine *p16* gene in previous studies[7,9,21,27] would also amplify the *p15* and *p14* genes. To examine the expression of *p16*, *p15*, and *p14* independently, it would be necessary to determine the whole sequence of exon 1 of the canine *p16* gene.

Using primers that would amplify not only *p16* but also *p15* and *p14*, 2 previous studies reported low or undetectable expression of these 3 genes in canine lymphoma[9,27]. Partial loss of chromosome 11, on which these genes are located, has also been reported in canine lymphoid malignancies using fluorescent in situ hybridization analysis and comparative genomic hybridization[9,12,41]. Based on these reports, the *p16*, *p15*, and *p14* genes are speculated to be partially or completely deleted in canine lymphoid malignancies, similar to human lymphoid tumors.

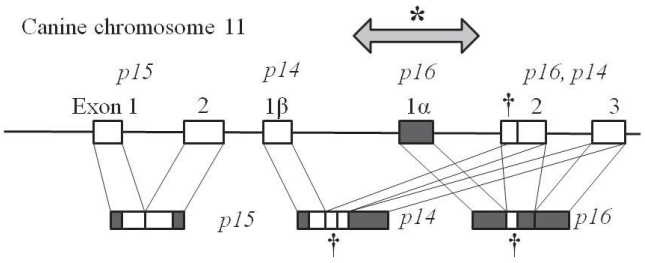

Fig. 1. Predicted gene locus of the *p16*, *p15*, and *p14* genes on canine chromosome 11 deduced from the gene locus on human chromosome 9. The identified and unidentified exons of *p16*, *p15*, and p14 are indicated by white and black squares, respectively. Arrow with asterisk (*) indicates a gap in canine genome database. Location of the primers in exon 2 of *p16* used in previous studies[21] are indicated by daggers (†).

The first purpose of this study was to determine the complete nucleotide sequence of the canine *p16* cDNA, in order to design primers specific to *p16*, *p15*, and *p14*. The second purpose was to examine the aberration of the *p16*, *p15*, and *p14* genes in the canine lymphoid tumor cell lines and primary tumor cells from dogs with various lymphoid tumors.

MATERIALS AND METHODS

Cells and patient samples: Six lymphoid tumor cell lines derived from dogs with naturally occurring lymphoid malignancies were examined. These included CLBL-1 (multicentric B-cell lymphoma)[35], GL-1 (B-cell acute lymphoblastic leukemia [ALL])[29], UL-1 (renal T-cell lymphoma)[44], CL-1 (mediastinal T-cell lymphoma)[28], Nody-1 (alimentary T-cell lymphoma)[15], and Ema (mediastinal T-cell lymphoma)[15]. Four of these cell lines (CLBL-1, GL-1, UL-1, and CL-1) were cultivated in RPMI-1640 (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (Biowest, Nuaillè, France), penicillin (100 units/ml), and streptomycin (0.1 mg/ml) (Sigma-Aldrich, St. Louis, MO). The other 2 cell lines (Nody-1, Ema) were cultivated in RPMI-1640 supplemented with 20% fetal bovine serum (Nichirei Biosciences Inc., Tokyo, Japan), penicillin (100 units/ml), and streptomycin (0.1 mg/ml). All cell lines were maintained in an atmosphere of 5 % CO_2.

Lymph node (LN) cells were obtained as a control from a healthy dog kept for blood transfusion in our teaching hospital via fine-needle aspiration (FNA). The dog was kept in accordance with the guidelines of Animal Care Committee of the Graduate School of Agricultural and Life Sciences, the University of Tokyo.

Dogs with lymphoid tumors were cytologically or pathologically diagnosed. Furthermore, clonal rearrangement of the *immunoglobulin heavy chain* (*IgH*) gene or the *T-cell receptor gamma chain* (*TCR*γ) gene in tumor cells confirmed using previously described primers[4,42] was examined to indicate their clonal origin and B- or T-cell linage. Fourteen dogs (dogs nos. 1–14) with IgH rearrangement were included and all were diagnosed

with multicentric high-grade lymphoma. Primary tumor cell samples were obtained from the LN of these dogs by FNA. Of the other 14 dogs (dogs nos. 15–28) showing *TCR*γ rearrangement, 11 dogs were diagnosed as having alimentary lymphoma, 1 with ALL, and 2 with chronic lymphocytic leukemia (CLL). Primary tumor cell samples were obtained by endoscopy from dogs with the alimentary lymphoma, and by peripheral blood mononuclear cell (PBMC) separation by gradient centrifugation[44] from the dogs with ALL and CLL. All primary tumor samples were obtained with owners' informed consent.

Full-length cloning of canine p16 complementary DNA: Total RNA was isolated using Illustra RNA Spin Mini (GE Healthcare, Little Chalfont, Buckinghamshire, UK), followed by DNase I (Invitrogen) treatment. Complimentary DNA (cDNA) was synthesized using PrimeScript® RT reagent Kit (TakaraBio, Shiga, Japan). All protocols were performed in accordance with the manufacturer's instructions. To amplify a part of exon 1 of canine *p16*, we designed degenerative primers, p16 EXON-F94 and p16 EXON-R293 (Table 1), based on the sequence of *p16* exon 1 from other species (human, chimpanzee, cattle, mouse, and rat). PCR amplification was performed with AmpliTaq gold® 360 (Applied Biosystems, Grand Island, NY) using normal LN cDNA as template. Cycle conditions included an initial denaturation step at 95℃ for 10 min, followed by 35 cycles of denaturation at 95℃ for 3 s, annealing at 68℃ for 30 s, and extension at 72℃ for 60 s, with a final extension step at 72 ℃ for 7 min. The PCR amplification products were electrophoresed on 2% agarose gel (BMBio, Tokyo, Japan), purified using Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI), and inserted into the pGEM®–T Easy Vector System (Promega). After transformation, plasmid DNA was extracted using NucleoSpin® Plasmid QuickPure (Macherey-Nagel, Düren, Germany) and subjected to sequence analysis using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and 3130xl Genetic Analyzer (Applied Biosystems).

The 5′ Rapid amplification of cDNA ends (5′ RACE) method was used to determine the full-length sequence of exon 1 of the *p16* gene using 5′-full RACE Core Set (TakaraBio). CL-1 cDNA template was generated using the p16 EXON2-R1481 primer phosphorylated at the 5′ end. After circularization using T4 ligase, the cDNA was amplified by nested-PCR using 4 primers: p16 EXON2-F1374 and p16EXON2-F1411, and p16 EXON 1-R169 and p16 EXON 1-R146 (Table 1). Three primers, p16

Table 1. Primer sequences used for full-length complementary DNA cloning and expression analysis of the *p16* gene in this study

Primer name	Primer sequence (5'-3')	Genbank no., nucleotide number	Amplicon size (bp)
p16 EXON-F94	GASGTGCGGGCRCTGCTSG	AB675384, nt. 94-112	200
p16 EXON-R293	GTGTCCAGGAAGCCCTCCCG	AB675384, nt. 293-274	
p16 EXON2-R1481	P-CAACTCAGATGTGGC	FM883643, nt. 1481-1466	NA
p16 EXON1-R169	ATGACCTGAATCGGGCTCCGA	AB675384, nt. 169-150	
p16 EXON1-R146	AGGCGGTTCGGCGCGTCGGGA	AB675384, nt. 146-126	
p16 EXON2-F1374	GCTGTCGCTGCGTACCTGCG	FM883643, nt. 1374-1385	
p16 EXON2-F1411	CCGAAAGTGGTAGCCACGCC	FM883643, nt. 1411-1430	
p16 EXON2-F153	GAGCCCGATTCAGGTCAT	AB675384, nt. 153-170	
p16 EXON1-F148	GGTCGGAGCCCGATTCA	AB675384, nt. 148-169	95
p16 EXON1-R242	ACGGGGTCGGCACAGTT	AB675384, nt. 242-226	
RPL13A-F	GCCGGAAGGTTGTAGTCGT	AJ388525, nt. 87-105	87
RPL13A-R	GGAGGAAGGCCAGGTAATTC	AJ388525, nt. 173-154	
p15 EXON1-F90	GCGGCAGCTCCTGGAAG	NM_00114629, nt. 90-106	140
p15 EXON1-R230	GGGTCGGCACAGTTGG	NM_00114629, nt. 230-215	
p14 EXON1-F330	GCCCTGGTGCTAAAGCTAGT	FM883643, nt. 330-349	154
p14 EXON1-R1230	TGACGGGGTCGGCACAGTT	FM883643, nt. 1230-1212	
GAPDH-F	TCACCAGGGCTGCTTTTAAC	AB038240, nt. 47-67	403
GAPDH-R	AGGAGGCATTGCTGACAATC	AB038240, nt. 448-429	

GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; NA, not applicable (primers used for 5' and 3' RACE); P, Phosphate group; R represents A or G, S represents C or G

EXON2-R1481, p16 EXON2-F1374, and p16 EXON-2F1411, were designed on the basis of the exon 2 sequences of the *p16/p14* genes in GenBank (GenBank accession no. FM883643)；the other 2 primers, p16 EXON1-R169 and p16 EXON1-R146, were designed on the basis of the partial sequence of *p16* exon 1 determined in this study (fragment amplified with p16 EXON-F94 and p16 EXON-R293 primers) (GenBank accession no. AB675384). Both the first and second PCR amplifications were performed using Takara LA Taq with GC Buffer (TakaraBio). Cycle conditions included an initial denaturation step at 94℃ for 1 min, followed by 25 cycles of denaturation at 94 ℃ for 30 s, annealing at 62℃ for 30 s, and extension at 72℃ for 60 s, with a final extension step at 72℃ for 5 min. The PCR product was cloned and sequenced as described above.

The 3′ RACE method was used to determine the universal exon 3 of the *p16/p14* genes using 3′-full RACE Core Set (TakaraBio). A primer, p16 EXON2-F153, was designed on exon 2 of the *p16/p14* genes (Table 1). The first strand of cDNA was synthesized from CL-1 RNA using oligo-dT primer containing an adaptor sequence. Subsequent PCR amplification was performed with the p16 EXON2-F153 primer and a supplied primer complementary to the adaptor sequence using AmpliTaq gold® 360. The protocol consisted of an initial denaturation step at 95℃ for 10 min, followed by 35 cycles of denaturation at 95℃ for 3 s, annealing at 60℃ for 35 s, and extension at 72℃ for 60 s, with a final extension step at 72℃ for 7 min. The PCR amplification product was cloned and sequenced as described above.

Real-time PCR analysis of p16 expression in canine lymphoid tumor cell lines: The primers *p16* EXON1-F148 and *p16* EXON1-R242 were designed to amplify 95 base pairs (bp) of *p16* exon 1 based on the *p16* sequence determined in this study (Table 1). The mRNA expression in each cell line was measured by real-time PCR using SYBR® Premix Ex Taq (TakaraBio). *Ribosomal protein L13A* (*RPL13A*) was used as the endogenous control (RPL13A F and RPL13A R；amplicon length 87 bp, Table 1). The cycle protocol was as follows：denaturation step at 95℃ for 10 s；40 cycles of denaturation at 95℃ for 5 s, and annealing/extension at 60 ℃ for 30 s；followed by the dissociation step at 95℃ for 5 s, 60℃ for 30 s, and 95 ℃ for 15 s. The PCR amplicons were electrophoresed on 12.5% polyacrylamide gel (ATTO, Tokyo, Japan).

The comparative cycle threshold (Ct) method was used to quantify *p16* transcript levels. ΔCt was determined by subtracting the Ct value of *RPL13A* from the Ct value of the *p16* gene. ΔΔCt was calculated by subtracting the ΔCt of the control sample from the ΔCt of the target sample. Normal LN was used as the control sample in this study. The levels of the *p16* transcripts relative to that in the normal LN was calculated as $2^{-\Delta\Delta Ct}$. To confirm the amplification efficacy of the primers, a standard curve was generated using serial dilutions of the CL-1 cDNA as template. All samples were evaluated in triplicate.

Expression analysis of the p15 and p14 genes in canine lymphoid tumor cell lines: Primers specific for *p15*, p15 EXON1-F90 and p15 EXON1-R230, and *p14*, p14 EXON1-F330 and p14 EXON1-R1230, were designed based on the sequences of *p15* and *p14* registered in GenBank (accession nos. NM_00114629 and FM883643), respectively (Table 1). GAPDH was used as the endogenous control gene (primers GAPDH-F and GAPDH-R；Table 1). Reverse transcriptase-polymerase chain reaction (RT-PCR) amplification was performed for the *p15* and *p14* genes using AmpliTaq gold® 360 and the cDNAs of the cell lines as templates in the following protocol：initial denaturation at 95℃ for 10 min, followed by 35 cycles of denaturation at 95℃ for 3 s, annealing at 60℃ for 30 s, and extension at 72℃ for 60 s, with a final extension step at 72℃ for 7 min. The PCR amplification products were electrophoresed on 12.5% polyacrylamide gel.

Amplification of the p15-p14-p16 gene locus on chromosome 11 by genomic PCR: Genomic DNA was extracted using QIAmp DNA Mini kit (Qiagen, Hilden, Germany). Eight pairs of primers (Table 2) covering a 100-kb region of canine chromosome 11, including the introns and exons of the *p16*, *p15*, and *p14* genes, were designed (Fig. 2). The primer sequences are listed in Table 2. The *GAPDH* gene was used as the endogenous control. PCR amplification was performed using AmpliTaq gold® 360 and the DNAs of cell lines and patient samples as templates. The PCR protocol was as follows：initial denaturation at 95℃ for 10 min, followed by 35 cycles with denaturation at 95℃ for 3 s, annealing at 60℃ for 30 s, and extension at 72℃ for 60 s, with a final extension step at 72 ℃ for 7 min. Electrophoresis was performed on 2% agarose gel. Amplification using distilled water was regarded as negative control.

RESULTS

F*ull-length cloning of canine p16 cDNA:* Using 5′ RACE and 3′ RACE methods, the nucleotide sequence of full-length canine *p16* cDNA was determined. A 705 – bp entire open reading frame encoding 151 amino acids could be identified (GenBank accession no. AB675384,

Table 2. Primer sequences used to amplify the *p15-p14-p16* genomic locus in this study

Primer name	Primer sequence (5'-3')	Genbank no., nucleotide number	Amplicon size (bp)
p15 usEXON1-F1	TGCCCTAACATTAATTTCTTCTCAG	AAEX02016331.1, nt. 54947-54971	399
p15 usEXON1-R1	GACCTTTGACCAGAATGAAGAGATA	AAEX02016331.1, nt. 54777-54753	
p15 usEXON1-F2	GGACATGTAATTTGAACCCAGATAG	AAEX02016331.1, nt. 44716-44740	303
p15 usEXON1-R2	TCCTAATCTCCCTTCCTAGACACTT	AAEX02016331.1, nt. 44462-44438	
p16 dsEXON3-F	CACCTTGTGGGAACTCTAGAAAGTA	AAEX02016329.1, nt. 1182-1206	529
p16 dsEXON3-R	TATTCAAAAGGGGTATTGGTCTGTA	AAEX02016329.1, nt. 802-778	
p15 usEXON1-F	GAAGAGGGCTGAAAGCTGACCT	AAEX02016331.1, nt. 13506-13522	561
p15 dsEXON1-R	CCAAACTTAAGTAGCGTTTGTTGTT	AAEX02016331.1, nt. 12984-12962	
p15 usEXON2-F	ATCAACAAAGTATAACGGACTCCTG	AAEX02016331.1, nt. 10872-10896	500
p15 dsEXON2-R	ACATTTGTAGCCTCCCTTCAACT	AAEX02016331.1, nt. 10169-10146	
p16 dsEXON1-F	CTCTCAAAATCCCTTCACACTTAAA	AAEX02016329.1, nt. 9417-9441	529
p16 dsEXON1-R	CTACCATTTTGAGGGTACCTCCTAT	AAEX02016329.1, nt. 8937-8913	
p16 dsEXON2-F	GTCATGATGATGGGCAGCACCCGCGT	AAEX02016329.1, nt. 7139-7164	1093
p16 dsEXON2-R	AATGGTTCTCCCTTCAGAAAAAG	AAEX02016329.1, nt. 6095-6072	
p16 usEXON3-F	TGAGGTGCCACATCTGAGTT	AAEX02016329.1, nt. 6831-6856	2600
p16 usEXON3-R	CAAGCATGCAGGGAAGAGTT	AAEX02016329.1, nt. 4555-4534	
GAPDHgen-F	TCACCAGGGCTGCTTTTAAC	AB038240, nt. 47-67	818
GAPDHgen-R	AGGAGGCATTGCTGACAATC	AB038240, nt. 448-429	

GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; R, Reverse

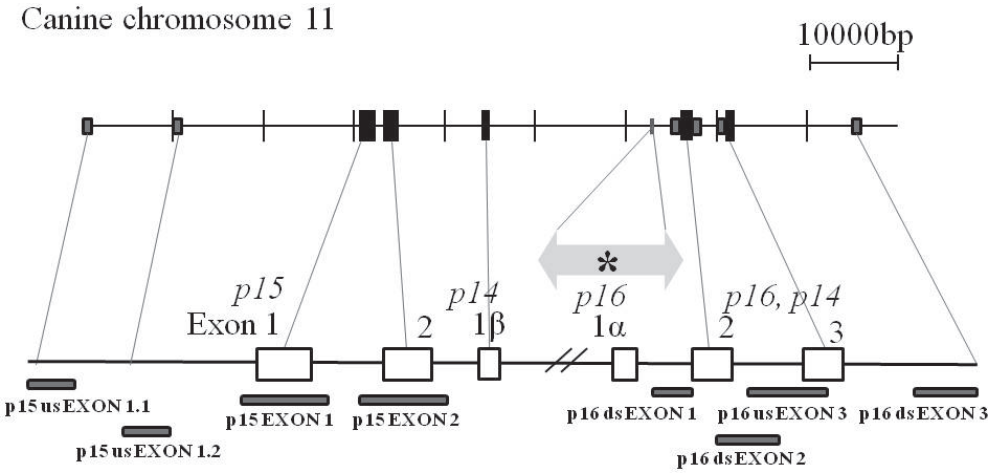

Fig. 2. Locations for the genomic PCR amplification on the *p15-p14-p16* gene locus. Arrow with asterisk (*) indicates a gap in the canine genome database. Black bars at the bottom represent the regions amplified by 8 primer pairs.

nucleotide [nt] 40–492). In the dog genome database, nt no. 166–472 and nt no. 473–705 of canine *p16* cDNA were found on canine chromosome 11 separately using BLAST (National Center for Biotechnology Information, Bethesda, MD) (nt no.166-472, 32195005–32195311 ; nt no. 473–705, 32192558–32192790). However, no sequence corresponding to the 5′ fragment of canine *p16* (nt no. 1–165) was found in the dog genome database. Because there is a gap region between exon 1β of the *p14* gene and exon 2 of the *p16/p14* genes where its exon 1α is expected to be locate (Fig. 1, 2) in the dog genome database, the 5′ fragment of canine *p16* (nt no. 1–165) was expected to be locate in this region. Nt no. 1–165 of canine *p16* was considered to be its exon 1α, and nt no. 166–472 and nt no. 473–705 were regarded as its exons 2 and 3 from the result of the alignment of the sequence (nt no.166–705) of canine *p16* cDNA using BLAST. Therefore, canine *p16* gene was assumed to be composed of 3 exons.

The determined full-length cDNA sequence and the deduced amino acid sequence of canine *p16* were compared with those of other species. The cDNA sequence of the coding region shared 85%, 86%, 86%, 79%, and 76% identities with human, chimpanzee, cattle, mouse, and rat sequences, respectively. The deduced amino acid sequence of canine *p16* was 81%, 81%, 82%, 61%, and 76% homologous to that of human, chimpanzee, cattle, mouse, and rat, respectively (Fig. 3). Exons 1 and 2 of canine *p16* were shown to contain 4 ankyrin repeats highly conserved among various species[34].

Expression analysis of p16, p15, and p14 genes using real-time RT-PCR or RT-PCR: The levels of *p16* mRNA were determined using real-time RT-PCR. The levels of *p16* mRNA in CLBL-1 and GL-1 cells were lower than that in normal LN cells, and below the limit of detection in UL-1, Nody-1, and Ema cells. The amount of *p16* mRNA in CL-1 was approximately 100 fold higher than that in normal LN cells (Fig. 4). Further, the levels of *p15* and *p14* expression were examined using RT-PCR. The levels of *p15* and *p14* mRNAs were uniformly high in 4 cell lines (CLBL-1, GL-1, UL-1, and CL-1) compared to that in normal LN cells. On the other hand, expression of *p15* and *p14* mRNAs was below the limit of detection in Nody-1 and Ema cells (Fig. 5). Amplification was not observed in these 2 cell lines even when the number of PCR cycles was increased from 35 to 40 (data not shown).

Amplification of the p15-p14-p16 gene locus by genomic PCR: To examine deletion in the genomic locus of *p16*, *p15*, and *p14* genes, we designed 8 primer pairs to amplify each region of the locus (Fig. 2). All the 8 primer pairs designed in the locus amplified the fragments of expected sizes in 4 cell lines (CLBL-1, GL-1, UL-1, and

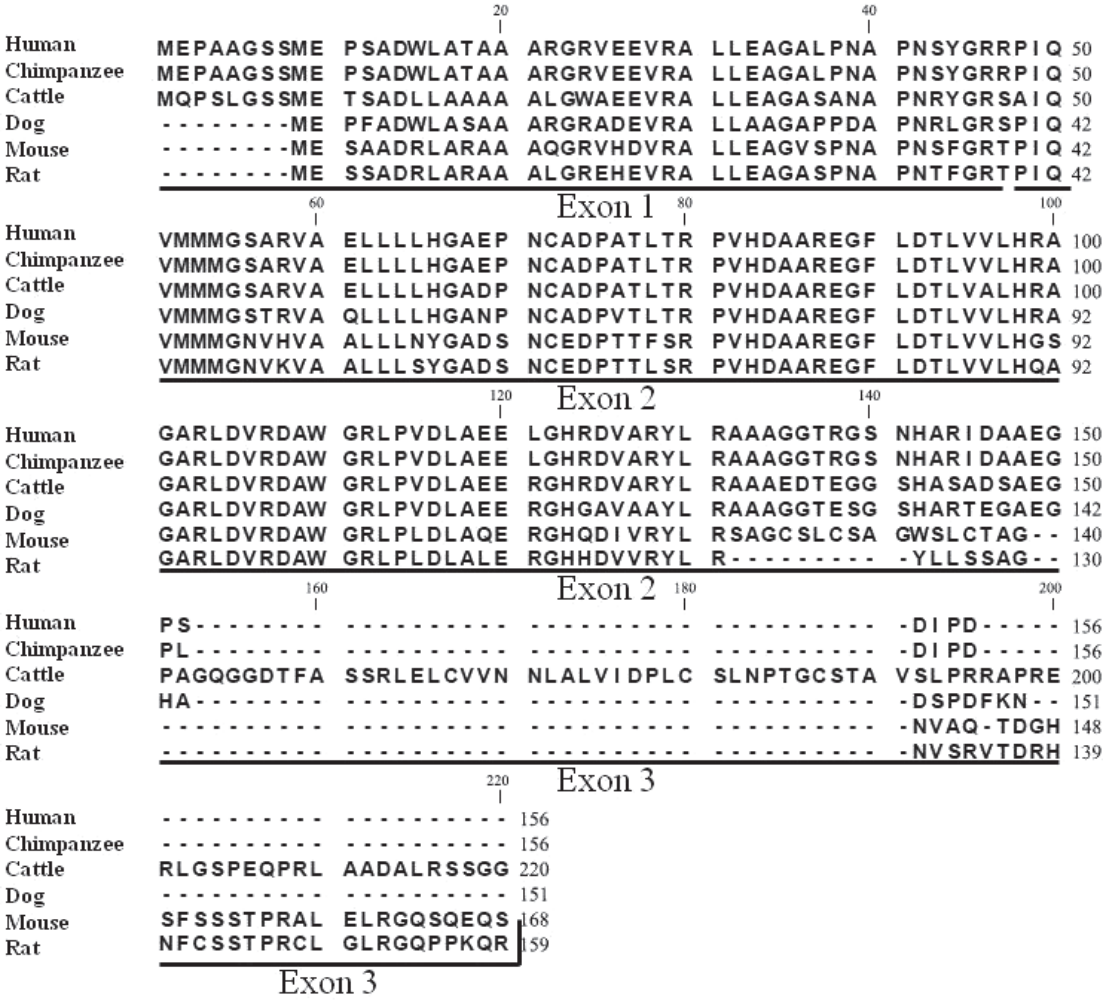

Fig. 3. Amino acid sequence comparison of the deduced canine P16 with P16 of human (GenBank accession no. NM_000077.3), chimpanzee (GenBank accession no. NM_001146290.1), cattle (GenBank accession no. XM_868375.4), mouse (GenBank accession no. NM_009877.2), and rat (GenBank accession no. NM_031550). The corresponding exons are indicated below the sequence. Dash denotes gap.

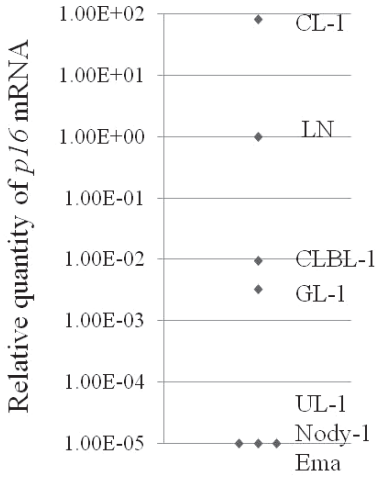

Fig. 4. Expression analysis of the *p16* gene in canine lymphoid tumor cell lines and a lymph node tissue using real-time polymerase chain reaction (RT-PCR). Levels of *p16* mRNA were measured relative to that in normal LN tissue as a control sample. The expression of *p16* mRNA is below the detection limit in UL-1, Nody-1, and Ema. Data are expressed as the mean value of triplicate assay.

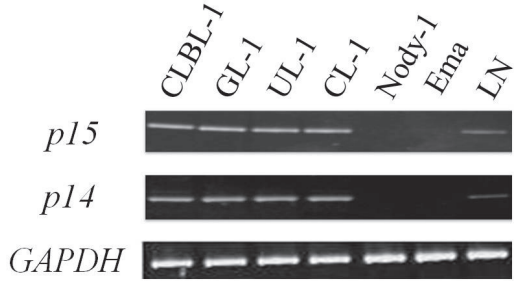

Fig. 5. Expression analysis of the *p15* and *p14* genes in canine lymphoid tumor cell lines and a lymph node tissue using RT-PCR. Thirty-five cycles of PCR reaction was performed for each gene. *GAPDH* (*glyceraldehyde- 3 -phosphate dehydrogenase*) was used as a control.

CL-1) and LN cells, whereas no amplification was observed with any of the 8 primer pairs in Nody-1 and Ema cells (Fig. 6).

Primary lymphoid tumor samples were also examined by genomic PCR. Due to limited DNA samples from lymphoid tumor patients, 5 primer pairs covering the exon regions of *p16*, *p15*, and *p14* were used to examine each gene loci. Faint bands were presumed to arise from non-tumor cells derived from the surrounding normal tissues.

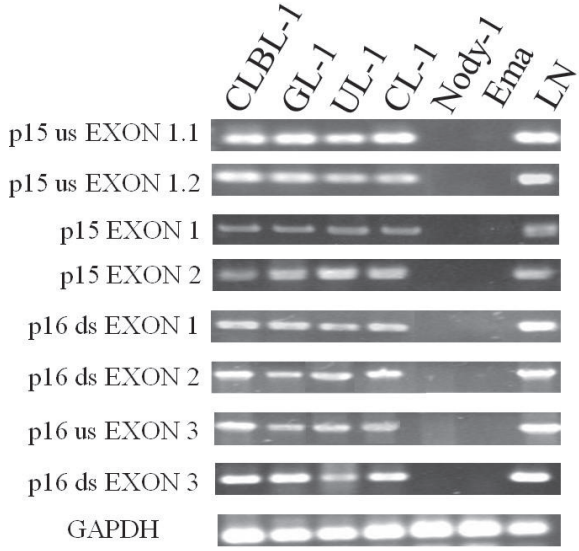

Fig. 6. Genomic PCR amplification of regions corresponding to the *p15-p14-p16* gene locus on chromosome 11. The location of each region is shown in Fig. 2. *GAPDH* gene was used as a control.

The 14 dogs diagnosed with multicentric high-grade lymphoma and harboring clonal IgH gene rearrangements all exhibited amplifications products of the expected sizes in the regions p15 EXON1, p16 dsEXON2, and p16 usEXON3 (Fig. 7). Amplified bands corresponding to p15 EXON2 and p16 dsEXON1 were faint to absent in 2 (dog nos. 4 and 9) and 1 (dog no. 7) of the 14 dogs, respectively. Eleven dogs showed successful amplifications with all the 5 primer pairs.

Among the 14 dogs with clonal *TCRγ* gene rearrangements (alimentary lymphoma, ALL, and CLL), amplifications of region p15 EXON1, p15 EXON2, p16 dsEXON1, p16 dsEXON2, and p16 usEXON3 were faint to absent in 2 (dog nos. 15 and 23), 3 (dog nos. 15, 18, and 23), 5 (dog nos. 15, 18, 19, 20, and 23), 2 (dog nos. 15, and 23), and 2 (dog nos. 15, and 23) dogs, respectively (Fig. 7). In 2 dogs (dog nos. 15 and 23), no or faint bands were detected with any of the 5 primer pairs. The results of genomic PCR in primary lymphoid tumor samples were summarized in Table 3.

DISCUSSION

In this study, we identified the full-length nucleotide sequence of the canine *p16* gene. As expected, canine *p16* was composed of 3 exons, sharing exons 2 and 3 with the *p14* gene. The deduced canine *p16* amino acid sequence was highly homologous to that of human, chimpanzee, and cattle throughout the coding region. On the

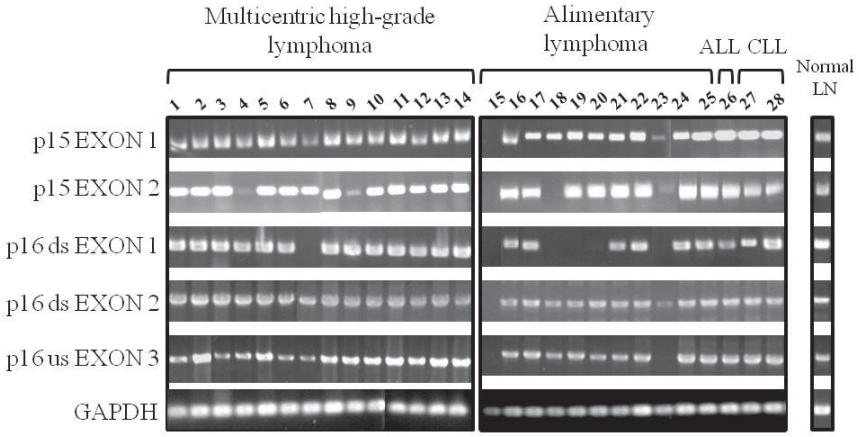

Fig. 7. Genomic PCR amplification for the *p15-p14-p16* gene locus in 28 canine primary tumor samples. The location of each region is shown in Figure 2. Band intensities fainter than that of normal LN were considered as absence of amplification. Dogs 1–14 were diagnosed as having multicentric high-grade lymphoma with clonal *immunoglobulin heavy chain* gene rearrangement. Dogs 15–25 were diagnosed with alimentary lymphoma, dog 26 with acute lymphoblastic leukemia (ALL), and dogs 27 and 28 with chronic lymphocytic leukemia (CLL). Dogs 15–28 exhibited clonal *T-cell receptor gamma chain* gene rearrangements. Genomic DNA purified from normal LN tissue and peripheral blood mononuclear cell (PBMC) was used as controls. The result of PBMC was similar to that of LN (data not shown). Amplification using distilled water was regarded as negative control.

Table 3. Results of genomic PCR to amplify the *p15-p14-p16* locus in primary lymphoid tumor samples

Dogs No.	Multicentric high-grade lymphoma														Alimentary lymphoma										ALL	CLL	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
p15 EXON 1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	±	+	+	+	+	+
p15 EXON 2	+	+	+	±	+	+	+	+	±	+	+	+	+	+	−	+	+	−	+	+	+	+	±	+	+	+	+	+
p16 ds EXON 1	+	+	+	+	+	+	−	+	+	+	+	+	+	+	−	+	+	−	−	±	+	+	−	+	+	+	+	+
p16 ds EXON 2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	±	+	+	+	+	+
p16 us EXON 3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	−	+	+	+	+	+

ALL, acute lymphoblastic leukemia ; CLL, chronic lymphocytic leukemia ; +, distinct band ; ±, faint band ; −, no band

other hand, the exon 3 sequence exhibited limited identity with that of mouse and rat, although exons 1 and 2 were relatively conserved. Exons 1 and 2 contained 4 ankyrin repeats, which are known to be highly conserved among various species[34]. The ankyrin repeat motif may be important for maintaining the functional conformation of P16.

The cloning of *p16* exon 1 enabled us to design primers that distinguished *p16* from *p15* and *p14*. Although exon 2 of *p15* and *p16/p14* were highly homologous, exon 1 of each gene shared only limited identity, and therefore it was possible to design primers specific to each gene. However, the primers specific for *p15* and *p14* generated several non-specific products, and could not be used for quantification. It was difficult to design another primer for *p15* because of the high homology of its exon 2 with that of the *p16/p14* genes (98%). Further, polymorphism in exon 1 of the *p15* gene was reported in normal dogs[1]. Indeed, the reported polymorphism in *p15* exon 1 was also observed in CLBL-1, GL-1, UL-1, CL-1, and LN tissues by sequence analysis (data not shown). Therefore, we did not quantify *p15* expression by real-time RT-PCR.

In this study, a part of the *p16* nucleotide sequence (GenBank accession no. AB675384, nt 1–91) was obtained from CL-1 cells but not confirmed in normal cells. Because *p16* expression was low in normal cells[39,46], it was difficult to obtain a clear band from normal tissue by RT-PCR and 5′ RACE. Instead, CL-1 cDNA was used as a template for PCR because this cell line exhibited remarkably high expression of the *p16* gene. Further, it was not possible to verify the sequence from the genome data-

base because *p16* exon 1α is located in the gap region. The remaining part of the *p16* gene (nt 92–705) was identical between normal LN and CL-1 cells. Although the sequence of 5′ end of canine *p16* cDNA should be verified in normal LN cells, the data gathered in this study conceivably reflected the sequence of canine normal *p16* cDNA.

The amount of *p16* mRNA expression was smaller in 5 (B-cell lines ; CLBL-1 and GL-1, and 3 T-cell lines ; UL-1, Nody-1, and Ema) of the 6 canine lymphoid tumor cell lines, compared to that in normal LN cells. *p15* and *p14* mRNAs were also undetectable in 2 lymphoid tumor cell lines (Nody-1 and Ema). On the other hand, *p16* mRNA was highly expressed in CL-1 (T-cell line). It has been reported that RB protein becomes inactivated when it is excessively phosphorylated, which elicits high expression of *p16* mRNA due to a feedback mechanism[14,26,31]. Examination of the phosphorylation status of RB in CL-1 cells will be needed. Furthermore, when some of the cell cycle regulators such as p21, p27, p57, and p53 are inactivated and the progression of cell cycle occurs, over expression of *p16* mRNA could be induced as a feedback mechanism. Since mediastinal lymphoma is very rare in dogs, we did not have an opportunity to examine the *p16* mRNA expression level in primary tumor samples from dogs with mediastinal lymphoma. In previous studies on the prognosis of canine lymphoma[38], the presence of mediastinal mass was shown as a poor prognostic factor. Further study is needed to examine the relationship between the augmented *p16* expression and the disease type or the biologic behavior of lymphoid tumors in dogs.

In Nody-1 and Ema, *p16*, *p15*, and *p14* mRNAs were not detected. Furthermore, genomic PCR amplification for the *p15-p14-p16* locus of the DNA samples with the 8 primer pairs did not generate any product in locus in Nody-1 and Ema. These results suggest the presence of wide-ranging deletions in chromosome 11 in Nody-1 and Ema. To confirm the exact status of the genetic deletions in these 2 cell lines, Southern blot analysis would be necessary. In the cells with such wide-ranging deletion, there should be a simultaneous loss of *p16*, *p15*, and *p14* resulting in the failure of cell cycle control. Inactivation of these genes, especially in their combinations, was implicated in the tumorigenesis and malignancy from the studies of the in vitro and in vivo mouse model systems with targeted disruption of the corresponding genes[23]. Induction of wild-type *p16* into *p16*-deficient human lymphoma cell lines leads to growth retardation and partial differentiation, indicating that *p16* deficiency might contribute to the malignant phenotype[23]. Mice with targeted disruption of both *p16*/p19 loci developed lymphomas and lymphoid leukemias with a low penetrance, as well as other tumors[36] From these findings, the simultaneous inactivation of *p16*, *p15*, and *p14* genes would be also associated with the tumorigenesis and malignancy in canine T-lymphoid tumor cells.

The amounts of *p16* mRNA in CLBL-1, GL-1, and UL-1 were shown to be lower than that in normal LN, whereas those of *p15* and *p14* mRNAs in these cell lines were similar to that in normal LN. If only the amount of *p16* mRNA is decreased despite the presence of *p15* and *p14* mRNAs as shown in normal LN, there is a possibility that deletion around *p16* gene exon 1 exists without wide-ranging deletion over *p15-p14-p16* locus. However, PCR amplification for the *p16* gene exon 1 generated a distinct band in these 3 cell lines using genomic PCR. It is possible that genetic aberrations exist in *p16* gene although not detected in this study, or epigenetic mechanism to suppress its expression might be related in these 3 cell lines.

We further examined whether genetic aberrations similar to that in Nody-1 and Ema exist in naturally occurring canine lymphoid tumor cases. Similar failure of amplification of the *p15-p14-p16* locus (p15EXON1, p15EXON2, p16dsEXON1, p16dsEXON2, and p16usEXON3) was observed in 2 dogs (dog nos. 15 and 23) with T-cell alimentary lymphoma, although several faint bands conceivably derived from concomitant normal cells were observed in dog no. 23. Such failure of amplification was not observed in any of the 14 multicentric B-cell lymphoma dogs.

Thomas et al.[41] indicated the frequent DNA copy number losses in *p15-p14-p16* locus of chromosome 11 in canine T-cell lymphomas but not in B-cell malignancies, using array-based comparative genomic hybridization. Although further studies are needed, deletion of *p15-p14-p16* locus would be an important candidate to understand the tumorigenesis in a subtype (possibly high-grade T-cell lymphoma) of canine lymphoid tumors.

Failure of amplification suggesting wide-ranging deletion of *p15-p14-p16* locus was observed in 2 canine lymphoid tumor cell lines, Nody-1 and Ema. Nody-1 was established from tumor cells obtained from a patient with alimentary lymphoma 15). Furthermore, similar failure of amplification was observed in 2 dogs (dog nos. 15 and 23) with alimentary lymphoma. Thus, such aberration of *p15-p14-p16* locus might be a type of genetic changes observed in canine alimentary lymphoma.

On the other hand, 6 of 28 primary lymphoid tumor samples exhibited faint amplification failure with only 1

or 2 of the 8 primer pairs (B-cell lymphoid tumor nos. 4, 7 and 9 ; T-cell lymphoid tumor nos. 18, 19, and 20) in this study, suggesting a different genetic aberration than in the tumor nos. 15 and 23. Failures of amplification could occur if mutation, single nucleotide polymorphisms at the primer-binding region, or heterozygous genetic deletion is present. Southern blotting or array comparative genomic hybridization would be required to reveal the cause of the failure of amplification.

In conclusion, the full-length canine *p16* cDNA was cloned and the levels of *p16*, *p15*, and *p14* expression in canine lymphoid tumor cell lines were determined. Failure of amplification suggesting wide-ranging deletions were observed in 2 canine T-cell lymphoid tumor cell lines lacking *p16*, *p15*, and *p14* expression. Similar failure of amplification was also found in 2 primary tumor samples from alimentary T-cell lymphoma. Deletion of the *p15-p14-p16* genomic locus could be one of the molecular aberrations in canine lymphoid tumor cells.

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