

MOLECULAR BASE OF COAGULATION FACTOR XI DEFICIENCY IN KERRY BLUE TERRIER

E. TCHERNEVA & U. GIGER

School of Veterinary Medicine, University of Pennsylvania,
Philadelphia, PA, USA

Summary

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Factor XI (FXI) deficiency has been reported in the Kerry Blue Terrier (KBT) breed and an isolated family of Great Pyrenees and English Springer Spaniel dogs (Knowler *et al.*, 1994). Affected dogs have a mild bleeding tendency, but many remain asymptomatic. The FXI deficiency seems to be inherited as an autosomal trait with incomplete penetrance. Here we describe the molecular defect responsible for FXI deficiency in KBT dogs. Five FXI-deficient KBTs with either no or mild bleeding tendencies, were studied. Their partial thromboplastin times, but not their prothrombin times, were markedly prolonged and their plasma FXI activities were <10% of normal healthy controls. Genomic DNA and cDNA were prepared and sequenced utilizing primers for the canine FXI sequence derived from the published canine genome sequence. All 15 exons of the sequenced canine FXI gene were identical between the normal KBTs' DNA and the published canine genome sequence. However, the 7th coding exon differs between normal and affected animals. It is normally 110 bp long, but in affected KBTs it contains a short interspersed nucleotide element (SINE) insertion. This exonic SINE is 90 bp long, consisting mostly of adenines coding for lysine which is presumed to affect the 3rd apple domain of the FXI gene. Further studies are in progress to characterize the effects of this SINE mutation on FXI function. Simple genomic DNA test has been developed to screen the KBT population to determine the mutant allele frequency within the breed and its association to any clinical bleeding tendencies.

Key words: FXI deficiency, Kerry Blue Terrier, short interspersed element (SINE)

INTRODUCTION

Coagulation proceeds by an intrinsic or intravascular pathway and by extrinsic or tissue juice pathway, both of which convert prothrombin to the active clotting enzyme thrombin. Thrombin converts fibrinogen to soluble fibrin monomers which, along with fibrin stabilizing factor (fibrinase, FXIII) and calcium becomes converted to the fully polymerized, insoluble fibrin clot. This final stage is the endpoint measured in most coagulation tests.

A few clotting factors participate in contact phase of intrinsic clotting (FXI, FXII, prekallikrein and high molecular weight kininogen).

Hereditary FXI deficiency has been reported in humans (Bolton-Maggs *et al.*, 1988; Seligson, 1993), dogs (Dodds & Kull, 1971; Knowler *et al.*, 1994), cats (Troxel *et al.*, 2002) and cattle (Kociba *et al.*, 1969; Brush *et al.*, 1987).

FXI deficiency is the most common hereditary coagulation disorder in cattle

but is relatively rare in other domestic animals. It has been identified in three breeds of dogs – English Springer Spaniels, Kerry Blue Terriers (KBT) and Great Pyrenees. It is an autosomal disease which produces a mild, spontaneous bleeding disorder (haematuria, gingival bleeding, epistaxis) but sometimes severe, protracted bleeding occurs 12 to 24 hours after surgical interventions. Plasma FXI activities appear to vary greatly in FXI deficient bleeding animals, and heterozygote dogs cannot be definitively differentiated from homozygote affected or normal dogs based on that assay.

The molecular defect causing FXI deficiency in dogs is not described so far. The canine model of FXI deficiency could be desirable for translational research in coagulation and investigation of novel therapeutic approaches. This paper presents the sequence of FXI gene in normal and affected KBT dogs and identification of disease causing mutation in a KBT family with hereditary defect. We evaluate DNA screening test for KBT dogs.

MATERIALS AND METHODS

Animals

Four related FXI-deficient KBTs (including mother and one of her male and female offspring).

One female FXI-deficient KBT from another family.

Other related KBTs and dogs of other breeds have been included as well.

Blood and cheek cells for RNA and DNA isolation were collected according to the ordinary practice in M. Ryan Hospital, School of Veterinary Medicine, University of Pennsylvania.

Sequencing of FXI cDNA

Total RNA was extracted from whole blood of normal KBT dog using RiboPure – Blood Kit (Cat.#1928, Ambion, Austin, Texas) and full length cDNA was made using the SMART cDNA synthesis kit (Clontech, Mountain View, CA, USA) following the manufacturer's protocol. The 5' and 3' ends of cDNA were synthesized by First Choice RLM-RACE Kit (Cat.#1700, Ambion, Austin, Texas). The full length cDNA was amplified with primers that were designed based on conserved amino acids residues among human, mouse, bovine and predicted canine cDNA (NCBI database: <http://www.ncbi.nlm.nih.gov/>). The PCR products were Roche purified (High Pure Product Purification Kit, Roche Diagnostics GmbH, Mannheim, Germany) and sequenced using the Applied Biosystems Big Dye Terminator version 3.1 Technology.

Isolation and characterization of mutant FXI gene

The FXI cDNA was utilized to search the dog genome database at NCBI (<http://www.ncbi.nlm.nih.gov/genome/guide/dog>) and the fifteen exons of the FXI gene were determined. Fifteen pairs of oligonucleotide primers (sequences and PCR conditions available upon request) were used to amplify the coding regions and exon-intron boundaries of the FXI, using genomic DNA from two affected and two normal KBT dogs. Sequencing of PCR-amplified products was performed following gel electrophoresis and gel purification (QIAquick gel extraction kit, Qiagen).

During DNA sequence analysis of the FXI gene from the two affected and two normal KBT dogs a disease causing mutation in coding exon 7 was observed.

DNA screening test

The nature of the mutation – 90 bp insertion makes the test easier. Our PCR test was designed for gDNA and it is presented on Fig. 3.

RESULTS

Sequencing of FXI cDNA

We used a PCR strategy based on conserved amino acid sequences among known FXI proteins from variety of species to isolate the FXI cDNA from canine blood RNA.

The protein sequence predicted from the cDNA results is mature protein of 624 amino acids and sharing 80 % identity with hFXI mature protein. A ClustalW alignment of cDNA sequence with the published sequences of FXI (NCBI Database; Human Acc.No:NM_000128; Mouse Acc.No: NM_028066 and Bovine Acc.No: NM_001008665) is shown on Fig. 1*. Using our cDNA sequence we located the FXI gene to the chromosome 16 of the dog genome by BLAST, with the gene comprised of fifteen exons, with exon-intron boundaries. Canine chromosome 16 shows conservation of synteny with human chromosome 4 (Lindblad-Toh *et al.*, 2005), where the hFXI gene is located (Asakai *et al.*, 1987).

Identification of short interspersed nucleotide element (SINE) mutation and evaluation of screening test

We analyzed FXI sequences from KBT dogs with a history of bleeding and several dogs from different breeds with normal coagulation behavior. Comparison of the

DNA sequences of the entire coding region and exon-intron junctions from the first and second group of dogs revealed an insertion of 90 bp (SINE) inside of coding exon 7 at +719 bp (the first exon of FXI gene is not coding; that way the terms coding exon 7 and exon 8 mean one and the same sequence).

The coding structure of the gene and SINE are presented on Fig. 2. Sequence analysis of coding exon 7 and boundaries revealed an insertion of the 3' end-repeat of the gene.

We investigated 27 KBT dogs. Most of them were homozygous carrying normal copies of the gene, 5 dogs were heterozygous, carrying one normal and one defective copies of the gene and 2 were homozygous and affected.

In addition to DNA sequence analysis, we developed a rapid detection method for the insertion mutation, based on PCR. We designed primers for 168 bp amplicon in normal gene; 258 bp respectively in gene with insertion (Fig. 3). The PCR test discriminates successfully between normal, affected and carrier dogs as seen from the figure.

DISCUSSION

FXI is a unique coagulation enzyme in that it contains four tandem apple domains (Ap), also known as PAN modules (Fujikawa *et al.*, 1986). These Ap show sequence homology with each other and other Ap in proteins such as hepatocyte growth factor and prekallikrein. FXI also contains a serine protease (SP) domain that is homologous to the serin protease domains of other coagulation factors.

FXI circulates as a dimer with two identical FXI monomers linked by non-covalent interactions between the Ap4 domains and by Cys321-Cys321 disulfide

* We used Lasergene 7.2 programme (DNASTAR Inc., Madison, WI) to do this investigation.

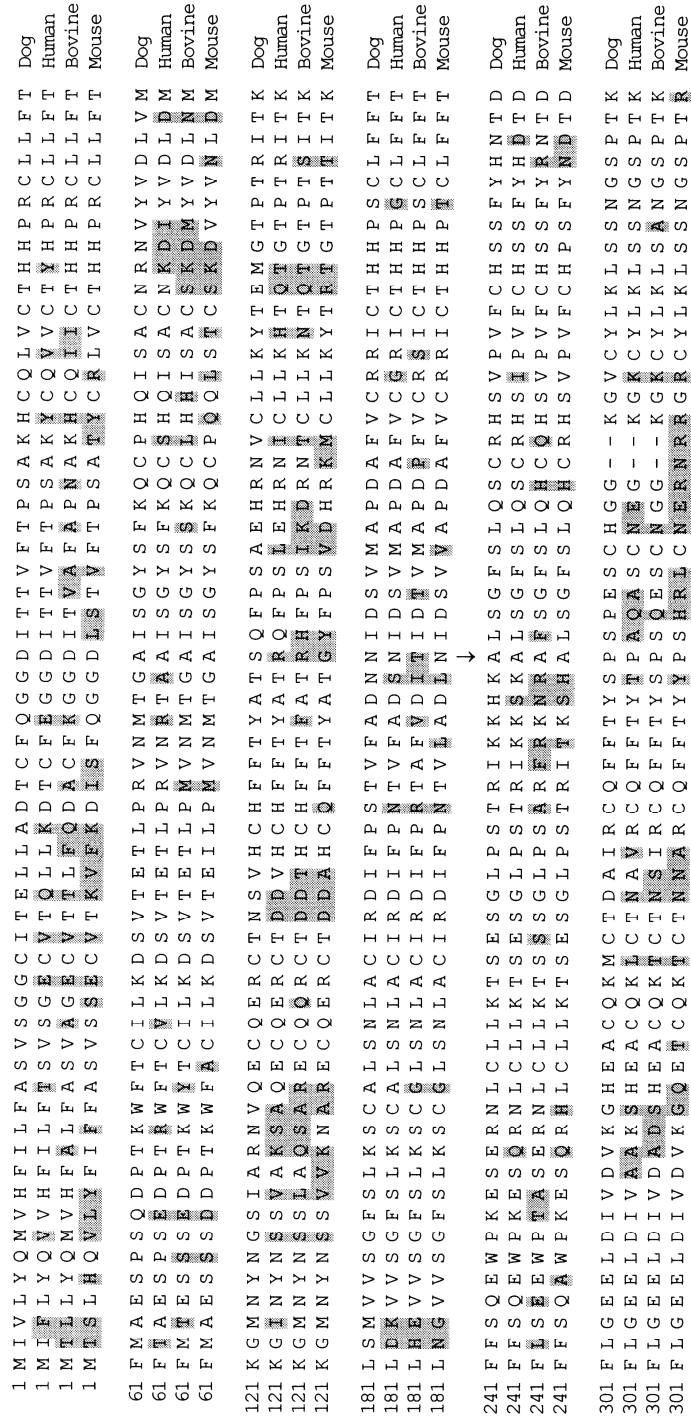


Fig. 1. Clustal W alignment report for Protein Consensus among four species. Residues 198 – 285 represent apple domain 3 of FXI. The point in which the SINE occurs in mutated gene is between 273 and 274 residues. Shadowed letters are for the different from the dog's FXI gene residues in the rest three species (continues on the next page).

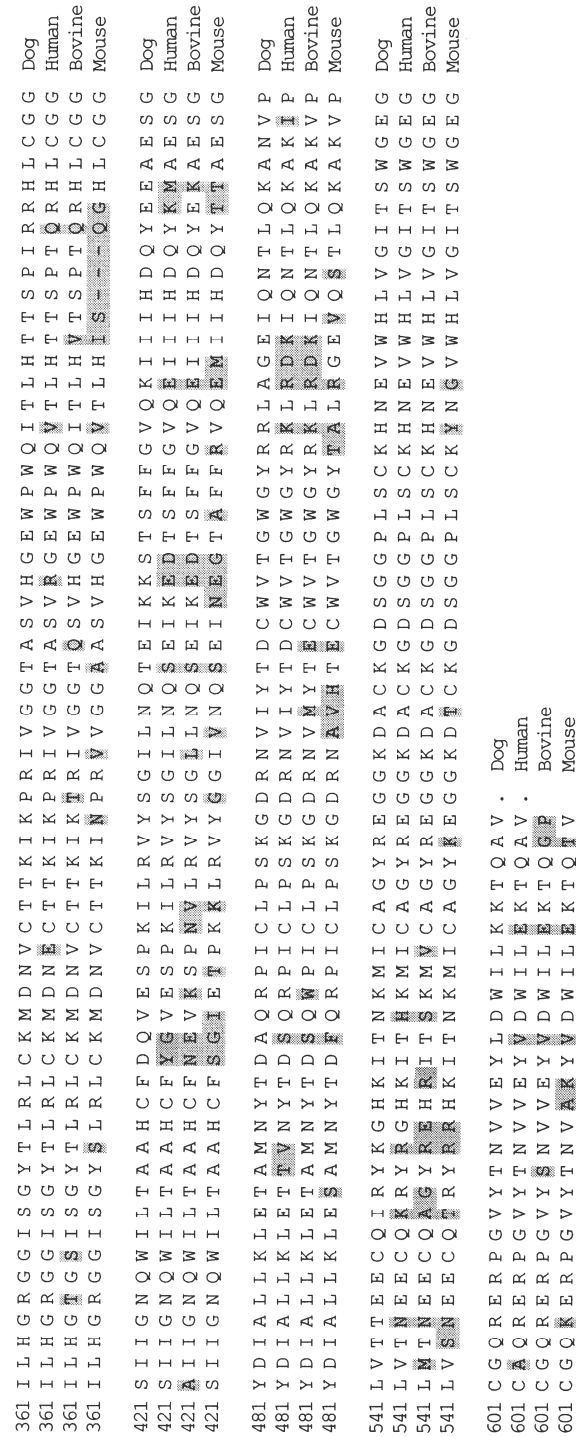


Fig. 1 (cont'd). Clustal W alignment report for Protein Consensus among four species. Residues 198 – 285 represent apple domain 3 of FXI. The point in which the SINE occurs in mutated gene is between 273 and 274 residues. Shadowed letters are for the different from the dog's FXI gene residues in the rest three species.

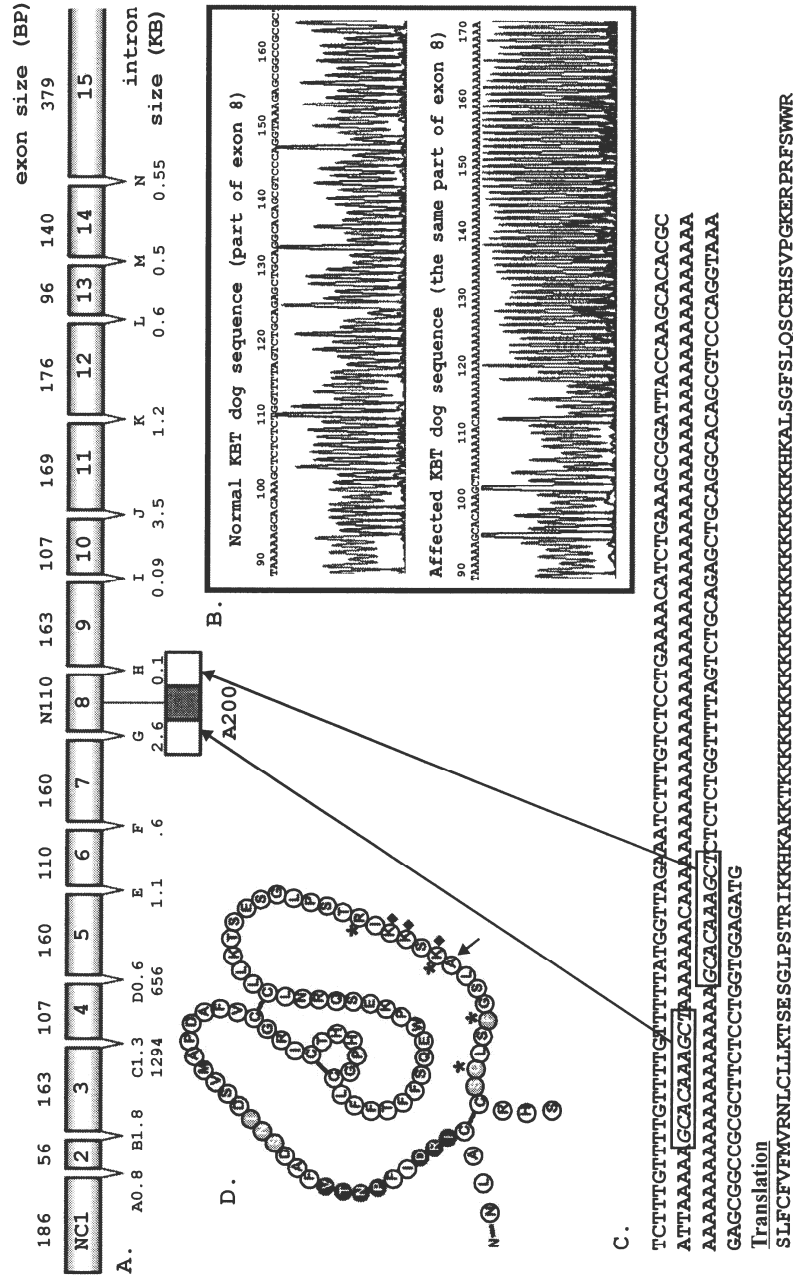


Fig. 2. Structure of FXI gene in normal and affected KBT. The gene consists of 15 exons. The first exon is not coding. The figures inside the boxes are for the numbers of exons. The figures above the boxes are for the nucleotides every exon contains. The box under the exon 8 (coding exon 7) represents the mutated stage containing SINE. A. Partial sequence of coding exon 7 (exon 8) from normal (up) and mutated (down) gene. B. Nucleotide sequence and translation of the exon, containing SINE. C. Exact place of the 90 bp SINE in the apple domain 3. D. Scheme of Ap3 (Sun M. F. *et al.*, 1999).

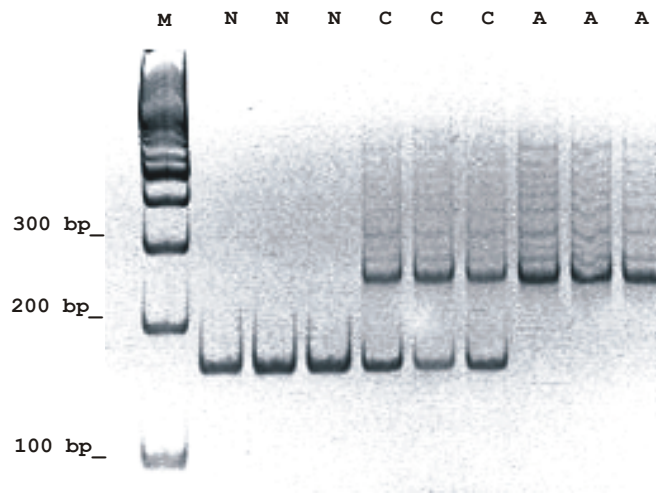


Fig. 3. PCR test for discrimination between normal, carriers and affected toward FXI deficiency in Kerry Blue Terriers. Non denaturing polyacrylamid gel (8%). Ethidium bromide staining and UV visualization. M – molecular size DNA marker; N – homozygote normal alleles; C – heterozygote (1 normal and 1 affected) alleles; A – homozygote affected alleles, containing SINE.

bond, although this bond is not essential for dimerization. FXI is activated when bound to activated platelets. The Ap3 domain within FXI binds to the platelet glycoprotein (GP) Ib-IX-V complex in the presence of high molecular weight kininogen (HK) and zinc ions or prothrombin and calcium ions.

Mutations within the FXI gene can cause FXI deficiency leading to a disorder with a variable clinical phenotype. Homozygotes toward defective FXI gene have low FXI activity (less than 20%), heterozygotes have 40 to 60 % FXI. At this stage we did not investigate the expression of FXI protein from normal and affected dogs.

The SINE mutation in the canine coagulation FXI gene, which is the object of our investigation, is the reason for a 30 amino acids insertion in the A3 domain of FXI serine protease (Fig.2D). The 30 residues, mostly lysine, are situated in the most important region of the A3 domain

which is responsible for interactions with heparin and platelets, between 237 and 238 residues. Probably the long lysine tail masks some of the interacting domains which causes lack of normal clotting ability. This mutation is in a very conservative region – between 273 and 274 residues as it seen from the Fig. 1. If we are in agreement with variants of SINEs impact on gene structure and expression suggested by Cordaux & Batzer (2006), that means the SINE we occurred is an exonization of short open reading frame without any shift from the normal sequence of the gene. This could be partial explanation of the mild clinical signs of FXI disorder in KBT.

Statistics of database coverage for FXI in human patients shows a frequency of 5% insertions (Saunders *et al.*, 2005). Genome modifications like SINE can occur during retrotransposition of a donor element (Kirkness *et al.*, 2003). When transcription past its normal termination it

can lead to the transduction of 3' sequences that flank donor element, as it is in the gene sequenced by us (Fig.2C). Data presented herein suggest that the oligo (dA)-rich tail length determines phenotype – a disorder in the coagulation way due to one coagulation protease. There are data that variable oligo(dA)-rich tail lengths associated with the SINE insertion are presented in different canine breeds (Clark *et al.*, 2006). But we show the first coagulation factor coding gene with SINE which is the reason for bleeding disorders in KBTs. There are two phenotypes of FXI deficiency described (Saunders *et al.*, 2005):

Type I is characterized by both low FXI coagulant activity (FXI:C) and low antigen (FXI:Ag). This indicates that the mutant protein is either present in lower amounts or absent in plasma suggesting that the mutation has a structural effect on the mutant protein - ie reducing translation, secretion or stability of FXI.

Type II is characterized by low FXI:C with normal FXI:Ag. This indicates that the mutant protein is present in normal amounts in plasma but has reduced or absent activity, suggesting that the mutation has a functional effect on the protein e.g. affecting substrate binding.

The mutant protein, described in this investigation, is more likely to phenotype II.

Characterization of this mutation and development of DNA-based screening test will aid in identification of dogs that are homozygous or heterozygous for this coagulation defect, allowing investigators to effectively eliminate this trait from dogs utilized in pharmacological and toxicological research studies. Conversely affected animals may be useful for those interested in novel approaches to the treatment of inherited coagulation disor-

ders, supporting the usefulness of this disease model in the study of therapeutic strategies.

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Correspondence:

Dr. Eva Tcherneva
INVITROGEN Co.
Environmental Diagnostic
7335 Executive Way
Frederick, MD-21704
e-mail: evatcherneva@hotmail.com