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

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ABSTRACT. 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 this study, we 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. We 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, we could not confirm the involvement of aberrant methylation events in the 5' CpG islands of the canine *FHIT* gene. We were 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 the present study, we 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.
KEY WORDS: cancer, canine, DNA methylation, gene expression, lymphoma/leukemia.

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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 [3, 11, 28]. 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, esophageal, breast and cervical tumors [11, 28]. 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 [17, 28]. The product of this gene, Fhit protein, is considered to be a tumor-suppressor protein and a modulator of the cell cycle and apoptosis [19, 20].

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 [16, 26]. 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 [30, 33, 39, 40]. 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 [30, 40]. Furthermore, homozygous or heterozygous deletion of the *FHIT*/FRA3B region has been reported in some cancer cells [1, 8, 18, 22, 27]. 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 [6, 18]. 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 [8].

Recently, we 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 [15]. 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 our knowledge, the relationship between the canine *FHIT* gene and tumor development in dogs has not been explored. In this study, we focused on canine lymphoma, which is one of the most common tumors in dogs. Canine lymphoma accounts for

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approximately 7%–24% of all canine tumors and 83% of all canine hematopoietic malignancies [32], and the inactivation of p16 cyclin-dependent kinase inhibitor has recently been shown to be associated with tumorigenesis in canine lymphoma [9]. 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, we examined the aberrations of the *FHIT* gene and Fhit protein expression by using canine lymphoma cell lines. Furthermore, we explored the hypermethylation of 5' CpG islands and the effects of *FHIT* allelic deletion.

MATERIALS AND METHODS

Cells: In this study, we 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) [23, 24, 35]. 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/m*l*) and streptomycin (100 μg/m*l*)] 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 hr 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, U.S.A.), and single-strand cDNA was synthesized from 2.5 μg of each total RNA sample by using SuperScript II RT (Invitrogen, Carlsbad, CA, U.S.A.) with an oligo-dT primer in accordance with the manufacturer's instructions. We prepared 3 primer pairs–e1/eL, BF1/eL and TF5/TF3 (Table 1)–to detect the canine *FHIT* genes; in our previous study, we had confirmed that these 3 primer pairs amplify the canine *FHIT* gene transcripts [15]. 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. 1A). The canine ribosomal protein L32 (RPL32), which is a stable housekeeping gene in canine lymphatic cells and tissues, was amplified as a control (Table 1) [25]. A thermal GeneAmp PCR system 9700 (Applied Biosystems, Foster, CA, U.S.A.) 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 a previous study [15]. 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/m*l* leupeptin and 2 μg/m*l* 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 hr, incubated with the antihuman Fhit antibody (diluted 1:500 in TBS-T) for 1 hr 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 hr 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, U.S.A.) 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, U.S.A.).

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 [30] 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 1). 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, U.S.A.) in accordance with the manufacturer's instructions. We used a HotStarTaq master mix kit (QIAGEN) and 50 *n*g 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, we 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 1). 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: We 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. 1B (a)]. Furthermore, we observed a faint band in the case of the UL-1 and GL-1 cell lines, but we did not detect any transcript in the case of the Ema and Nody-1 cell lines. In the case of CL-1, we detected a smear and 2 small transcripts with lengths of 500 bp and 600 bp [Fig. 1B (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. We used the e1/eL primer pair to analyze the transcripts from CL-1; however, we were 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. 1B (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. 1B (c)]. Sequence analysis of the ORF in the Ema and Nody-1 cell lines showed

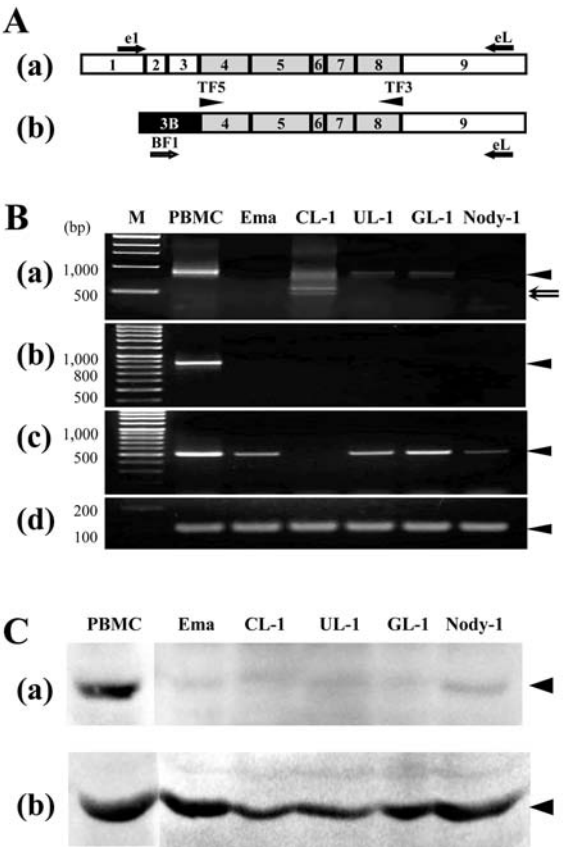

Fig. 1. A. The 2 types of canine *FHIT* transcripts. (a) The wild-type canine *FHIT* transcript is composed of 9 exons and is amplified by the primers e1/eL (black arrows). (b) The alternative splicing form of the canine *FHIT* transcript (Type B) has a unique 5' non-coding sequence, exon 3B, and is amplified by primers BF1/eL (white and black arrows, respectively). These 2 types of transcripts share the same ORF, which corresponds 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 lymphoma cell lines. The expression of canine Fhit protein was detected using an antihuman Fhit polyclonal antibody (clone ZR44; Invitrogen) (a). As a control, an anti-human actin polyclonal antibody (clone C-11; Santa Cruz Biotechnology) was used to reprobe the membrane (b).

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 our previous study, 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 [15]. We performed immunoblot

Table 1. Oligonucleotide primers for PCR amplification

Primers	Sequences (5'–3')
FHIT transcripts detection primers	
e1	TCACTTCCCAGCTGCCAAGATC
eL	TAAGTACATAGCCCAGGAAGTGTGGAAG
BF1	CTGTGGACAGAAACATCCCACCTG
TF5/ TF3[a)]	TAGGATCCGCTTCAACCGTGAGGAAATG/ ACGATATCTTTCATGCCTGTAAAGTCA
RPL32-F/ RPL32-R	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.

analysis by using this antibody in order to evaluate expression of the Fhit protein in canine lymphoma cell lines. As shown in Fig. 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. 2A); in contrast, use of the primers UpF1/UpR1 yielded amplified unmethylated DNA from all the cell lines and the control PBMCs (Fig. 2B), 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. 2C). 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

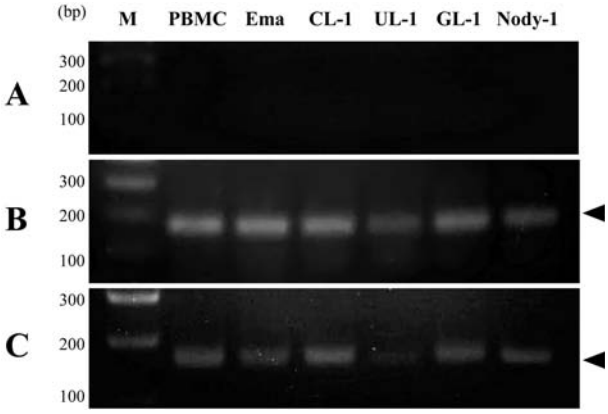

Fig. 2. Methylation-specific PCR (MS-PCR) analysis of canine lymphoma cell lines. DNA extracted from the 5 lymphoma cell lines and the PBMCs isolated from a healthy dog were subjected to bisulfite modification followed by PCR amplification using primers MpF1/MpR1 (A) or UpF1/UpR1 (B), which amplify methylated *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).

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 1). We did not detect any deletions between exons 3 and 3B in the Ema, UL-1, GL-1 and Nody-1 cell lines. However, we 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. 3). 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. 3). In contrast, we 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. 3B). 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 [2, 13, 16, 26, 39]. Furthermore, a decrease in Fhit protein expression has been correlated with poor clinical prognosis of some human tumors,

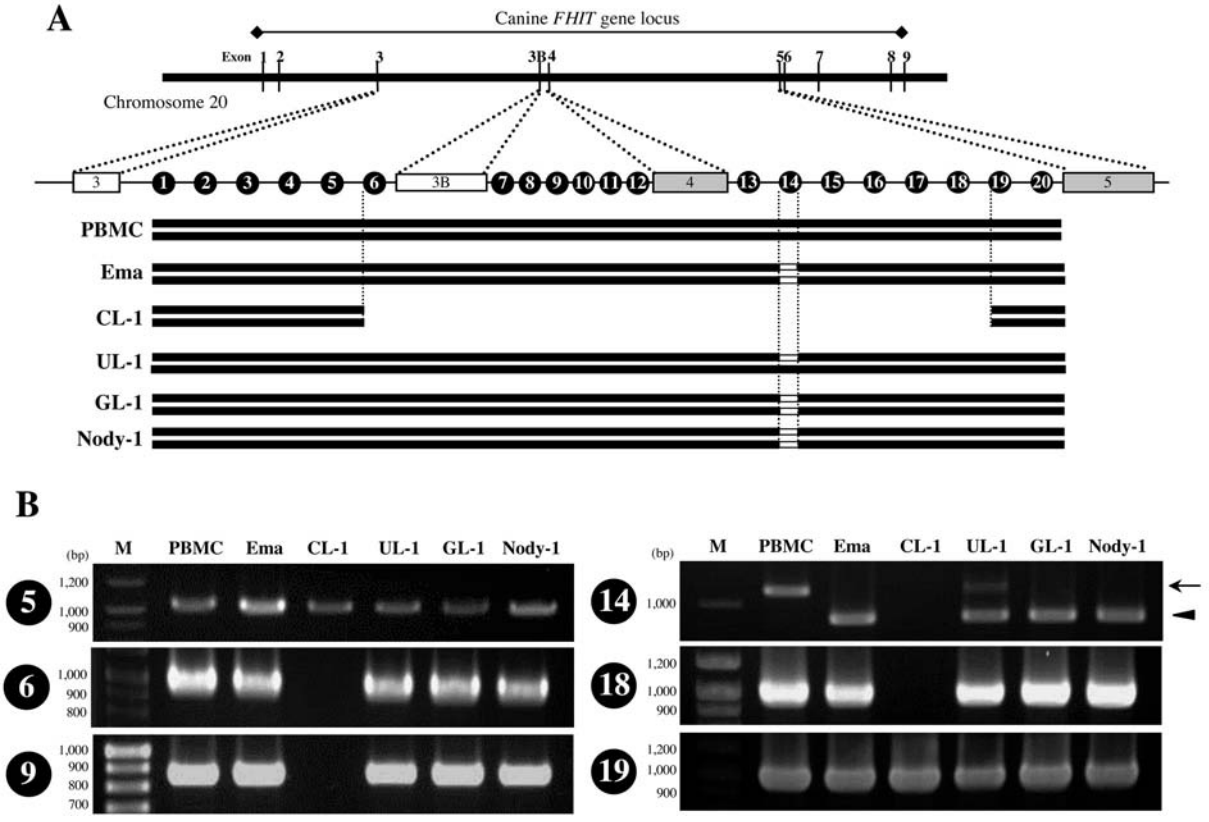

Fig. 3. A. Genomic deletions in the canine lymphoma cell lines. In order to identify the deletions of the canine *FHIT* genomic locus in the cell lines, we prepared 20 primer sets (shown in black circles) corresponding to the intronic region between exon 3 and the type B-specific exon 3B (1–6), exon 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, we 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.

including lung, oral and breast cancers [4, 12, 31]. 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 [7, 38]. 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 [19, 20]. Other studies have described that Fhit suppresses target genes such as *cyclin D1, axin2*, *Mmp-14* and *survivin* by directly binding to β-catenin [34].

Aberrant *FHIT* transcripts lacking regions between exons 3–9 have been detected in human hematopoietic malignancies [13, 21, 26, 29], although the wild-type transcript is also expressed along with these transcripts [13, 21, 26, 29]. In contrast, complete loss of *FHIT* transcripts is detected infrequently [13, 21, 26, 29]; nevertheless, Fhit protein is frequently absent in these tumor cells [13, 26]. In this study, we 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. 1B (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. 1B (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. 3). Furthermore, in the RT-PCR analysis using the e1/eL primers, we obtained approximately 500-bp- and 600-bp-long aberrant *FHIT* transcripts from the CL-1 cDNA [Fig. 1B (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, we performed RT-PCR amplification of the cDNA between exons 1 and 5 by using the e1 primer along with a newly designed antisense primer. We 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. 3A). 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, we detected a reduction (or absence) of Fhit protein expression in the CL-1 cell line (Fig. 1C). 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 [18]. 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.

It has recently 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 [15]. 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. We 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 [30, 33, 39, 40]. The 5' CpG island of the human *FHIT* gene is located in a 460-bp region around exon 1 (from –95 to +365 bp) [30]; 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 [30]: GC content, 66.5%; CpG density, 7.8%; and observed/expected CpG index, 0.7. We 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, we 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 [30, 40]. Furthermore, murine *FHIT* gene analysis has revealed the presence of cancer- or tissue-specific methylation patterns [14]. In the present study, we 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, we 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 [6, 18]. Human exon 5 corresponds to canine exon 4, which contains the initiation site of the ORF in the canine *FHIT* gene [15]. 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, we 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, we could not confirm the involvement of aberrant methylation events in the 5' CpG island of the canine *FHIT* gene. However, we were 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 [5, 21, 26, 36], and the incidence of reduction or absence of Fhit protein expression has been shown to be approximately 58%–76% [2, 5, 13]. 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 [37, 39], and allelic loss of the *FHIT* gene locus has been described in chronic lymphocytic leukemia [10]. In the present study, we detected decreased expression of wild-type *FHIT* transcripts and concurrent expression of aberrant *FHIT* transcripts in canine lymphoma cell lines; we 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, we 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.

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