Molecular Information of the Canine *FHIT* Gene and Its Aberrations in Canine Lymphoma Cell Lines

(犬の*FHIT*遺伝子の分子情報とリンパ腫細胞株におけるその異常)

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CONTENTS

GENERAL INTRODUCTION

In the small animal veterinary medicine, many types of tumors have developed as in humans, and the hematopoietic malignancies are one of the most common diseases in the clinic. However, myelodysplastic syndromes (MDSs) are extremely rare disease in dogs. MDSs are characterized by prominent dysplastic feature in the multiple cell lines of the blood and bone marrow, and suggested the induction of the genetic alterations in hematopoietic stem cells [Weiss et al., 2000; Blue, 2003; Weiss, 2003]. In the human MDSs, some genetic abnormalities have been reported including some chromosomal deletions such as chromosomes 5, 7, 11, 12, 13 and 20, and some aberrations of tumor associated genes including Ras, p53 and p15 [Hirai, 2003]. However, the genetic etiology has not been clarified in the dog.

The common fragile sites (CFSs) have been identified as genomic unstable regions that tend to form gaps and breaks on the metaphase chromosomes when the cells are exposed to replication impedance, such as by aphidicolin, 5-azacytidine and bromo-deoxyuridine [Glover, 2006; Smith et al., 2007]. In the CFSs, a FRA3B (3p14.2) region is one of the most frequently affected regions, which is extended for 4 M base pairs (bp) in the human

chromosome 3, and allelic loss or homozygous deletions in the region have been reported in many types of cancers including in lung, renal, esophageal, breast, and cervical tumors [Glover et al., 2006; Smith et al., 2007]. Regardless of the unstable region, some genes are located in FRA3B region, and in particular the *FHIT* gene has been well investigated [Iliopoulos et al., 2006; Smith et al., 2007].

Aberrant *FHIT* gene expression and the down-regulation of its protein, Fhit, expression have been reported in many types of human solid tumors including lung, cervical, esophageal, and breast cancers, and hematopoietic malignancies including myelocytic leukemia, lymphocytic leukemia, and myelodysplastic syndrome [Huebner et al., 2003; Peters et al., 1999; Brenner et al., 2002]. It was also identified that *FHIT* -/- and +/- mice developed spontaneous or N-nitrosomethylbenzylamine (NMBA)-inducing tumors [Fong et al., 2000; Zanesi et al., 2001]. Furthermore, the decreased expression of Fhit has also been described to correlate with the clinical poor prognosis in some human cancers [Gayther et al., 1997; Virmani et al., 2001; Arun et al., 2005; Guerin et al., 2006]. These facts may provide supportive evidence that the *FHIT* gene is regarded as the tumor-associated

gene.

In dogs, many types of tumors have developed in the same manner as in humans. As same as in a human cancer, the *FHIT* gene may also be prospective for playing the "key" gene of the cancer development in the dog. However, the role of the *FHIT* gene and Fhit protein has not been explored in canine tumors. Recently, a canine whole genome sequence was clarified by the Whitehead Institute/ MIT Center for Genome Research (WICGR) [Lindblad-Toh et al., 2005] and disclosed in the NCBI genomic databank. However, the canine *FHIT* gene has not been registered.

From these backgrounds, a series of the study was carried out to understand the genetic abnormalities in canine hematopoietic tumors. In chapter 1, a clinical case report of the canine MDS, which had been my motive throughout this study were introduced. In chapter 2, I clarified the basic information of canine *FHIT* gene structure and its protein expression. Finally, in chapter 3, I explored the aberrations of the *FHIT* gene and its protein expression in canine lymphoma cell lines, since canine lymphoma is one of the most common hematopoietic tumors in dogs, and some canine lymphoma cell lines could be available for *in vitro* studies.

CHAPTER 1

A dog with myelodysplastic syndrome

: chronic myelomonocytic leukemia

Summary

An eleven-year-old female pug was referred to Yamaguchi University Animal Hospital for evaluation of anemia and thrombocytopenia. The cytological examination of the peripheral blood showed some giant monocytic lineage blast cells. A few granulocytes and platelets had dysplastic features. On day 7, in addition to increasing the monocytic lineage cells, the dysplastic features of the blood had also increased compared to the initial examination. I performed bone marrow aspiration upon her death. The bone marrow revealed dysplastic features in all three hematopoietic cell lines, and an increase in the monocytic cell line. Based on the features of the bone marrow and the peripheral blood, this case was confirmed to be myelodysplastic syndrome - Chronic myelomonocytic leukaemia (MDS-CMML).

Myelodysplastic syndromes (MDSs) are a heterogeneous group of acquired hematological disorders that are characterized by prominent dysplastic features in multiple cell lines in the blood and bone marrow [Jain et al., 1991; Kouides et al., 1996; Raskin, 1996; Germing et al., 2000; Blue, 2003], and are extremely rare disease in dogs. In humans, some genetic etiology have been described in MDSs including some chromosomal deletion and translocation, genetic mutations such as Ras and p53, and epigenetic alteration, one of which is the promoter methylation of the p15 gene [Hirai, 2003]. In contrast, the initiating cause of primary MDSs in dogs has not yet been identified [Jain et al., 1991; Blue, 2000], but alterations in the hematopoietic stem cells are also suggested [Weiss et al., 2000; Blue, 2003; Weiss, 2003].

The classification of the canine MDS is debatable, however, the French-American-British (FAB) system has usually applied in dogs [Blue, 2000; Blue, 2003]. Based on this system, MDS categorized into five subtypes as follows; refractory anemia (RA), refractory anemia with ringed sideroblast (RARS), refractory anemia with excess blast (RAEB), refractory anemia with excess blast in transformation (RAEB-t) and chronic myelomonocytic leukemia (CMML). In this chapter, I describe the clinicopathological features and clinical outcome of a dog with

MDS-CMML, since no case reports have been documented in dogs with MDS-CMML.

An eleven-year-old female pug was referred to Yamaguchi University Animal Hospital for evaluation of progressive anemia and thrombocytopenia, which had not responded to seven days treatment with prednisolone (3 mg/kg, once daily). Upon physical examination, fever (40°C), pale mucous membranes and mild dyspnea were identified (Day 1). Mild bronchitis and splenomegaly were confirmed by radiological examination. The hemogram indicated a normocytic (mean corpuscular volume, 69.5 fl), normochromic (mean corpuscular hemoglobin concentration, 32.7 g/dl), non-regenerative anemia (hematocrit, 16%, reticulocyte production index, 0.15) and thrombocytopenia (48 × 10^3 /μl). The leukocyte count was within the normal range (14,949 /μl, Table 1). The cytological examination of the peripheral blood showed a left shift neutrophilia, some giant monocytic lineage blast cells and a small number of myeloid blast cells. These blast cells were distinguished by peroxidase and alpha-naphthyl butyrate esterase stain. A few granulocytes had dysplastic features, including bizarre nuclear shape, and asynchronous maturation of nuclei and cytoplasm. Large platelets were also seen. Differential diagnoses for this case included MDSs and acute myeloid leukemia (AML). Therefore, we suggested bone marrow aspiration to make a final diagnosis. However, the owner did not consent to

perform the bone marrow aspiration. Consequently, we started treatment with oral cyclosporine (7 mg/kg, once daily) and blood transfusion as supportive therapy. However, on day 7, the dog's condition worsened and the red blood cell and platelet counts decreased in spite of a blood transfusion administered six days earlier (hematocrit, 15% and platelet counts, 4×10^3 /μl). The leukocyte count slightly decreased, but it was still within the normal range (13,450 /μl, Table 1). In the peripheral blood, the number of promonocytes and monocytes were clearly increased (Table 1 and Fig. 1a). These monocytic lineage cells stained positive for alpha-naphthyl butyrate esterase (Fig. 1b) with sodium fluoride inhibition (data not shown). The dysplastic features of the blood— lobulated rubricytes (Fig. 1c), granulocytes with pseudo-Pelger-Huët anomaly and hypersegmentation, large platelets (Fig. 1d) and micromegakaryocytes (Fig. 1e)—had increased compared to those observed at the initial examination. The patient was administered supportive care, including blood transfusion, but she died on the same day. The cause of her death was not determined because we could not do autopsy. Immediately, upon her death, we performed bone marrow aspiration with the owner's consent. Examination of the bone marrow revealed normocellularity and the ratio of myeloid cells relative to erythroid cells (M: E) was 1.9:1 (Table 2 and Fig. 2a). The ratio of blast cells to all

nucleated cells (ANC) was 8.5%. There were few mature leukocytes, and an increase in the monocytic lineage cells was observed which accounted for 13.1% of ANC in the bone marrow (Table 2 and Fig. 2b). The most of mature monocytic lineage cells stained positive for alpha-naphtyrate butyrate esterase with sodium fluoride inhibition. Furthermore, there were certain dysplastic features in all three hematopoietic cell lines. These dysplastic changes included doughnut-shaped neutrophil precursors (Fig. 2d), asynchronously maturated rubricytes and rubricytes with multi-nuclei (Fig. 2e), mononuclear megakaryocytes and micromegakaryocytes. Additionally, macrophage phagocytosis was observed (Fig. 2c). Based on the features of the bone marrow and the peripheral blood, this case was confirmed to be MDS-CMML.

MDS-CMML is classified in the MDSs category by the FAB Cooperative Group, but it has been excluded from the human MDSs classification of the World Health Organization (WHO) scheme [Germing et al., 2000; Blue, 2003; Weiss, 2003]. Recently, the WHO system replaced the initial FAB classification for humans due to its improved prognostic discrimination [Germing et al., 2000]. In the field of veterinary medicine, however, its use has not been critically evaluated in large number of cases [Blue, 2000; Weiss, 2003]. It is also thought that CMML is included

in MDSs for its mutilineage dyshematopoiesis and cytopenias, and its high tendency to progress to AML [Rosati et al., 1996]. In this case, in addition to severe erythrocytopenia and thorombocytopenia, the patient had serious dysplastic features in all three cell lines of the blood and bone marrow. Consequently, this case was diagnosed as MDS-CMML based on the FAB system. MDSs are regarded as preleukemic disorders, and some cases that progressed to AML have also been reported in dogs [Couto et al., 1984; Weiss et al., 1985; Mcmanus et al., 1998; Weiss et al., 2000; Fujino et al., 2003]. In the present case, the circulating monocytic lineage cells had been increasing; however, at the time of death, blast cells constituted less than 30% of total marrow cells. Therefore, the condition had not progressed to AML. In dogs, MDSs tends to progress to AML, and therefore, the prognosis is poor. Although many drugs have been evaluated for the treatment of MDSs, an appropriate treatment protocol has not yet been established. Since canine primary MDSs are extremely rare disease, the etiology and genetic background have been unexplained. More case reports and the assessment for the molecular based treatment are required in the future.

CHAPTER 2

Molecular Cloning of the Canine Fragile Histidine Triad (*FHIT*) Gene and Fhit Protein Expression in Canine Peripheral Blood Mononuclear Cells

Summary

A fragile histidine triad (*FHIT*) gene has been studied as a tumor-associated gene in humans. The aberrant *FHIT* gene and its protein expression have been reported in many types of human cancers. The present chapter explored the canine *FHIT* gene structure and its protein expression in the peripheral blood mononuclear cells of healthy dogs by RT-PCR, RACE and immunoblot analysis. The obtained canine *FHIT* gene contained nine small exons and was located on canine chromosome 20. Furthermore, I identified an alternative splicing form of the *FHIT* transcript. The deduced amino acid sequence was well conserved between species, and anti-human Fhit antibody could be used to detect the canine Fhit protein. These findings will be useful for future research.

Introduction

The common fragile sites (CFS) are genomic unstable regions that tend to form gaps and breaks on metaphase chromosomes when cells are exposed to replication impedance, such as by aphidicolin, 5-azacytidine and bromo-deoxyuridine [Glover, 2006; Smith et al., 2007]. The FRA3B (3p14.2) region is one of the most frequently affected regions in the human genome, in which the instability region extends for 4 megabase pairs (Mbp), and allelic loss or homozygous deletions have been reported in many types of cancer, including lung, renal, esophageal, breast and cervical tumors [Glover, 2006; Huebner et al., 2003; Smith et al., 2007]. Regardless of the unstable region, some genes are located in FRA3B, and in particular, a fragile histidine triad (*FHIT*) gene has been investigated extensively [Iliopoulos et al., 2006; Smith et al., 2007]. In the human genome, this gene spans 1.5 Mbp within the FRA3B region; however, the final transcript contains only 1.1 kbases coding for a 17 kDa Fhit protein and is composed of 10 exons [Ohata et al., 1996]. Thus, this gene is composed of small exons and extremely large introns.

Aberrant *FHIT* gene expression and down-regulation of Fhit protein expression have been reported in many types of human tumors. The aberration of the *FHIT*

gene includes some exonic deletions and small sequence insertions in its genome [Druck et al., 1997; Ohata et al., 1996; Peters et al., 1999]. Moreover, aberrant methylation in the CpG island around exon 1 of the human *FHIT* gene has been found in numerous cancers, and this aberrant methylation has been shown to be associated with the inactivation of the *FHIT* gene and decreased expression of the Fhit protein [Tanaka et al., 1998; Vitmani et al., 2001; Zöchbauer-Müller et al., 2001]. Decreased expression of the Fhit protein has been found in approximately 50% to 83% of human solid tumors, including those of lung, cervical, esophageal and breast cancers, and 40% to 70% of hematopoietic malignancies, including myelocytic leukemia, lymphocytic leukemia and non-Hodgkin's lymphoma [Huebner et al., 2003; Peters et al., 1999; Sugimoto et al., 1997]. It has also been revealed that *FHIT* -/- and +/- mice develop spontaneous or N-nitrosomethylbenzylamine (NMBA)-induced tumors [Fong et al., 2000; Zanesi et al., 2001]. Therefore, the *FHIT* gene is regarded as a tumor-associated gene. The function of the Fhit protein is considered to be related to modulation of the cell cycle and apoptosis. Some studies have reported that Fhit modulates the mid-S phase DNA checkpoint response through regulation of the checkpoint proteins Hus1 and phosphoChk1 [Ishii et al., 2006; Ishii et al., 2007]. Other

studies have suggested that Fhit suppresses target genes, such as *cyclin D1*, *axin2*, *Mmp-14* and *survivin*, by binding directly to β-catenin [Weiske et al., 2007]. However, the precise function of the Fhit protein has not been clarified.

In dogs, many types of tumor seem to develop in the same manner as in humans. However, the roles of the *FHIT* gene and Fhit protein have not been explored in canine tumors. Recently, the complete canine genome sequence was clarified by the Whitehead Institute/MIT Center for Genome Research (WICGR) [Lindblad-Tho et al., 2005] and disclosed in the NCBI genomic databank. However, the canine *FHIT* gene has yet to be identified, probably because it may be difficult to identify extremely small exons that are surrounded by large introns. In another genomic database, Ensembl, a 661-base fragment of the canine *FHIT* cDNA sequence has been predicted, but the sequence is incomplete because the open reading frame (ORF) of the *FHIT* gene does not contain the start codon. Therefore, this study explored the canine *FHIT* gene structure. Moreover, a commercially available anti-Fhit antibody was examined for detection of canine Fhit protein expression in order to obtain basic information for future studies concerning the *FHIT* gene and Fhit protein.

Materials and Methods

Cells: Peripheral blood mononuclear cells (PBMC) were prepared from three healthy beagle dogs. Briefly, the heparinized whole blood was centrifuged, and the buffy coat was suspended in the phosphate-buffered saline (PBS) [2.7mM KCL, 0.14M NaCl, 1.5mM KH_2PO_4, 8.1mM $Na_2HPO_4 \cdot 12H_2O$]. The PBMC was isolated by gradient centrifugation using Lymphoprep (Fresenius Kabi Norge, Oslo, Norway). The PBMC was further purified by overlaying on bovine serum albumin (BSA) and by centrifugation in order to remove contaminated platelets.

RNA extraction, RT-PCR and sequence analysis: Total RNA was extracted from the PBMC using TRI Reagent (Molecular Research Center, Cincinnati, OH, USA), and single-strand cDNA was synthesized using SuperScript™ II RT (Invitrogen, Carlsbad, CA, USA) with an oligo-dT primer. The detection primers (FHIT-F1 and RP1: 5'- CAACATCTCATCAAGCCCTC -3' and 5'- CATTTCCTCCTCGG ATCTCCA -3', respectively) were designed within the predicted coding region based on the human (NCBI accession No: NM_002012), bovine (NM_001040646), mouse (NM_010210) rat (NM_021774), and canine *FHIT* gene sequences in the Ensembl database (Ensembl accession No: ENSCAFG00000007194). Using the primers and recombinant *Taq*DNA polymerase (Invitrogen), RT-PCR was

performed with 30 cycles of denaturation (94°C for 1 min), annealing (55°C for 1 min), and polymerization (72°C for 2 min) in the reaction mixture recommended by the manufacture. The resulting PCR product was subjected to direct sequencing using a BigDye Terminator v3.0 Ready Reaction Cycle Sequencing kit (Applied Biosystems, Foster, CA, USA).

5' and 3' RACE analysis: Based on obtained canine partial *FHIT* cDNA sequence, 5' and 3' extension primers were designed (5' and 3' RACE: 5'-CGAAGGGGACACACCAGGACATG -3' and 5'- TGAAGCATGT TCACGTCACGTTC -3', respectively). Each 1μg of mRNA was newly prepared from a healthy dog's PBMC using the FastTrack 2.0 kit (Invitrogen, Carlsbad, CA, USA) and 5' and 3' RACE analyses were performed using a SMART RACE cDNA Amplification kit (Clontech, Mountain View, CA, USA) according to the manufactures' instructions for both procedures.

Immunoblot analysis: The ORF of the canine *FHIT* gene was introduced into a mammalian expression vector, pcDNA 3.1 (+) (Invitrogen), and transfected transiently into the human bladder cancer cell line KK47. Briefly, the canine *FHIT* coding region was amplified by RT-PCR using cDNAs from the PBMCs of healthy dogs with the following primers: 5'- TAGGATCCGCTTCAACCGTGA

GGAAATG -3' (TF5) and 5'- ACGATAT CTTTCATGCCTGTAAAGTCA -3' (TF3), which contain restriction enzyme cutting sites for *Bam*HI and *Eco*RV, respectively. After enzyme treatment, the amplified *FHIT* gene was ligated into pcDNA3.1(+). The subcloned Fhit expression plasmid (pcDNA-Fhit) was used to simultaneously transfect KK47 cells with a plasmid containing a puromycin-resistance gene, pBabePuro, at a molar ration of 15:1, using Lipofectamine 2000 (Invitrogen). Concurrently, pcDNA3.1(+) was also transfected under the same conditions as an empty vector control (pcDNA). After incubation for 6 h, the cells were washed with PBS, cultured in fresh complete medium for 24 h, and then placed into fresh complete medium. Puromycin (2.5 μg/ ml) was added to the medium 24 h later. Successfully transfected cells enriched by tratment with puromycin for 24h were washed with PBS and harvested. Untransfected KK47 cells, KK47 cells transfected with pcDNA-Fhit, KK47 cells transfected with pcDNA and three PBMC samples isolated from the three above-mentioned healthy dogs were lysed in NP-40 lysis buffer [1% NP-40, 50 mM Tris (pH 8.0), 150 mM NaCl, 1% SDS, 2 μg/ml leupeptin and 2 μg/ml aprotinin]. The lysates were cleared by centrifugation at 15,000 rpm for 10 min at 4°C. Portions of the lysates (10 μg for each KK47 sample and 50 μg for each

PBMC sample) were then separated by 15% SDS-polyacrylamide gel electrophoresis (PAGE) and blotted onto a nitrocellulose membrane. The membrane was incubated in blocking buffer consisting of 5% (wt/vol) nonfat dry milk in TBS-T [20 mM Tris-HCl (pH 7.4) and 150 mM NaCl containing 0.05% Tween-20] for 1 h, incubated with the anti-human Fhit antibody (clone ZR44, Invitrogen; diluted 1:500 in TBS-T) for 1–2 h at room temperature, rinsed with TBS-T and then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (diluted 1:4000 in TBS-T, Invitrogen) for 1 h at room temperature. After washing with TBS-T, the antibody-antigen complex was visualized using Western Lightning Chemiluminescence Reagent Plus (PerkinElmer) and an LAS-3000mini chemiluminescence detection system (Fujifilm, Tokyo, Japan).

Results

The wild-type canine FHIT transcript: To clarify the sequence of canine wild-type *FHIT* transcript, a 391-base canine PBMC cDNA was amplified by RT-PCR using the F1/Rp1 primers, which was designed based on the *FHIT* gene sequence of other species. The direct sequence of obtained product revealed that it was well conserved compared to the human and bovine *FHIT* gene sequences (similarity scores of 89.88% and 89.49%, respectively). Moreover, the obtained sequence was completely identical to the canine chromosome 20 sequence according to an NCBI database search. The 3' region of the *FHIT* cDNA was obtained from the polyA sequence by 3' RACE analysis, and it was well conserved among the species examined. In contrast, the 5' non-coding sequence, which corresponded to the human, bovine and mouse exon 1 sequences, could not be obtained by 5' RACE. Therefore, canine exon 1 was determined from canine chromosome 20 based on the human and bovine exon 1 sequences using BLAST searches, and a sense primer was designed within this region (e1: 5'-TCACTTCCC AGCTGCCAAGATC -3'). In addition, an antisense primer was also designed within the final exon, which was clarified by 3' RACE (eL: 5'-TAAGTACATAGCCCAGGAAGTGTGGAAG -3'). Using these e1/eL primers,

RT-PCR and sequence analysis were performed to obtain 86.6% of the canine *FHIT* cDNA sequence. As a result, the 924-base sequence of canine *FHIT* was obtained, excluding the primer sequences (canine wild-type *FHIT* transcript; deposited in NCBI_accession No. AB469369). Based on this cDNA sequence for *FHIT* and the canine chromosome 20 sequence, the canine *FHIT* gene was predicted to contain nine short exons (30-306 bases) separated by extremely large introns (2,333-494,789 bases, Fig. 3). Moreover, based on the ORF sequence of the wild-type *FHIT* transcript, the amino acid sequence was deduced. The presumed canine Fhit protein was composed of 149 amino acid residues and was shown to have high similarities with those of other species (98.7%, 98.0%, 98.0 and 96.6% for the rat, mouse, bovine and human, respectively).

The alternative splicing form of the FHIT transcript: Interestingly, the 5' non-coding sequence of the cDNA obtained using primers e1/eL was different from that of the cDNA obtained by 5' RACE in the upstream region of exon 4, which contains the start codon. Hereafter, the later types of the transcripts are defined as 'type B' transcript. In a database search, a different 141-base sequence was identified between wild-type exons 3 and 4 in canine chromosome 20 and was named exon 3B (Fig. 3B). Direct sequence analysis of these products confirmed

that the wild-type and type B transcripts were only different within the 5' non-coding region, and the type B transcript had the same sequence after exon 4 as the wild-type transcript (deposited in NCBI_accession No. AB469370). Then, the sense primer was subsequently designed within exon 3B (BF1: 5'- CTGTGG ACAGAAACATCCCACCTG -3') to confirm expression pattern of the two types of *FHIT* transcript in the different individuals and tissues. Preparing the cDNA from PBMCs, as descrived above, from the PBMCs of three healthy dogs and from five tissue samples from the lung, intestine, liver, spleen and thymus of another healthy dog, RT-PCR was performed by wild type- and type B-specific primers (e1/eL and BF1/eL, respectively). As a result, the wild-type transcript was detected in all eight samples, whereas the type B transcript was identified in the three PBMCs, intestine and spleen but not in the lung, liver and thymus (Fig. 4A).

Canine Fhit protein expression and the availability of anti-human Fhit antibody: A commercially available anti-Fhit antibody (clone ZR44, Invitrogen) was examined for the detection of the canine Fhit protein expression. Immunoblot analysis was performed using three PBMC samples isolated from healthy dogs, and KK47 cells transfected with pcDNA-Fhit, KK47 cells transfected with pcDNA, as well as untransfected KK47 cells. A protein of approximately 17 kDa was

identified in all of the canine PBMC samples examined (Fig. 4B). Moreover, the Fhit protein was also identified in pcDNA-Fhit-transfected KK47 cells, whereas the Fhit protein was not detected in either the untransfected KK47 cells or the empty vector transfected KK47 cells (Fig. 4B).

Discussion

By RT-PCR using the e1/eL primers, and RACE analysis, the 924-base sequence of canine wild-type *FHIT* transcript was identified in this study. The obtained canine wild-type *FHIT* gene has been composed of nine exons, whereas the human *FHIT* gene has been shown to contain 10 exons [Ohata et al., 1996]. The difference in the exon numbers between human and canine *FHIT* resulted from differences in the 5' non-coding region, in which the human and canine *FHIT* genes contain four and three exons, respectively. Exons 1 and 2 of the canine *FHIT* gene have sequence similarities with those of humans, but exons 3 and 4 of the human *FHIT* gene do not have any similarity with canine *FHIT*, which contains only one exon corresponding to the human exon 3 and 4 regions. In contrast, exons 4 to 9 of the canine *FHIT* gene correspond to exons 5 to 10 of the human *FHIT* gene, which contain the ORF and 3' non-coding region, and were well conserved between the species. Furthermore, the deduced canine amino-acid sequence of the Fhit protein was composed of 149 residues. In contrast, the human Fhit protein is composed of 147 amino acid residues and represents the second branch of the histidine triad (HIT) protein superfamily, which is characterized by a His-X-His-X-His-XX (X: hydrophobic amino acid) motif

[Brenner, 2002]. This motif was conserved completely as His-Val-His-Val-His-Val-Leu (a.a. 96-100) in canine Fhit. Amino acids 1-75 in human Fhit, which are required for binding to β-catenin, were also well conserved in canine Fhit [Weiske et al., 2007]. These results suggest that the canine *FHIT* gene has presumably similar functions to those in other species.

In this study, two types of *FHIT* transcripts, wild type and type B, were detected. Because the type B transcript was obtained by 5' RACE analysis, it is possible that the 5' regions were not synthesized completely during the 5' RACE procedures. However, RT-PCR using the e1/eL primers amplified only a single band (Fig. 4A). Moreover, direct sequence analysis of the product showed that it was the wild-type transcript. These results suggest that the type B transcript is an alternative splicing form of *FHIT* that shares exons 4 to 9, but not exon 1, with the wild-type transcript. In the human *FHIT* gene, alternative splicing forms have also been reported in normal lymphocytes and other tissues, including the lung, liver, trachea and bronchial epithelium [Fong et al., 1997; Gayther et al., 1997; Sugimoto et al., 1997; Peters et al., 1999; Yang et al., 1999]. These transcripts resulted from the loss of entire exons generating splice junctions between the following exons: exons 3 and 6, exons 3 and 9, exons 3 and 8, exons 4 and 8 or

exons 7 and 9 [Fong et al., 1997; Sugimoto et al., 1997; Peters et al., 1999]. More importantly, small sequence insertions previously identified in human lymphocytes were also identified in the canine type B transcript obtained in the present study. In these transcripts, a small 47 or 72 base sequence was inserted between exons 4 and 5, although the sequences of the small insertions have not been reported and could not be determined using their loci in the human genome [Peters et al., 1999]. However, these small insertions and exon 3B in the canine type B transcript do not seem to influence the ORFs of the *FHIT* genes because the insertions were found in the 5' non-coding region.

In contrast, type B transcript was detected in all three PBMC samples and the spleen and intestine, but not from lung, liver and thymus. As described above, alternative splicing forms of *FHIT* transcripts have been reported frequently in human lymphocytes and PBMCs, which contain lymphocytes [Sugimoto et al., 1997; Peters et al., 1999; Yang et al., 1999]. This may also apply to the spleen, which expressed the type B transcript in the present study, because the spleen is part of the lymphatic system. Despite belonging to the same system, the thymus of the dog did not express the type B transcript. Moreover, to the best of my knowledge, the alternative splicing forms of *FHIT* transcripts have not been

isolated from the human intestine. The frequency of expression of alternatively splicing forms may differ between tissues, individuals and species. In the present study, the type B transcript was obtained by 5' RACE, and its existence was confirmed by RT-PCR using the BF1 primer (located in exon 3B). Therefore, further studies are necessary to clarify the complete 5' non-coding sequence of the type B transcript as well as its tissue distribution using Northern blot analysis, screening with a cDNA library and immunohistochemistry.

In immunoblott analysis, it was also evaluated the usefulness of the anti-human Fhit polyclonal antibody. It was used because it recognizes both human and rat Fhit protein, as described in the manufacturer's data sheet, and the deduced amino acid sequence of the canine Fhit protein obtained in the present study was shown to have high sequence similarity with that of human Fhit protein (96.6%). Indeed, the size of the approximately 17-kDa protein obtained by immunoblotting was the expected size of canine Fhit. Moreover, the anti-human Fhit polyclonal antibody distinguished the canine Fhit protein in pcDNA-Fhit-transfected KK47 cells, and had not recognized in either the untransfected KK47 cells or the empty vector transfected KK47 cells. It may suggest that the anti-human Fhit polyclonal antibody is available tool for the detection also in canine Fhit protein.

Although two types of canine *FHIT* transcript were identified from the PBMCs of the healthy dogs, no other size variants of Fhit protein were observed. This was not inconsistent with my prediction because the type B transcript also contains the same ORF as the wild-type transcript. Thus, the type B transcript may have an additive effect on Fhit protein expression and coexist with the wild-type transcript in normal canine lymphocytes. In humans, it has also been reported that alternative transcripts coexist with wild-type transcripts in lymphocytes, and only a single Fhit protein has been detected [Peters et al., 1999]. The wild-type *FHIT* transcript in human tumor cells has been reported to silence by CpG-island methylation in the upstream region of exon 1 [Tanaka et al., 1998]. Therefore, the alternative splicing form of the *FHIT* transcript including the type B transcript obtained in the present study may play a role in an alternative Fhit-expressing system. Further studies are necessary to clarify this point.

This study clarified the canine *FHIT* gene structure and its protein expression pattern. The basic form of the canine *FHIT* transcript had been shown to have small exons separated by very large introns, and this unique structure is basically conserved in human, bovine, rat and mouse *FHIT* genes. Moreover, its amino acid sequence was extremely similar to those of other species. In the human and mouse,

the *FHIT* gene is located in the unstable fragile regions of the genomes, FRA3B and Fra14A2, respectively [Shiraishi et al., 2001; Smith et al., 2007]. A previous study reported the canine folate/thymidine depleted and aphidicolin-inducible fragile site using PBMCs from the Boxer and Doberman Pinscher breeds. The chromosomal fragile sites varied depending on breed and age, but chromosome 20, which contains the canine *FHIT* gene locus, was not identified as a fragile site [Stone et al., 1991]. However, the FRA3B/Fra14A2 loci are conserved in AT-rich sequences with numerous short and long repeats throughout the region [Inoue et al., 1997; Shiraishi et al., 2001]. The intronic regions of the canine *FHIT* gene also have AT-rich short repeat sequences (data not shown). This observation suggests that the canine *FHIT* gene is also located in a fragile chromosomal site. Furthermore, the present study demonstrated that the anti-human Fhit antibody can be used for study of canine Fhit protein expression. In humans, more than 700 papers have been published concerning *FHIT* gene expression and Fhit protein function because loss of Fhit protein has been observed in many types of human cancer [Huebner et al., 2007]. Recently, a *FHIT* gene replacement therapy using an adenoviral vector has also been studied at a preclinical stage [Ishii et al., 2001; Pichiorri et al., 2006]. The basic information about the canine *FHIT* gene and Fhit

protein obtained in this study will be useful in clarifying the relationship between *FHIT* gene expression and cancer development in dogs.

CHAPTER 3

Aberrations of the *FHIT* Gene and the Fhit Protein in Canine Lymphoma Cell Lines

Summary

The fragile histidine triad (*FHIT*) gene is a tumor-associated gene, and aberrant *FHIT* gene and protein expression have been described in many types of human tumors. Furthermore, it has been reported that *FHIT* gene inactivation is induced by hypermethylation of 5′ CpG islands in the gene or by genomic deletion around the open reading frame (ORF). In chapter 3, I explored the aberrations in the canine *FHIT* gene and Fhit protein expression and assessed the methylation status and genomic deletions by using 5 canine lymphoma cell lines. I found that the decrease in the expression of the Fhit protein in canine lymphoma cell lines was similar to that in human tumors. The expression of the wild-type *FHIT* transcript was reduced in all 5 cell lines. However, I could not confirm the involvement of aberrant methylation events in the 5′ CpG islands of the canine *FHIT* gene. I was able to identify homozygous or heterozygous deletions in the canine *FHIT* genes in all 5 cell lines. Moreover, a widespread genomic deletion of the *FHIT* gene, which included the ORF region, was detected in 1 cell line. In chapter 3, I detected aberrations in the *FHIT* gene and Fhit protein expression in all 5 canine lymphoma cell lines, and this phenomenon might be an important factor in promoting canine lymphoma.

Introduction

Common fragile sites (CFSs) are unstable genomic regions that tend to form gaps and breaks on metaphase chromosomes when the cells are exposed to replication impedance [Arlt et al., 2006; Glover, 2006; Smith et al., 2007]. Among CFSs, FRA3B (3p14.2) is one of the most frequently affected regions in the human genome, and allelic loss or homozygous deletions in this region have been found in many types of cancers, including lung, renal, esophagus, breast and cervical tumors [Glover, 2006; Smith et al., 2007]. Regardless of the unstability of this region, some genes are located at FRA3B, and one such gene—the fragile histidine triad (*FHIT*) gene—has been extensively investigated [Iliopoulos et al., 2006; Smith et al., 2007]. The product of this gene, Fhit protein, is considered to be a tumor-suppressor protein and a modulator of the cell cycle and apoptosis [Ishii et al., 2007; Ishii et al., 2006].

In humans, aberrant *FHIT* gene expression and downregulated Fhit protein expression have been reported in many types of solid tumors, including lung, cervical, esophageal and breast tumors, and in hematopoietic malignancies, including myelocytic leukemia, lymphocytic leukemia and non-Hodgkin's

lymphoma [Huebner et al., 2003; Peters et al., 1999]. Aberrant methylation of the CpG islands around exon 1 of the *FHIT* gene has been reported in numerous cancers, including esophageal, lung, breast and cervical tumors, and lymphocytic leukemia [Tanaka et al., 1998; Virmani et al., 2001; Zheng et al., 2004; Zöchbauer-Müller et al., 2001]. Aberrant methylation is associated with inactivation of the *FHIT* gene, and studies on esophageal and non-small lung cancer cell lines have shown that demethylation of hypermethylated cells induces re-expression of the *FHIT* gene [Tanaka et al., 1998; Zöchbauer-Müller et al., 2001]. Furthermore, homozygous or heterozygous deletion of the *FHIT*/FRA3B region has been reported in some cancer cells [Ahmadian et al., 1997; Fong et al., 1997; Inoue et al., 1997; Larson et al., 1997; Shridhar et al., 1996]. These reports have described allelic losses around exon 5 of the human *FHIT* gene, which is a region containing the start codon of the open reading frame (ORF) of *FHIT*; however, the deletion points vary between the types of cancer [Corbin et al., 2002; Inoue et al., 1997]. In some human lung cancer cell lines, homozygous deletions have been shown to be associated with loss of the wild-type *FHIT* transcripts and expression of an aberrant transcript [Fong et al., 1997].

In chapter 2, I demonstrated the canine *FHIT* gene structure and its protein

expression in peripheral blood mononuclear cells (PBMCs) and other tissues, including tissues of the lungs, intestine, liver, spleen and thymus, in healthy dogs. The canine *FHIT* gene is located in an approximately 1.4-megabase pair (Mbp) region of canine chromosome 20; the wild-type transcript is composed of 9 small exons (30–306 bp) that are separated by extremely large introns (2,333–494,789 bp), and the ORF corresponds to exons 4 to 8. Furthermore, an alternative splicing form of the *FHIT* transcript (named type B) has been found in PBMCs and other tissues. The 5′ noncoding region of the type-B transcript was differentiated from the wild-type transcript by the presence of a novel exon—exon 3B—that was present in the region between exons 3 to 4 of the wild-type transcript. However, the type-B transcript shows the same exons as the wild-type *FHIT* transcript after exon 4; therefore, the type-B transcript does not induce any aberrant expression of the Fhit protein in dog PBMCs. In dogs, many types of tumors develop in the same manner as they do in humans. However, to the best of my knowledge, the relationship between the canine *FHIT* gene and tumor development in dogs has not been explored. In this study, I focused on canine lymphoma, which is one of the most common tumors in dogs. Canine lymphoma accounts for approximately 7%–24% of all canine tumors and 83% of all canine hematopoietic malignancies

[Vail et al., 2007], and the inactivation of p16 cyclin-dependent kinase inhibitor has recently been shown to be associated with tumorigenesis in canine lymphoma [Fosmire et al., 2007]. However, the precise molecular mechanisms of tumorigenesis in cases of canine lymphoma remain to be elucidated. Clarification of the correlation between canine lymphoma and the aberrations of the *FHIT* gene and Fhit protein expression may be helpful in analysis of cancer etiology in the veterinary field. Therefore, I examined the aberrations of the *FHIT* gene and Fhit protein expression by using canine lymphoma cell lines. Furthermore, I explored the hypermethylation of 5′ CpG islands and the effects of *FHIT* allelic deletion.

Materials and Methods

Cells: In this study, I used 2 newly established canine lymphoma cell lines (the Ema line and the Nody-1 line) and 3 previously reported canine lymphoma cell lines (CL-1, UL-1 and GL-1) [Momoi et al., 1997; Nakaichi et al., 1996; Yamazaki et al., 2008]. The Ema and Nody-1 lines were established from the pleural effusion of a patient with thymic lymphoma and the ascites fluid of a patient with alimentary lymphoma, respectively; the T cell lineages of the 2 cell lines were confirmed by T cell-receptor gene rearrangement analysis and surface-antigen analysis using a flow cytometer. All the cell lines were maintained in a complete medium [RPMI-1640 containing 10% fetal bovine serum (FBS), penicillin (100 U/ ml) and streptomycin (100 μg/ ml)] and grown in an atmosphere containing 5% CO_2. PBMCs were obtained from a healthy beagle dog. Briefly, the heparinized whole blood was centrifuged, and the buffy coat was suspended in phosphate-buffered saline (PBS; 2.7 mM KCl, 0.14 M NaCl, 1.5 mM KH_2PO_4, 8.1 mM $Na_2HPO_4 \cdot 12H_2O$). PBMCs were isolated by gradient centrifugation using Lymphoprep (Fresenius Kabi Norge, Oslo, Norway) and further purified by overlaying them on FBS and performing centrifugation to remove contaminated platelets. The cells were cultured in the complete medium

for 2 h to remove monocytes. Microscopic analysis was performed, and approximately 80% of the cells were confirmed to be lymphocytes; these cells were used as the control throughout the present study.

RNA extraction, Reverse transcriptase-polymerase chain reaction (RT-PCR) and sequence analysis: Total RNA was extracted by using TRI Reagent (Molecular Research Center, Cincinnati, OH, USA), and single-strand cDNA was synthesized from 2.5 μg of each total RNA sample by using SuperScript II RT (Invitrogen, Carlsbad, CA, USA) with an oligo-dT primer in accordance with the manufacturer's instructions. I prepared 3 primer pairs—e1/eL, BF1/eL and TF5/TF3 (Table 3)—to detect the canine *FHIT* genes; in chapter 2, I had confirmed that these 3 primer pairs amplify the canine *FHIT* gene transcripts. These primer pairs were used to amplify exons 1 to 9 of the canine wild-type *FHIT* transcript, exons 3B to 9 of the alternative splicing form (type B), and exons 4 to 8 of the common ORF of these transcripts (Fig. 5A). The canine ribosomal protein L32 (RPL32), which is a stable housekeeping gene in canine lymphatic cells and tissues, was amplified as a control (Table 3) [Peters et al., 2007]. A thermal GeneAmp PCR system 9700 (Applied Biosystems, Foster, CA, USA) was used for PCR amplification, which was performed by using the

following protocol: denaturation at 95°C for 5 min; 35 cycles of 94°C for 1 min, 58–65°C for 1 min and 72°C for 2 min; and then a final incubation at 72°C for 10 min. The PCR products were electrophoresed on 1.5% agarose gels and stained with ethidium bromide. The amplified products were further analyzed by direct sequencing using a BigDye Terminator v3.0 Ready Reaction Cycle Sequencing kit (Applied Biosystems); further analysis was performed at the DNA Core facility of the Center for Gene Research, Yamaguchi University.

Immunoblot analysis: Immunoblot analysis was performed by using a rabbit antihuman Fhit polyclonal antibody (clone ZR44; Invitrogen) that had been confirmed to detect the canine Fhit protein in chapter 2. Each sample was lysed in NP-40 lysis buffer [1% NP-40, 50 mM Tris (pH 8.0), 150 mM NaCl, 1% SDS, 2 μg/ml leupeptin and 2 μg/ml aprotinin] and was sonicated to degrade the genomic DNA. The lysates were cleared by centrifugation at 15,000 rpm for 10 min at 4°C. The lysates (50 μg per sample) were diluted with SDS sample buffer [2% SDS, 10% glycerol, 60 mM Tris (pH 6.8), 5% β-mercaptoethanol, 0.01% bromophenol blue], separated by using 15% SDS-polyacrylamide gel electrophoresis (PAGE) and blotted onto a nitrocellulose membrane. The membrane was incubated in a blocking buffer consisting of 5% (wt/vol) nonfat dry milk in Tris-buffered saline

containing Tween 20 (TBS-T) [20 mM Tris-HCl (pH 7.4) and 150 mM NaCl containing 0.05% Tween 20] for 1 h, incubated with the antihuman Fhit antibody (diluted 1:500 in TBS-T) for 1 h at room temperature, rinsed with TBS-T and incubated with horseradish peroxidase-conjugated goat antirabbit IgG (Invitrogen; diluted 1:4000 in TBS-T) for 1 h at room temperature. The antibody-antigen complex was washed with TBS-T and visualized by using a Western Lightning Chemiluminescence Reagent Plus kit (PerkinElmer, Waltham, MA, USA) and an LAS-3000mini chemiluminescence detection system (Fujifilm, Tokyo, Japan). The membrane was reprobed with an antihuman actin polyclonal antibody (clone C-11; Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Bisulfite Modification of DNA and Methylation Specific-PCR (MS-PCR): MS-PCR was performed to assess the methylation status in the canine lymphoma cell lines. The CpG sites of the canine *FHIT* gene were determined by using the human *FHIT* CpG site map [Tanaka et al., 1998] to examine the region around exon 1 of the *FHIT* genomic sequence, and 3 primer pairs were designed by using the Methyl Primer Express software ver.1.0 (Applied Biosystems). The primer pairs MpF1/MpR1 and UpF1/UpR1 were used to amplify methylated *FHIT* DNA and unmethylated *FHIT* DNA, respectively (Table 3). The third primer

pair—WpF1/WpR1—was used to amplify the control, wild-type *FHIT* DNA, which had not undergone bisulfite modification. The DNA samples were extracted by using a QIAamp DNA Mini kit (QIAGEN, Hilden, Germany), and 1 μg of each DNA sample was used as the template for bisulfite modification, which was performed by using a CpGenome DNA Modification kit (Chemicon International, Temecula, CA, USA) in accordance with the manufacturer's instructions. I used a HotStarTaq master mix kit (QIAGEN) and 50 ng of each DNA sample for PCR amplification. The following conditions were employed for PCR amplification: 95°C for 15 min; 35 cycles of 94°C for 30 sec, 55–65°C for 30 sec and 72°C for 1 min; and then 72°C for 10 min. The amplified products were electrophoresed on 2% agarose gels and stained with ethidium bromide.

Deletion analysis: In order to identify the deletions in the canine *FHIT* genomic locus in the cell lines, I designed 20 pairs of primers corresponding to the intronic region between exon 3 and the type-B-specific exon 3B (1F/R–6F/R), exons 3B and 4 (7F/R–12F/R) and exons 4 and 5 (13F/R–20F/R; Table 3). These primers were designed to amplify 800–1,000 bp products, which were randomly selected within each intronic region. The DNA samples were prepared as described above, and PCR amplification was performed by using a HotStarTaq

master mix kit (QIAGEN). The following conditions were employed for PCR analysis: 95°C for 15 min; 35 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec and extension at 72°C for 2 min. The amplified products were electrophoresed on 2% agarose gels and visualized by using ethidium bromide staining.

Results

Expression of FHIT transcripts in canine lymphoma cell lines: I explored the expression pattern of the *FHIT* gene transcripts in canine lymphoma cell lines by using specific primers to perform RT-PCR amplifications of the wild-type, type B and ORF transcripts of canine *FHIT*. The expected 872-bp product of the wild-type transcript band was observed in the case of the control PBMCs [Fig. 5B (a)]. Furthermore, I observed a faint band in the case of the UL-1 and GL-1 cell lines, but I did not detect any transcript in the case of the Ema and Nody-1 cell lines. In the case of CL-1, I detected a smear and 2 small transcripts with lengths of 500 bp and 600 bp [Fig. 5B (a)]. Direct sequence analysis showed that the amplified transcripts in the UL-1 and GL-1 cell lines showed 100% similarity with the wild-type *FHIT* transcript. I used the e1/eL primer pair to analyze the transcripts from CL-1; however, I was unable to identify the sequences of the smear or the 2 small-sized transcripts. The type-B transcript could not be amplified from any of the cell lines [Fig. 5B (b)]. The expression of the ORF region seemed to be reduced in all the cell lines, especially in the Ema and Nody-1 cell lines, and no bands were detected in the CL-1 cell line [Fig. 5B (c)]. Sequence analysis of the ORF in the Ema and Nody-1 cell lines showed that these

2 cell lines had sequences that were the same as that obtained from the wild-type transcript.

Expression of the Fhit protein in canine lymphoma cell lines: In chapter 2, a rabbit antihuman Fhit polyclonal antibody (clone ZR44, Invitrogen) was used to detect the canine Fhit protein (approximately 17 kDa) in the PBMCs of healthy dogs. I performed immunoblot analysis by using this antibody in order to evaluate expression of the Fhit protein in canine lymphoma cell lines. As shown in Figure 1C, expression of the Fhit protein in all 5 cell lines was lower than that observed in PBMCs isolated from a healthy dog.

Methylation status of CpG islands in canine lymphoma cell lines: The human CpG-island profile was used to search for CpG islands around exon 1 (–1,000 to + 2,000 bp) of the canine *FHIT* gene. Canine CpG islands have been presumedly found at a 437-bp locus around canine *FHIT* exon 1 (–126 to +312). Amplification of the methylated DNA by using the MpF1/MpR1 primers did not yield any bands for any of the cell lines or the control PBMCs (Fig. 6A); in contrast, use of the primers UpF1/UpR1 yielded amplified unmethylated DNA from all the cell lines and the control PBMCs (Fig. 6B), suggesting that aberrant methylation could not have occurred in this region. Furthermore, amplification using the primers

WpF1/WpR1, which detect unmodified wild-type DNA, yielded faint bands in all the samples (Fig. 6C). However, the reduced level of PCR amplification by the WpF1/WpR1 primers in comparison with the amplification by the UpF1/UpR1 primers suggests that the DNA was at least partially modified by the CpGenome DNA Modification kit and that aberrant methylation had not occurred in any of the canine lymphoma cell lines.

Homozygous or heterozygous deletion of the FHIT locus in canine lymphoma cell lines: Homozygous or heterozygous deletion of the canine *FHIT* gene locus in canine lymphoma cell lines was evaluated by using 20 pairs of primers (Table 3). I did not detect any deletions between exons 3 and 3B in the Ema, UL-1, GL-1 and Nody-1 cell lines. However, I did not detect any bands for the 6F/R amplified region in the CL-1 cell line, suggesting that a homozygous deletion had occurred (Fig. 7). This deletion was also detected in the midstream region between exons 4 and 5 (amplified by primers 18F/R), although amplified DNA was obtained from the downstream region, which was amplified by using the primers 19F/R (Fig. 7). In contrast, I did not detect any deletion in the other cell lines, except for that observed in the region amplified by using the primers 14F/R. In this region, amplification yielded a 1099-bp band in the control PBMCs, while an 830-bp

band was detected in the Ema, Nody-1 and GL-1 cell lines (Fig. 7B). Furthermore, both of these bands were observed in the UL-1 cell line. Sequence analysis of the 830-bp band from the Ema cell line revealed that this region contained intermittent deletions consisting of a large deletion (200 bp), several small deletions (11, 3 and 2 bp) and 8 single-point mutations. Moreover, the 830-bp bands from the UL-1 and Ema cell lines had the same sequence. In contrast, the sequence of the 1099-bp band from the UL-1 cell line was identical to that of the PBMC samples.

Discussion

In humans, decreased expression of Fhit protein has been observed in approximately 50%–83% of solid tumors and in 40%–76% of hematopoietic tumors [Albitar et al., 2001; Hallas et al., 1999; Huebner et al., 2003; Peters et al., 1999; Zheng et al., 2004]. Furthermore, a decrease in Fhit protein expression has been correlated with poor clinical prognosis of some human tumors, including lung, oral and breast cancers [Arun et al., 2005; Guerin et al., 2006; Toledo et al., 2004]. In the present study, Fhit protein expression decreased in all the canine lymphoma cell lines examined, suggesting that decreased expression of the Fhit protein may be associated with tumorigenesis in canine lymphoma.

It has been suggested that the *FHIT* gene and its protein play an important role in tumorigenesis in human tumors. Indeed, it has been reported that Fhit-deficient mice develop spontaneous or N-nitromethylbenzylamine (NMBA)-induced tumors more frequently than mice with normal expression of Fhit [Fong et al., 2000; Zanesi et al., 2001]. Nevertheless, the precise function of the Fhit protein has not been clarified. However, some studies have revealed that Fhit modulates the mid-S phase DNA checkpoint response by regulating the checkpoint proteins Hus1 and phosphoChk1 [Ishii et al., 2007; Ishii et al., 2006]. Other studies have

described that Fhit suppresses target genes such as *cyclin D1*, *axin2*, *Mmp-14* and *survivin* by directly binding to β-catenin [Weiske et al., 2007].

Aberrant *FHIT* transcripts lacking regions between exons 3–9 have been detected in human hematopoietic malignancies [Hallas et al., 1999; Iwai et al., 1998; Peters et al., 1999; Sugimoto et al., 1997], although the wild-type transcript is also expressed along with these transcripts [Hallas et al., 1999; Iwai et al., 1998; Peters et al., 1999; Sugimoto et al., 1997]. In contrast, complete loss of *FHIT* transcripts is detected infrequently [Hallas et al., 1999; Iwai et al., 1998; Peters et al., 1999; Sugimoto et al., 1997]; nevertheless, Fhit protein is frequently absent in these tumor cells [Hallas et al., 1999; Peters et al., 1999]. In this study, I observed that expression of the wild-type *FHIT* transcript was reduced in 2 canine lymphoma lines (UL-1 and GL-1) and absent in the other 2 lines (Ema and Nody-1) [Fig. 5B (a)]. Moreover, the expression of the ORF region decreased in all the cell lines, and the *FHIT* ORF expression was completely absent in CL-1 [Fig. 5B (c)]. We detected a homozygous deletion between the midstream region of intron 3 and the region before exon 5 of the *FHIT* gene locus in the CL-1 cell line (Fig. 7). Furthermore, in the RT-PCR analysis using the e1/eL primers, I obtained approximately 500-bp- and 600-bp-long aberrant *FHIT* transcripts from

the CL-1 cDNA [Fig. 5B (a)]. These findings suggested that the aberrant transcripts of the CL-1 cell line might have resulted from the absence of exon 4 in the *FHIT* cDNA. In order to clarify the deletion in the CL-1 cDNA, I performed RT-PCR amplification of the cDNA between exons 1 and 5 by using the e1 primer along with a newly designed antisense primer. I selected 3 amplified bands with different sizes and performed direct sequencing analysis. A complete sequence was obtained in 1 of the 3 sequence reactions, while the sequences could not determined in the other 2 reactions. This sequence was shown to be 135 bp shorter than the wild-type *FHIT* transcript. In this sequence, the 3′ end of exon 2 was connected to a 58-bp sequence that was located between intron 4 of the *FHIT* gene locus, followed by the 5′ initiation site of exon 5 (Fig. 7A). Although the results of PCR analysis of the *FHIT* gene locus (data not shown) confirmed the existence of exon 3 on the CL-1 genome, exons 3 and 4 were absent from the CL-1 cDNA. Moreover, I detected a reduction (or absence) of Fhit protein expression in the CL-1 cell line (Fig. 5C). Homozygous or heterozygous deletion, which results in loss of the Fhit protein if the deletion contains the ORF region, of the *FHIT* gene locus has also been reported in many types of human cancer cells [Inoue et al., 1997]. These data suggest that the reduction (or absence) of Fhit protein

expression in CL-1 cells can be attributed to the absence of exon 4, which contains the start codon of the ORF, and that homozygous deletion of the *FHIT* gene is also associated with tumorigenesis in canine lymphoma.

In chapter 2, it has been shown that an alternative splicing form—the type-B *FHIT* transcript—is coexpressed with the wild-type *FHIT* transcript in the PBMCs, spleen and intestine of healthy dogs and that it may play a role in an alternative Fhit-protein-expressing system that is activated when expression of the wild-type transcript is reduced. In this study, the type-B transcript could not be detected in any of the canine lymphoma cell lines. Exon 3B, which is a specific exon for the type-B transcript, was identified in the genome of all the cell lines, except for CL-1. These observations suggest that expression of the type-B transcript is induced in normal cells but not in canine lymphoma cell lines.

In the Ema and Nody-1 cell lines, the wild-type transcript could not be amplified, but the ORF region was expressed weakly. In contrast, the wild-type transcript was weakly expressed in the UL-1 and GL-1 cell lines, which certainly contained the ORF region. I had confirmed that all the cell lines contained exons 1, 2 and 3 in their genome and exons 6 through 9 in their cDNA (data not shown); therefore, the abovementioned observation may have resulted from incomplete

cDNA synthesis during the RT reaction in these cell lines. Alternatively, it could have resulted from the existence of an alternative splicing form(s) other than type B in these lines. Further studies are necessary to clarify these observations.

In humans, aberrant methylation of 5′ CpG islands in the *FHIT* gene has been reported in some solid tumors, including esophageal, cervical, lung and breast cancers and in hematopoietic malignancies, showing that methylation causes reductions in the expression levels of the *FHIT* gene and Fhit protein [Tanaka et al., 1998; Virmani et al., 2001; Zheng et al., 2004; Zöchbauer-Müller et al., 2001]. The 5′ CpG island of the human *FHIT* gene is located in a 460-bp region around exon 1 (from –95 to +365 bp) [Tanaka et al., 1998]; however, the 5′ CpG island of the canine *FHIT* gene has not been explored previously. Human 5′ CpG islands have been detected using the following criteria [Tanaka et al., 1998]: GC content, 66.5%; CpG density, 7.8%; and observed/expected CpG index, 0.7. I used these criteria to identify 5′ CpG islands in the region around exon 1, ranging from –1,000 to +2,000 bp, of the canine *FHIT* gene. A presumed canine CpG island, which was shown to have a GC content of 65.98% and CpG density of 6.64%, was detected in a 437-bp region around canine *FHIT* gene exon 1 (–126 to +312). In the present study, I designed sense and antisense primers corresponding to this

region. While aberrant methylation could not be detected by using these primers, the Fhit protein expression level was decreased in all the canine lymphoma cell lines examined. In human tumors, it has been reported that a primer set designed to detect 5′ CpG-island methylation of the *FHIT* gene in esophageal cancer could not detect it in lung and breast cancers [Tanaka et al., 1998; Zöchbauer-Müller et al., 2001]. Furthermore, murine *FHIT* gene analysis has revealed the presence of cancer- or tissue-specific methylation patterns [Han et al., 2004]. In the present study, I could not detect aberrant methylation in any of the cell lines; however, further studies are necessary to conclude that there is no relationship between the 5′ CpG-island methylation status and the Fhit protein expression level in canine lymphoma cell lines.

In this study, I identified widespread genomic deletions that contained the ORF region in the CL-1 cell line and homozygous (Ema, GL-1 and Nody-1 cell lines) and heterozygous deletions (UL-1 cell line) in intron 4 of the canine *FHIT* genomic sequence. Sequence analysis revealed that the heterozygous deletion pattern had intermittent deletions consisting of a large deletion (200 bp) and 3 small deletions (46, 3 and 2 bp). In humans, the tumor-specific deletion around exon 5 of the *FHIT* gene, spanning the region between exon 4 and the proximal

50-kbp region of intron 5, has been well described [Corbin et al., 2002; Inoue et al., 1997]. Human exon 5 corresponds to canine exon 4, which contains the initiation site of the ORF in the canine *FHIT* gene. The identified deletion point of the canine *FHIT* gene is located in the proximal 37-kbp region of canine *FHIT* intron 4, which corresponds to the human *FHIT* deletion site. However, this aberration of canine *FHIT* intron 4 has no apparent effect on the ORF of the *FHIT* gene. Further analysis is necessary to clarify the relationship between this deletion and the expression of the *FHIT* gene and/or the Fhit protein.

In this study, I have shown that the reduction in Fhit protein expression in the 5 canine lymphoma cell lines was similar to that observed in human tumor cells. The *FHIT* gene aberrations that resulted in expression of the aberrant *FHIT* transcripts were detected in the CL-1 cell line, but were not observed in the other cell lines. Expression of the wild-type *FHIT* transcript seemed to be reduced in all the cell lines; however, I could not confirm the involvement of aberrant methylation events in the 5′ CpG island of the canine *FHIT* gene. However, I was able to identify homozygous or heterozygous deletions in the canine *FHIT* genome in all the canine lymphoma cell lines, suggesting that the canine *FHIT* gene locus is also a fragile site in the genomes of lymphoma cells.

In human lymphocytic malignancies, the incidence of loss (decrease) of wild-type *FHIT* gene transcripts and co-expression of aberrant *FHIT* gene transcripts have been reported to be approximately 37%–46% and 27%–80%, respectively [Chen et al., 2004; Iwai et al., 1998; Peters et al., 1999; Yang et al., 1999], and the incidence of reduction or absence of Fhit protein expression has been shown to be approximately 58%–76% [Albitar et al., 2001; Chen et al., 2004; Hallas et al., 1999]. Moreover, hypermethylation of the *FHIT* promoter region has been reported in 40% of acute lymphoblastic (ALL) leukemia cell lines and 27% of pediatric ALL patients [Yang et al., 2006; Zheng et al., 2004], and allelic loss of the *FHIT* gene locus has been described in chronic lymphocytic leukemia [Gartenhaus, 1997]. In the present study, I detected decreased expression of wild-type *FHIT* transcripts and concurrent expression of aberrant *FHIT* transcripts in canine lymphoma cell lines; I also observed decreased expression of the Fhit protein in all the cell lines. While aberrant hypermethylation of the canine *FHIT* 5′ CpG island could not be detected in all the cell lines, I did detect some homozygous or heterozygous deletions of the canine *FHIT* gene locus in them. Therefore, the decreased expression level of the canine *FHIT* gene and the Fhit protein might be an important factor in tumorigenesis in canine lymphoma in

a manner similar to that observed in the case of human hematopoietic tumors. The molecular mechanism of tumorigenesis in dog tumors has not been completely clarified. Further studies using clinical tumor specimens are necessary to identify the relationship between aberrations of the *FHIT* gene and tumorigenesis in dogs.

CONCLUSION

In dogs, many types of tumors have developed in the same manner as in humans, and hematopietic tumors are especially common. However, MDSs are extremely rare diseases compared with lymphoid malignancies in dogs, and their clinical information has been lacked. Therefore, I reported the case, which was suffered from primary canine MDS-CMML in chapter 1. MDSs divide into two categories, primary and secondary MDSs. In incidence rates, the canine primary MDSs are fewer than the secondary MDSs [Blue, 2000; Weiss et al., 2001]. The secondary MDSs have been induced by other diseases such as the tumor, immune-mediated or infectious disease, some drugs and chemical agent [Blue, 2000; Weiss et al., 2001; Weiss, 2003]. In contrast, the primary MDSs have been shown to result from the genetic alternations in hematopoietic stem cells in humans, although it has not been clarified in dogs. In the human cancer development, numerous genetic alterations have been well studied including some chromosomal aberrations, and the aberrations of some tumor associated genes such as oncogenes and tumor suppressor genes. The CFSs have been identified as genomic unstable regions [Glover, 2006; Smith et al., 2007]. In the CFSs, a FRA3B region is one of the most frequently affected regions, and allelic loss or homozygous deletions in the region have been reported in many types of human cancers

[Glover et al., 2006; Smith et al., 2007]. Regardless of the unstable region, some genes are located in FRA3B region, and in particular the *FHIT* gene has been well investigated [Iliopoulos et al., 2006; Smith et al., 2007]. From these backgrounds, I focused on the *FHIT* gene as a candidate for canine CFSs in dog tumors.

A fragile histidine triad (*FHIT*) gene has been well described in the human as a tumor-associated gene. More than 700 papers have been published concerning *FHIT* gene expression and Fhit protein function, because the loss of Fhit protein was observed in many types of human solid tumors and hematopoietic malignancies [Huebner et al., 2003; Peters et al., 1999]. In chapter 2, the canine *FHIT* gene structure and its protein expression in healthy dogs' PBMC were examined by RT-PCR, 5' and 3' RACE and immunoblot analysis. Based on my research, the obtained canine wild-type *FHIT* gene contained nine small exons surrounded by large introns. This structure was well conserved between the other species. Moreover, the deduced amino-acid sequence was shown to have high similarities with those of the other species, and anti-human Fhit antibody was found to be available for the detection of the canine Fhit protein by immunoblot analysis. In this chapter, an alternative splicing form of the *FHIT*, called type B, was also identified. In human, some alternative splicing forms of *FHIT* transcripts

have also been reported in normal lymphocytes and other tissues [Fong et al., 1997; Gayther et al., 1997; Sugimoto et al., 1997; Peters et al., 1999; Yang et al., 1999]. The obtained canine *FHIT* type B transcript showed to be different from the wild-type transcript in the 5' upstream region. However, the ORF of the type B transcript was the same with that of the wild-type transcript, and no aberrant form of the Fhit protein was observed.

In chapter 3, I evaluated the aberration of the *FHIT* gene and its protein in five canine lymphoma cell lines based on the basic information concerning the canine *FHIT* gene and the protein obtained in chapter 2. Moreover, I also examined the aberrant methylation status in 5' CpG and the genomic deletion on the canine *FHIT* allele. In all the canine lymphoma lines examined, the expression of the Fhit protein was decreased in a similar manner to in human tumors. Expression of the wild-type *FHIT* transcript was reduced in all the cell lines. However, the involvement of aberrant methylation events in 5' CpG islands of the canine *FHIT* gene could not be confirmed. In contrast, homozygous or heterozygous deletions in the canine *FHIT* gene were identified in all five cell lines. Moreover, a widespread genomic deletion of the *FHIT* gene, which included the ORF region, was detected in one cell line. These results suggested that aberrations of the *FHIT*

gene and Fhit protein expression may also play an important role in tumorigenesis in canine lymphoma.

In the past, a few studies have been reported concerning the canine CFSs [Stone et al., 1991]. However, chromosome 20, which contains the canine *FHIT* gene locus, was not identified as a fragile region. In my study, the AT rich repetitive region, which is the characteristic finding in the CFSs of the human and mouse, was also found on the canine *FHIT* locus, and the genomic deletion in all canine lymphoma cell lines were confirmed. These findings suggest that the canine *FHIT* gene may also be encompassed in the chromosomal fragile site. If it is true, the inactivation events of the canine *FHIT* gene may tend to occur frequently, and result in tumor development. This is a first report showing the association of the aberration of the *FHIT* gene and the Fhit protein expression with the tumorigenesis in dogs, and suggesting the existence of the *FHIT* gene on the canine CFSs. These observations indicate a new insight of the inactivation of the tumor associate gene, which locates in CFSs in tumorigenesis in dogs. I hope the present series of studies will contribute the future research to explore the canine tumor development.

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Table 1. Results of hematological examination at day 1 and day 7

Complete blood count	Day 1	Day 7	Reference range*
RBC [a] ($\times 10^6$/μl)	2.3	2.4	5.5-8.5
Hb [b] (g/dl)	5.3	5.5	12.0-18.0
Hematocrit (%)	16	15	37-55
MCV [c] (fl)	69.5	67.9	60-77
MCHC [d] (g/dl)	32.7	34.2	32-36
Platelet ($\times 10^3$ /μl)	48	4	200-500
WBC [e] (/μl)	14,949	13,450	6,000-17,000
Myelocyte	151	145	
Metamyelocyte	2,265	290	
Band Neutrophil	3,473	1,160	0-300
Segmented Neutrophil	7,701	4,785	3,000-11,500
Eosinophil	0	0	100-1,250
Basophil	0	0	
Lymphocyte	604	725	1,000-4,800
Monoblast	151	145	
Promonocyte	302	2,175	
Monocyte	302	4,025	150-1,350
Nucleated RBC (/μl)	151	870	

a) RBC: red blood cell.
b) Hb: hemoglobin.
c) MCV: mean corpuscular volume.
d) MCHC: mean corpuscular hemoglobin volume.
e) WBC: white blood cell.
* Schalm's Veterinary Hematology 5th ed.

Table 2. Myelogram on day 7

Cell type	Percentage (%)
Rubriblast	0.2
Basophilic rubricyte	0.0
Polychromatic rubricyte	16.9
Metarubricyte	10.4
Myeloblast	5.7
Promyelocyte	0.0
Myelocyte	22.4
Metamyelocyte	14.4
Band neutrophil	7.0
Segmented neutrophil	1.5
Band eosinophil	2.3
Segmented eosinophil	0.0
Band basophil	0.0
Segmented basophil	0.0
Monoblast	2.1
Promonocyte	0.2
Monocyte	10.8
Lymphoblast	0.0
Lymphocyte	5.3
Megakaryoblast	0.0
Megakaryocyte	0.8
M: E ratio [a)]	1.9
Blast ratio	
ANC [b)]	8.5
NEC [c)]	11.7

a) M: E ratio; myeloid cells relative to erythroid cells ratio

b) ANC; all nucleated cells

c) NEC; non-erythroid cells

Table 3. Oligonucleo Table 4. Oligonucleotide primers for PCR amplification

Primers	Sequences (5'-3')
FHIT transcripts detection primers	
e1	TCACTTCCCAGCTGCCAAGATC
eL	TAAGTACATAGCCCAGGAAGTGTGGAAG
BF1	CTGTGGACAGAAACATCCCACCTG
TF5/ TF3[a)]	TAGGATCCGCTTCAACCGTGAGGAAATG/ ACGATATCTTTCATGCCTGTAAAGTCA
RPL32F/ RPL32R	TGGTTACAGGAGCAACAAGAAA/ GCACATCAGCAGCACTTCA
MS-PCR primer pairs	
MpF1/ MpR1	GTGGGTATACGTTTAGGCGTC/ TATAAAAACCAAACGCGCC
UpF1/ UpR1	TTAGTGGGTATATGTTTAGGTGTT/ TATAAAAACCAAACACACCTAA
WpF1/ WpR1	GTGGGCACACGCCCAGGCGCC/ TGTAAGGACCAGACGCGCC
Deletion detection primer pairs	
1F/R	AATTGAGTTACAGGCTTACTTGTGG/ TTGGTTTTGGTATAGGTCACAATCT
2F/R	TAGATGAGAAGAATTTTGGAACAGG/ CTCAATAAACCTGATGGCTACTTGT
3F/R	GAGCACATAACTTCGTTTTACGTTT/ TAATGGTCCATACAGGTGAAATCTT
4F/R	CTCTACAGTGGGTGAGGTTATGTCT/ TGGATAGTTTTCTTGGATACCACAT
5F/R	AGAGAGCTTAACTTTCGTTTTAGGC/ CATGAGGATGATTTCTCTGATTTCT
6F/R	GTGCTGTAGGGCATTCTACTGTATT/ ATTCATTTCCCCATCTCACATACTA
7F/R	CACATCTGAAGACCTCTAGGAGAAG/ TCATGCTAGTGCTCTCTCTCAAATA
8F/R	ACTCTTCAAAACAAAACAAACCAAG/ TTTGGGATTTCATTCAATAAGTGAT
9F/R	GCAGCTTTTCTAGCTCATTTACACT/ ACAACACTATGAGGAATGTGTTTCA
10F/R	TGACCATCCTACAAAAGTACTGACA/ TCTAGGGTTGAGGTCTCCTTAGACT
11F/R	AAAGCAAAGAACTAGGAGAAAGAGG/ GTTTAAACCTCTGTTCCACAAAGAA
12F/R	ATTATGAAAAAGTGAAGCTTTGTGG/ GCCCTTCCTACCATGTCTATTTATT
13F/R	CTCTGGAAGAGTGAGAAGTAACAGG/ CTGGTGTTACTCAAGTTTCTCCATT
14F/R	AGTGAAAGAAGCCAGACACTAAAGA/ TTTAAATGTGGGTATCAAGGAAAAA
15F/R	CCTGGCAAGACTTAGTAAACACATT/ GACGTAAGTCTTTCTAATGCAGAGC
16F/R	CATCTAGGAGCTCATTAAAAATGGA/ AGAGGGGAGATACCTGCTAAATAGA
17F/R	ATGTAATAATTCCTGGAAGGGAGAC/ GGTTTATACAGATTGGATGATTTGC
18F/R	TTGTTGGGAAATCATAACAGATTTT/ ATTCCTGTATACTTCTGCCTGTGAC
19F/R	GGCAAAATCAAAGATGAATAAGCTA/ GCTCATGGTCAGTACACACTTTATG
20F/R	AACAACCGAAATGTTATTGATGGTG/ GTCGCCTGAAACAAATCTGCCACTTC

a) These primers contain restriction enzyme cutting sites for *Bam*HI (TF5) and *Eco*RV (TF3), respectively.

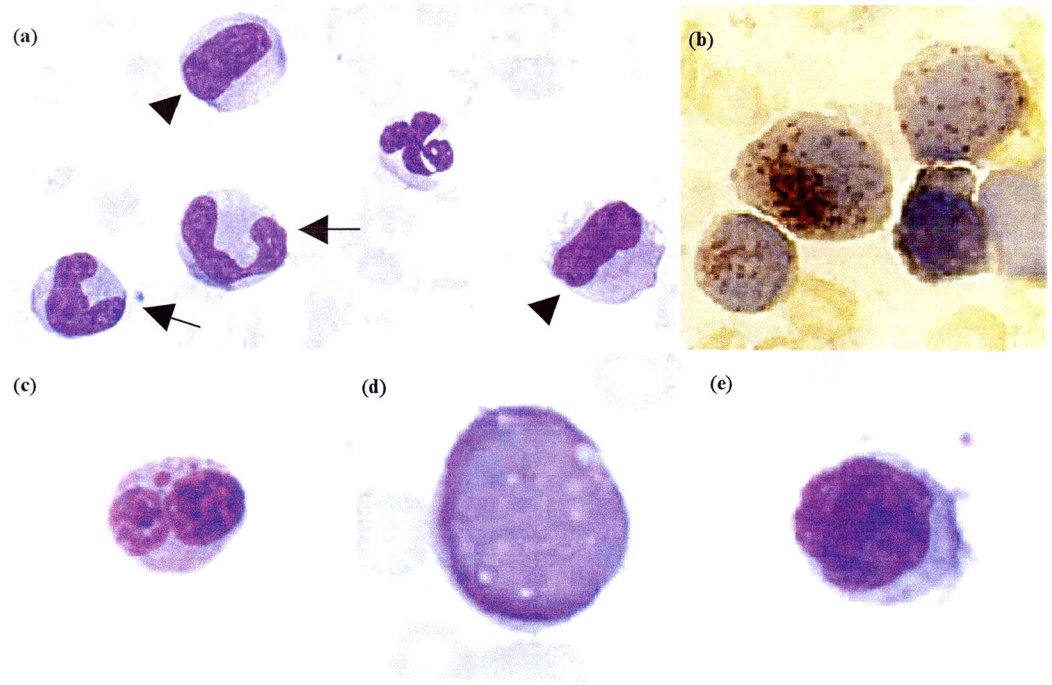

Fig. 1. Photomicrographs of the peripheral blood on day 7. The numbers of monocytic lineage cells were increased than those of day 1, and dysplastic features are obtained in all three cell lines. (a) Promonocytes (arrowheads) and monocytes (arrows), (b) positive staining for alpha-naphthyl butyrate esterase, (c) lobulated rubricytes, (d) large palatets and (e) micromegakaryocytes.

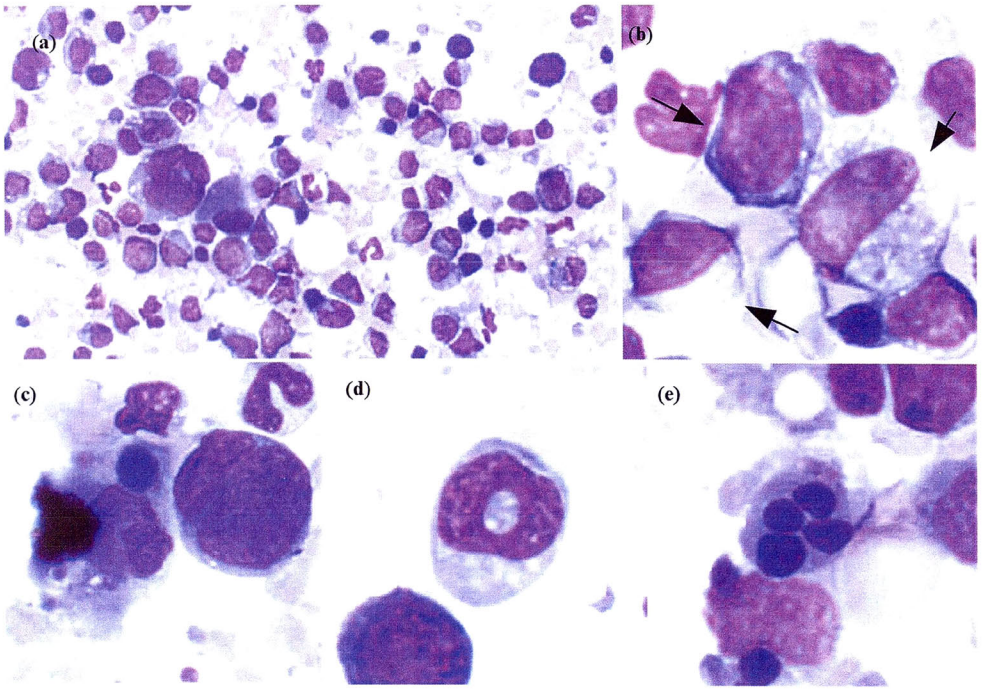

Fig. 2. Photomicrographs of the bone marrow aspirate on day 7. (a) Low magnification of the marrow smear, (b) monocytic lineage cells (arrows), (c) macrophage phagocy tosis and unequal division of blast cell, (d) doughnut-shaped neutrophil precursors and (e) rubricytes with multi-nuclei.

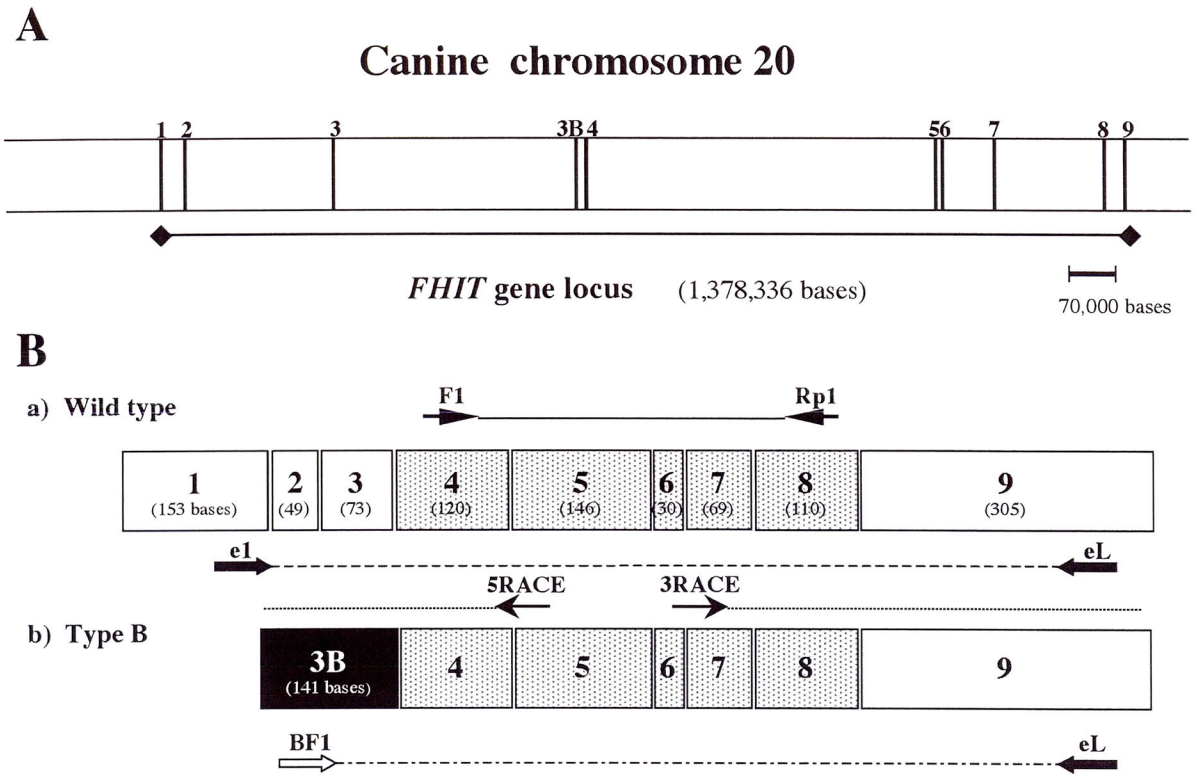

Fig. 3. **A.** *FHIT* gene locus on canine chromosome 20. The *FHIT* gene exons are surrounded by extremel y large introns. Each exon is shown by a black box containing the number of the exon. A specific exon in the type B transcript, exon 3B, is present between exons 3 and 4 of the wild-type transcript. **B.** Two types of canine *FHIT* transcript were obtained. The wild-type transcript consists of nine exons and shows a basically similar structure to the *FHIT* genes in other species (a). Comparison of the two transcripts shows that the type B transcript includes an exon, exon 3B, in the 5' non-coding region that is not found in the wild-type transcript (b). The open reading frames of these two transcripts (shaded boxes) share the same amino acid sequence. The numbers below the exon numbers indicate the length of each exon, and the arrows indicate the primers used in this study.

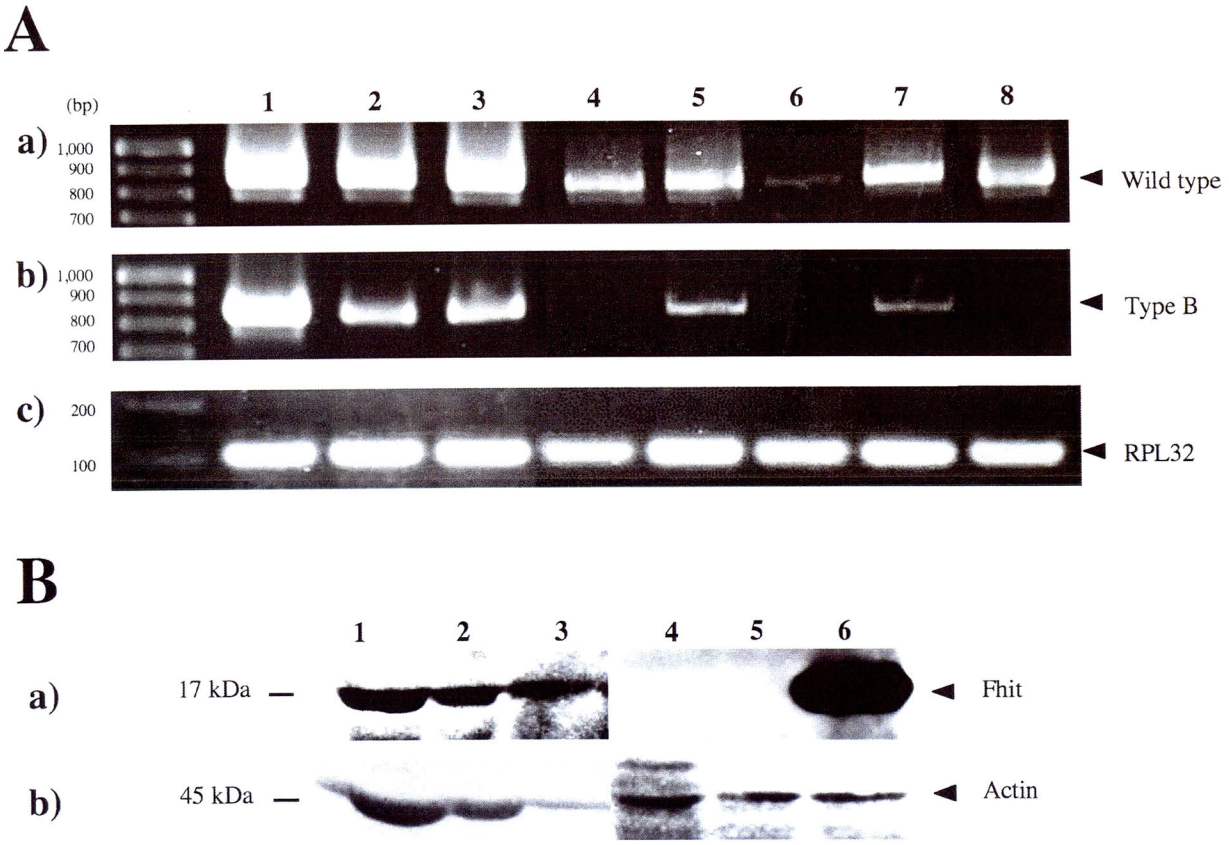

Fig. 4. **A.** RT-PCR analysis of the *FHIT* gene in canine PBMCs and other tissues. The wild-type and type B transcripts of the *FHIT* gene were amplified using e1/eL primers (a) and BF1/eL primers (b), respectively. As an internal control, canine ribosomal protein L32 (RPL32), which is a stable housekeeper gene, was used for detection of the canine lymphatic cells and tissues (c) [Peters et al., 2007]. Lanes 1-3, PBMCs from different dogs; lane 4, lung; lane 5, intestine; lane 6, liver; lane 7, spleen; and lane 8, thymus.

B. Immunoblotting of the Fhit protein from canine PBMCs and from a canine *FHIT* gene-transfected cell line. Immunoblot analysis was performed using an anti-human Fhit polyclonal antibody (clone ZR44, Invitrogen) (a). Anti-human actin polyclonal antibody was used for reprobing (b). Lanes 1-3, PBMCs from different dogs; lane 4, parental KK47 cells; lane 5, empty vector-transfected KK47 cells; and lane 6, canine *FHIT*–transfected KK47 cells.

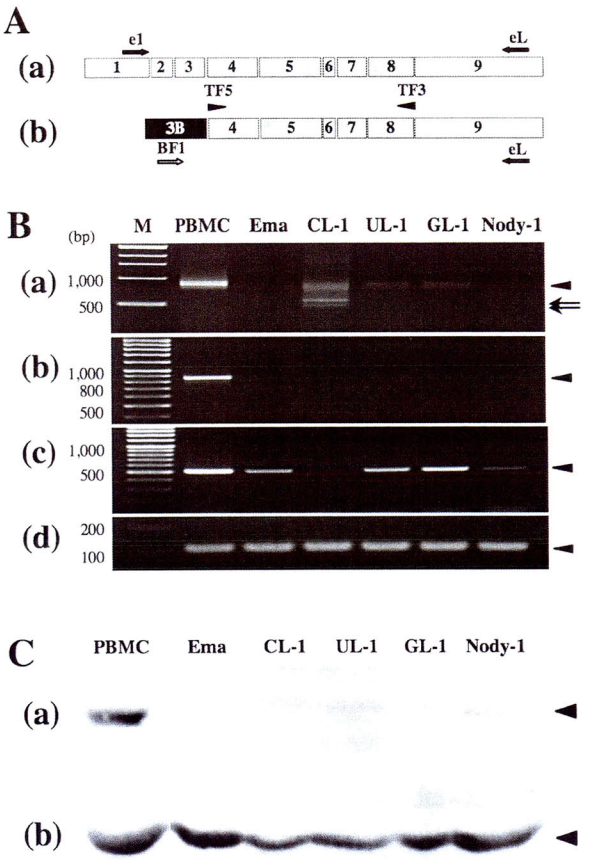

Fig. 5. **A.** The 2 types of canine *FHIT* transcripts. (a) The wild-type canine *FHIT* transcript is composed of 9 exons and is amplified by the primers e1/eL (black arrows). (b) The alternative splicing form o f the canine *FHIT* transcript (Type B) has a unique 5′ non-coding sequence, exon 3B, and is amplified by primers BF1/eL (white and black arrows, respectively). These 2 types of transcripts share the same ORF, which corre sponds to exons 4–8 (shaded boxes), and the TF5/TF3 primer pair (arrowheads) amplifies this region. **B.** RT-PCR analysis of the *FHIT* gene transcripts in the canine lymphoma cell lines. Expression of the wild-type *FHIT* transcript (a), the type-B transcript (b) and the ORF region (c) was determined by RT -PCR. The canine ribosomal protein L32 (RPL32) was amplified as an internal control (d). The arrowheads indicate the expected bands, and arrows indicate the unexpected bands observed in the CL-1 cell line. **C.** Immunoblot analysis for detection of Fhit protein in the canine lymphom a cell lines. The expression of canine Fhit protein was detected using an antihuman Fhit polyclonal antibody (clone ZR44; Invitrogen) (a). As a control, an antihum an actin polyclonal antibody (clone C-11; Santa Cruz Biotechnology) was used to reprobe the membrane (b).

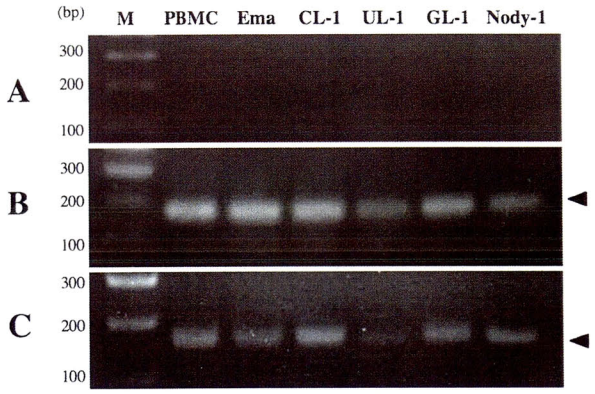

Fig. 6. Methylation-specific PCR (MS-PCR) analysis of canine lymphoma cell lines. DNA extracted from the 5 lymphoma cell lines and the PBMCs isolated from a healthy dog were subjected to bisulfite modification followed by PCR amplification using primers MpF1/MpR1 (A) or UpF1/UpR1 (B), which amplify methylated *FHIT* DNA or unmethylated *FHIT* DNA, respectively. As a control, wild-type *FHIT* DNA, which had not undergone bisulfite modification, was also amplified by the primers WpF1/WpR1 (C).

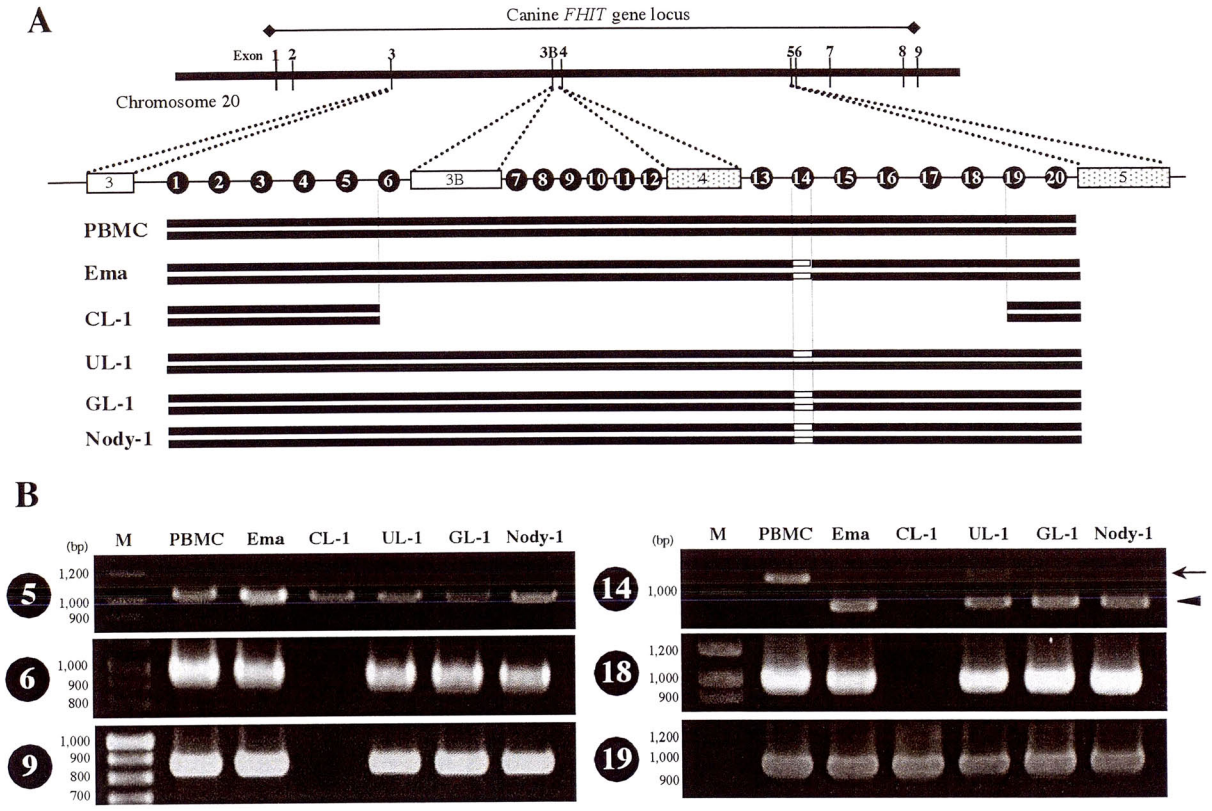

Fig. 7. **A.** Genomic deletions in the canine lymphoma cell lines. In order to identify the deletions of the canine *FHIT* genomic locus in the cell lines, I prepared 20 primer sets (shown in black circles) corresponding to the intronic region between exon 3 and the type B-specific exon 3B (1–6), exon 3B and exon 4 (7–12) and exon 4 and exon 5 (13–20) on canine chromosome 20. The allelic information of the 5 lymphoma cell lines and the PBMCs isolated from a healthy dog is shown by black bars. The white bars shown at the 14th position represent intermittent deletions containing the 200-, 11-, 3- and 2- bp deletions (see text). **B.** Representative results of PCR analyses for detection of allelic information in the canine lymphoma cell lines. The DNA samples from the normal control PBMCs and the 5 lymphoma cell lines were subjected to PCR amplification to identify homozygous or heterozygous deletions. Each black circle with a white number represents an amplified region. In the 14th position, I observed an expected band (an arrow) and a smaller band (an arrowhead), and sequencing analysis was used to confirm that the latter band resulted from intermittent deletions containing 200-, 11-, 3- and 2-bp.