A Deletion Mutation Causes Hemophilia B In Lhasa Apso Dogs

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Hemophilia B is a bleeding disorder caused by a deficiency of clotting factor IX (FIX). A colony of FIX deficient Lhasa Apso dogs has been established and the molecular basis of hemophilia B has been determined. The plasma factor IX levels were <1% of normal canine levels in affected dogs. A complex deletion mutation at nucleotides 772-777 was found when hepatocyte cDNA from a hemophilia B dog was sequenced. The sequence was identical to the normal canine sequence except for a deletion including nucleotides 772-776 and a C → T transition at nucleotide 777. The mutation results in mRNA instability and a premature termination codon in the nucleotide sequence encoding the activation peptide. The mutation was verified by sequencing genomic DNA from an FIX-deficient dog. A genetic test for the detection of heterozygous animals was established using heteroduplex analysis. Although hemophilia B has been described in many dog breeds, this is only the second mutation to be sequenced. The Lhasa Apso dog model should be valuable for evaluating novel strategies for treating hemophilia B such as gene therapy.

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Materials and Methods

Experimental animals. Normal, heterozygous, and FIX-deficient dogs were healthy and disease free at the time of study. Experimental animals were housed in a facility accredited by the American Association for Laboratory Animal Care. The experimental protocols were approved by the institutional Animal Care and Concerns Committee.

Activated clotting time (ACT). Two milliliters of blood was collected into a prewarmed (37°C) ACT tube containing 6 to 10 mg siliceous earth (Becton Dickinson VACUTAINER Systems, Rutherford, NJ); the sample was mixed by inverting the tube 5 times and was incubated at 37°C. After a 1 minute incubation, the tube was removed from the heating block at 15 second intervals to observe the appearance of the first visible clot. The normal range for dogs is 64 to 95 seconds. 

Activated partial thromboplastin time (APTT). Plasma samples were obtained from blood collected with 0.109 mol/L trisodium citrate at a 9:1 ratio. One hundred microliters of actin cephaloplastin (Baxter, Miami, FL) was prewarmed to 37°C to which 100 μL of the test plasma was added and the mixture was incubated at 37°C for an additional 3 minutes. One hundred microliters of prewarmed 0.02 mol/L calcium chloride (Baxter) was added and the time to initial clot formation determined with a fibrometer (Becton Dickinson and Co, Cockeysville, MD). The normal range for dogs is 14 to 20 seconds.

Canine FIX antigen assay. Ninety-six well enzyme-linked immunosorbent assay (ELISA) plates were coated with 100 μL/well of a mouse antihuman factor IX monoclonal antibody (Boehringer Mannheim, Indianapolis, IN) diluted 1:100 in 0.1 mol/L sodium bicarbonate (pH 9.4) for 2 hours at room temperature, rinsed 5 times with 0.05% Surfact-Amps Tween 20 in phosphate-buffered saline (PBS), and then blocked for 1 hour at room temperature using Super Block (Pierce, Rockford, IL). One hundred microliters of plasma was then applied to each well and incubated at 4°C overnight, rinsed with 0.05% Surfact-Amps Tween 20 in PBS, incubated with 100 μL of a 1:50 dilution of a rabbit antihuman FIX antibody (Accurate, Westbury, NY) for 2 hours at room temperature, rinsed, and incubated with 100 μL of a peroxidase-conjugated goat anti-rabbit IgG (H + L) (Accurate) for 2 hours at room temperature. The plates were developed with 100 μL of 1 mg/mL 2,2’-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium...
salt for 30 minutes or until sufficient color developed, then the reaction was stopped using 50 μL of 1% sodium dodecyl sulfate (SDS). The assay was read at 405 nm on an ELISA plate reader (SLT Lab Instruments, Tecan, Research Triangle Park, NC). The assay sensitivity was 0.4% of normal canine plasma FIX levels.

**Southern blot analysis.** Mononuclear cells were isolated from 10 mL of whole blood using 30 mL of a red blood cell lysis buffer (155 mmol/L NH₄Cl, 10 mmol/L NH₄HCO₃, 0.1 mmol/L EDTA). The mononuclear cells were centrifuged and then resuspended in 3 mL of DNA extraction buffer (10 mmol/L Tris, 0.1 mmol/L EDTA, 0.5% SDS, pH 8.0) containing 400 μg of proteinase K and incubated overnight at 60°C. The digest was then subjected to two rounds of phenol:chloroform:isoamyl alcohol (25:24:1) extraction and one chloroform:isoamyl alcohol extraction (24:1). Genomic DNA was then precipitated with isopropanol/sodium acetate for 30 minutes or until sufficient color developed, then the DNA was resuspended in 3 mL of DNA extraction buffer (24:1) (GIBCO-BRL, Gaithersburg, MD) extraction and one ch1oroform:isoamyl alcohol (25:24:1). Genomic DNA was then precipitated with a one-fifth volume of 10 mmol/L NH₄Cl, 10 mmol/L NH₄HC03, 0.1 mmol/L EDTA). The mixture was extracted once with phenol:chloroform:isoamyl alcohol (25:24:1), precipitated with isoopropanol/sodium acetate and resuspended in diethylpyrocarbonate (DEPC) treated water and RNasin (Promega, Madison, WI). Total hepatic RNA was isolated using standard procedures. Briefly, 3 g of liver was homogenized with 10 mL of 4 mol/L guanidine isothiocyanate (GTC) and 7.8 μL of β-mercaptoethanol, N-lauryl sarcosinate and sodium citrate were then added to a final concentration of 0.5% and 25% mol/L, respectively. The mixture was extracted once with phenol:chloroform:isoamyl alcohol (25:24:1), precipitated with isopropanol/sodium acetate and resuspended in diethylpyrocarbonate (DEPC) treated water and RNasin (Promega, Madison, WI). Total hepatic RNA was separated by formaldehyde gel electrophoresis and subjected to Southern blot analysis. The probe, a purified 501 bp polymerase chain reaction (PCR) amplicon (Six2-AIX5) from canine FIX cDNA, was labeled with 32P-dCTP using the random hexamer method with a Random Prime-it kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The blot was washed to a final stringency of 0.1 × SSC and 0.1% SDS at 65°C.

**Northern blot analysis.** Total hepatic RNA was isolated using standard procedures. Briefly, 3 g of liver was homogenized with 10 mL of 4 mol/L guanidine isothiocyanate (GTC) and 7.8 μL of β-mercaptoethanol, N-lauryl sarcosinate and sodium citrate were then added to a final concentration of 0.5% and 25% mol/L, respectively. The mixture was extracted once with phenol:chloroform:isoamyl alcohol (25:24:1), precipitated with isopropanol/sodium acetate and resuspended in diethylpyrocarbonate (DEPC) treated water and RNasin (Promega, Madison, WI). Total hepatic RNA was separated by formaldehyde gel electrophoresis and subjected to Northern analysis using the same probe as used for Southern blot analysis. The blot was stripped by boiling in 0.1 × SSPE, 1% SDS for 30 minutes and rehybridized with a mouse β-actin probe to estimate RNA loading.

Reverse transcription (RT) and DNA amplification. Synthesis of single-stranded cDNA from total hepatic RNA of a FIX-deficient dog was performed using the Superscript RT kit (GIBCO-BRL), 6 μg total hepatic RNA, and 0.1 μmol/L of sequence specific primer Aix1 (5’-CCCCAGAAAGAGAAAAACACG-3’). The RNA and primer were combined, heated to 90°C, and then cooled to 70°C for primer annealing. SuperScript reverse transcriptase, dNTPs, synthesis buffer, and dithiothreitol (DTT) were then added and the reaction tube incubated at 45°C for approximately 2 hours. The reaction was then added to a final volume of 100 μL containing 1 × PCR buffer (50 mmol/L KCl, 10 mmol/L Tris-HCl [pH 9.0 at 25°C], 0.1% Triton X-100 [Sigma, St Louis, MO]), 2.5 mmol/L MgCl2, 0.2 mmol/L deoxynucleoside triphos-phates, sequence specific primers (each at a concentration of 0.1 μmol/L), and 2.5 U of thermostable Taq DNA polymerase (Promega). Amplification was performed in a PTC-100 thermal cycler (MJ Research, Inc, Watertown, MA) with an initial denaturing step of 95°C for 10 minutes, followed by 40 cycles of annealing at 55°C for 1 minute, extension at 72°C for 2 minutes, and denaturing at 94°C for 1 minute.

**Cloning and DNA sequencing.** The FIX cDNA sequence from a hemophilia B dog was determined from overlapping PCR amplicons. The Taq polymerase was removed from PCR reaction mixtures using Stratagene resin (Stratagene) and the products were then desalted and concentrated using Amicon spin columns (Amicon, Inc, Beverly, MA). The termini of the PCR products were filled in using 3 U of Klenow DNA polymerase (NEB, Beverly, MA) and phosphorylated using 4 U of T4 polynucleotide kinase (NEB). The SIX5-AIX2 and GSIX4-GAIIX4 amplicons were then blunt-end ligated into the Smal I digested, dephosphorylated pGem-4Z vector. SIX1-AIX6, SIX4-AIX4, and SIX2-AIX5 PCR amplicons were self-ligated to form concatenators, digested with the appropriate restriction enzyme to generate homologous sticky ends, and then ligated into the dephosphorylated pGem-4Z vector, which had been digested with the same restriction enzyme. Competent DH5α cells (GIBCO-BRL) were transformed with the ligation reactions and plated on ampicillin/X-Gal coated LB agar plates. Positive clones were identified by PCR using sequence specific primers and sequenced with the Sequenase version 2.0 T7 DNA polymerase sequencing kit (US Biochemical, Cleveland, OH). The sequencing reactions were performed according to manufacturer's instructions. The nucleotide sequences were compared to the previously published normal canine sequence and discrepancies between the normal canine factor IX sequence and that obtained from the hemophilic animal were verified by sequencing genomic DNA. At least 2 clones of each amplicon, except for SIX4-AIX4 which contained the mutation, were sequenced on the sense and antisense strands and were identical to the normal canine FIX cDNA sequence. Two clones from the SIX4-AIX4 amplicon and 2 GSIX4-GAIIX4 genomic clones were sequenced in both directions and were identical to the normal canine sequence except for the mutation.

**RESULTS**

Clotting parameters. The coagulation tests in the two affected male dogs were consistent with FIX deficiency. The APTT clotting times were 47.5 and 60 seconds (normal range 14 to 20 seconds) and the ACT times were 5.4 30' and 3' 15' (normal range 64 to 95 seconds) for dogs A and B, respectively. Both dogs had FIX levels of <1% in a FIX specific APTT, whereas the other intrinsic clotting factor activities were normal or increased. The hemophilia B dogs also had <1% of the normal canine plasma FIX antigen level.

**Southern and Northern analysis.** Southern blot and Northern blot analysis were performed to determine the nucleotide level for the severe FIX deficiency. The restriction fragment length digestion patterns for the two FIX-deficient dogs and two normal dogs were similar in DNA digested with BamHI and EcoRI indicating there were no major rearrangements, additions, or deletions in the FIX gene (data from www.bloodjournal.org by guest on October 31, 2017. For personal use only. null)
Fig 1. Northern blot analysis of FIX mRNA. Total hepatic RNA, either 5 μg (lanes 1 and 2) or 10 μg (lanes 4 and 5) from hemophilia B (lanes 2 and 5) and normal animals (lanes 1 and 4) was analyzed by formaldehyde gel electrophoresis. Hybridization was performed using a 32P-labeled canine FIX probe. The same blot was stripped and reprobed with 32P-labeled β-actin to verify equal loading of RNA. Lane 3, empty.

not shown). Northern blot analysis of total hepatic RNA from a normal and hemophilic dog showed a single 3 kb band when probed with the canine FIX cDNA (Fig 1). Although the apparent size of the FIX mRNA was similar in the normal and hemophilic dog, the quantity of FIX mRNA was significantly decreased in the hemophilic dog (Fig 1, lanes 2 and 5), even though the blot was loaded equivalently as determined by probing for β-actin mRNA (Fig 1).

DdeI mutation analysis. Factor IX deficiency in the Chapel Hill hemophilia B dog colony is due to a G → A transition at nucleotide 1477. The G → A transition results in the creation of a new restriction site for DdeI, which is the basis for a PCR diagnostic test. Amplification of canine genomic DNA, using specific sense and antisense primers, results in a 133 bp product in both normal and mutant DNAs. However, DdeI digestion of mutant DNA generates bands of 54 bp and 79 bp, whereas normal DNA is resistant to DdeI digestion. The 133 bp amplicon from both FIX-deficient Lhasa Apso dogs was resistant to DdeI digestion (data not shown) indicating that the FIX deficiency in these animals is caused by a different mutation than the previously published G → A transition.

FIX-mutation in Lhasa Apso dogs. The entire coding region of the FIX-deficient cDNA from a FIX-deficient Lhasa Apso dog (Dog A) was sequenced by the dideoxy-nucleotide chain termination method with PCR amplified hepatocyte cDNA. The sequence was identical to that previously reported, except for a 5 bp deletion at nucleotides 772-776 and a C → T transition at nucleotide 777 resulting in a transcriptional frameshift and a termination codon at amino acid 146 of the mutant sequence (Fig 2). This mutation was verified by sequencing genomic DNA from a second FIX-deficient dog (Dog B).

Heteroduplex analysis. Genomic DNA from normal, heterozygous, and affected dogs was amplified using FIX primers GSIX4 and GAIX4, which flank the 5 bp deletion. The expected amplicon sizes are 181 and 176 bp in normal and FIX-deficient dogs, respectively. The normal and FIX deficient dogs had single bands at 181 bp and 176 bp, respectively, but surprisingly, a distinct band appeared at approximately 225 bp in heterozygous dogs and with a mixture of DNA from normal and FIX-deficient dogs (Fig 3). The upper band in DNA from heterozygous dogs and mixed DNA from normal and affected dogs disappeared after S1 nuclease digestion (Fig 3, lanes 6 and 8).

Fig 2. The FIX mutation in Lhasa Apso dogs (A). Polyacrylamide gel electrophoresis of sequencing reactions from mutant genomic DNA. The normal and mutant nucleotide sequence and deduced amino acid sequence are shown. (B) Also shown is a schematic depiction of the mutation and primer locations in the FIX protein and cDNA.
The decreased amount of FIX mRNA on the Northern blot suggested either a promoter/enhancer regulatory defect resulting in decreased transcriptional initiation or mRNA instability caused by premature termination. Because the mutation causes the expression of a premature termination codon at amino acid 146, it is likely that the decreased mRNA levels are caused by transcript instability. This conclusion is supported by the fact that mRNA stability for triosephosphate isomerase, β-hexosaminidase A, β-globin, histones, and pyruvate kinase were decreased when the coding region was shortened or lengthened in association with either a frameshift or nonsense mutation.

Mutations in the human FIX gene have been extensively characterized and provide insight into the patterns and distribution of mutations occurring within a gene. Of the 1,380 patient entries in the sixth edition of the hemophilia B database, there were 86 deletions, 21 additions, and 6 entries involving both deletions and additions of <30 nucleotides.\(^5\) More than 25% of the independent mutations observed in the factor IX gene are transitions at the dinucleotide CpG.\(^5\) Transitions at the dinucleotide CpG occur approximately 24-fold more frequently than transitions at non-CpG dinucleotides.\(^22\) Analysis of mutations in the human FIX gene supports the hypothesis that the Lhasa Apso mutation is caused by a 5 bp deletion at position 772-776 with cytosine deamination at position 777. There have been no additions or deletions reported at position 777 in the human gene but there have been 23 cases of C → T transition occurring at this nucleotide position in the human gene.\(^5\) Alternatively, the mutation could result from a 6 bp deletion with an insertion of a single T. The exact mechanism for the dog mutation is not known but a purine rich consensus sequence common to deletion hot spots in 5 different human genes, a direct repeat element (CACG\(_{6}\)CACG\(_{6}\)) and an inverted palindrome (CACG\(_{6}\)CACG\(_{6}\)) are adjacent to or involved in the deletion mutation (bold nucleotides). The purine rich and secondary structure elements are implicated as mutational hot spots caused by polymerase α arrest and slipped mispairing loops during DNA replication.\(^22\)

The nonsense mutation found in the Lhasa Apso dog model results in the expression of a termination codon at amino acid position 146 and thus a predicted protein of considerably shorter length as compared to 414 amino acids for the wild-type protein. The deletion/deamination mutation in the Lhasa Apso dog occurs immediately preceding the activation peptide with the termination codon being the first codon encoded in the activation peptide. The truncated protein would lack the catalytic domain and should not have enzymatic activity. There are 5 frameshift mutations in the human database just 5′ to the Lhasa Apso dog mutation which also result in FIX activity and FIX antigen levels that are <1%.\(^3\) The canine FIX mutation that was previously reported by Evans et al\(^1\) has not been identified in the human FIX gene. The missense mutation at nucleotide 1477 (G → A) is located in the catalytic domain of the FIX gene. The Chapel Hill mutation is thought to cause steric hindrance, which prevents proper folding of the molecule and structural instability. The mutations in the Chapel Hill and Auburn hemophilia B dogs are different but both result in severe hemophilia B.

The Chapel Hill mutation resulted in the creation of a new restriction enzyme recognition site for Ddel, which was used to develop a genetic test that could distinguish normal, mutant, and heterozygous animals.\(^9\) The 5 bp deletion in the Lhasa Apso dogs did not result in the appearance of a new restriction enzyme site, so heteroduplex analysis was used to establish a genetic test. Heteroduplex analysis is a rapid and reliable method for detecting single base changes, deletions, and additions.\(^26\) A 181 bp PCR amplicon (GSIX4-GAIX4) from genomic DNA consistently formed a heteroduplex with DNA from heterozygous animals, thus allowing rapid detection of carrier animals. Mixtures of DNA from normal and mutant animals also generated the novel 225 bp heteroduplex band, confirming that PCR amplification of DNA coding for the normal and mutant alleles results in spontaneous heteroduplex formation. Heteroduplex analysis has also been used to screen for single base changes in exon 6 of the human FIX gene.\(^29\)

The Lhasa Apso hemophilia B model should be valuable for investigating novel methods for treating hemophilia B such as gene therapy. Characterization of the molecular basis of hemophilia B in Lhasa Apso dogs showed a complex deletion/transition mutation that results in severe hemophilia B.

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