A Novel Missense Mutation in Exon 4 of the Factor VIII:C Gene Resulting in Moderately Severe Hemophilia A

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A new point mutation due to C → T transition at codon 189 (TCA) of the factor VIII:C gene was found in a Chinese patient with moderately severe hemophilia A. This mutation abolishes the EcoRI site (GAATTC) in exon 4 and can be directly detected by polyacrylamide gel electrophoresis of amplified genomic DNA.

Hemophilia A, the most common congenital bleeding disorder, affects 1 in 10,000 to 20,000 males. The molecular lesions are heterogeneous and only 10% of specific defects had been characterized so far. These included deletions and point mutations.1 Most of the point mutations detected involved the recognition site (TCGA) for the restriction enzyme TaqI and of these, most are associated with methylation-induced C → T transitions at CpG dinucleotides in either the coding or complementary strand of DNA.1-2 The two exceptions were respectively G to C transversion in exon 22 and A to G substitution in exon 7.3

We report a mutation of the EcoRI site (GAATTC) in exon 4 owing to C → T transition at codon 189 (TCA) of the factor VIII:C (FVIII:C) gene, confirmed by direct sequencing of polymerase chain reaction (PCR)-amplified genomic DNA.

Materials and Methods

The patient (L.K.C.) was a 28-year-old Chinese man with moderately severe hemophilia A. His factor VIII (FVIII:C) level as measured by clotting assay was 0.035 IU/mL and vWF factor antigen (vWF:Ag) level by immunoassay was 0.85 IU/mL. FVIII:Ag determined by enzyme-linked immunosorbent assay (ELISA) method was 0.087 IU/mL (normal control 0.82 IU/mL, performed by Dr I.R. Peake). No FVIII inhibitor was found in this patient. His mother, who had a brother and a nephew with hemophilia A, had a ratio of VIII:C to vWF:Ag of 0.29.

Restriction enzyme analysis. DNA was extracted from peripheral blood leukocytes of the patient, and 5-μg amounts were digested with the restriction enzymes TaqI, EcoRI, and SstI, respectively, under conditions recommended by the manufacturer (New England Biolabs, Beverly, MA). DNA from other hemophilia A patients and normal male subjects was run at the same time. The DNA digests were separated by agarose gel (0.8%) electrophoresis, transferred to nitrocellulose filters, and hybridized to 32P-labeled FVIII:C cDNA probes by standard techniques.3 The hybridized filters were washed under stringent conditions and autoradiographed for 2 to 5 days. The FVIII:C cDNA probes used were (a) probe A, 1.8-kilobase (kb) fragment containing exons 1 through 12 of the human FVIII:C cDNA cloned into KpnI-SacI sites of PUC 18 (Genetics Institute, Cambridge, MA) and (b) a 5-kb BglII-EcoRI FVIII:C DNA fragment that contains part of exon 14 to exon 26 and the 3' end of the FVIII:C gene (pF8-100; Chiron, Emeryville, CA).

Enzymatic gene amplification. PCR amplification of genomic DNA from patient L.K.C. and a normal male subject were performed with the thermostable DNA polymerase from Thermus aquaticus (Taq polymerase, Perkin Elmer Cetus, Norwalk, CT).4 The reaction mixture containing genomic DNA (1 μg), primers, deoxynucleotide triphosphate (dNTP), and enzyme buffer (concentrations as recommended by the manufacturer) was heated at 95°C for 5 minutes to separate the DNA strands, and 2 to 3 U Taq polymerase were added. The mixture was sealed under 40 μL mineral oil and incubated through three temperature cycles: first at 60°C for 1 minute, then at 94°C for 1 minute 15 seconds, and finally at 48°C for 1 minute 15 seconds. This heating cycle was repeated 35 times. The two amplification primers encompassing both ends of exon 4 of the FVIII:C gene were: forward primer 5'-GCTGAA TATA 3' and reverse primer 5'-TTC TCT CAG A 3' (19 mer) and 5'-GTT CAG AGT TAG C 3' (19 mer). The amplified fragment, which is approximately 213 base pairs (bp), was isolated from 6% polyacrylamide gel.

Confirmation of mutation in exon 4. Approximately 200 ng isolated PCR-amplified exon 4 fragment was digested with EcoRI according to the manufacturer's instructions (New England Biolabs) and fractionated on 12% polyacrylamide gel at 45 mA for 45 minutes. The gel was stained with ethidium bromide for visualization under ultraviolet light (302 nm).

Direct genomic sequencing of PCR-amplified exon 4. Direct sequencing of the amplified fragment was performed using 32P-deoxycytidine triphosphate (α-32P-dATP, Amersham International, Amersham, England), T7 DNA polymerase, and reagents of the Sequenase kit (United States Biochemical, Cleveland, OH) with the modifications described previously.5 Approximately 100 ng template was used, and sequencing in both the sense and antisense strands were made using an excess (8 pmol) of the primary primers (PCR primers). The samples were analyzed on 8 mol/L urea and 6.5% acrylamide gel (60 cm) at 60 W.

Results

Restriction endonuclease digestion and hybridization with FVIII:C cDNA probes. Hybridization of EcoRI digest from patient L.K.C. with probe A showed loss of a 1.9-kb fragment and the presence of an extra 4.4-kb fragment (Fig 1) as compared with similarly treated DNA from 29 unrelated hemophilic patients and 81 unrelated normal male subjects. The former fragment corresponded to EcoRI sites in intron 3 and exon 4 of FVIII:C gene. Loss of the EcoRI site in exon 4 would result in a longer fragment of 4.4 kb, extending from the EcoRI site in intron 3 to the next site in intron 4. Hybridization of TaqI and SstI digests of genomic DNA from patient L.K.C. and the 29 unrelated hemophilic patients revealed no abnormal fragments as compared with normal male subjects.
EXON 4 MISSSENSE MUTATION OF FVIII:C GENE

Confirmation of loss of EcoRI site in exon 4. EcoRI digests of the PCR-amplified exon 4 fractionated in 12% polyacrylamide gel are shown in Fig 2. In the normal subject, digestion with EcoRI yielded 173- and 40-bp fragments, whereas in patient L.K.C., as a result of a mutation of the EcoRI site, no cleavage was possible and the 213-bp fragment corresponding to an uncut exon 4 was observed.

Genomic DNA sequencing of PCR-amplified exon 4. After PCR amplification of a 213-bp exon 4 of the FVIII-C gene, sequence analysis of both the sense and antisense strands showed a mutation at codon 189 from TCA -> TTA (serine -> leucine) (Fig 3). (The initiation codon ATG is regarded as codon 1, corresponding to the beginning of the signal peptide.) This indicated that the normal EcoRI recognition site GAATTC was altered to GAATTTC in patient L.K.C. No abnormality was detected in the exon 4 sequence of the normal male subject.

DISCUSSION

In the present study, a novel missense mutation, C -> T at codon 189 (TCA) in exon 4 of the FVIII:C gene was identified. Although most other point mutations associated with hemophilia A affect the TaqI site, this is the first mutation described that affects the EcoRI site. Most TaqI site mutations involved C -> T transition in the CpG dinucleotide and are mediated by methylation-induced deamination of cytosine to thymine in either the coding or complementary strand of the DNA, resulting in either a stop codon (TGA) or a missense mutation of arginine (CGA) to glutamine (CAA). Thus, CpG dinucleotides are well-recognized mutational hotspots in the human genome and

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Fig 1. Autoradiograph of EcoRI-digested genomic DNA from hemophilia A patients hybridized to probe A. Fragment sizes in kilobases (kb) and their corresponding exons (numbers in parentheses) are shown. In lane 3, patient L.K.C. showed loss of a 1.9-kb fragment and generation of a 4.4-kb fragment (*). Bottom: Expanded portion of the FVIII:C gene around exon 4; EcoRI sites (E) (arrows).

Fig 2. Polyacrylamide gel electrophoresis of amplified exon 4 (213 bp) of the FVIII:C gene from patient L.K.C. (lanes 1 and 2) and a normal male (lanes 3 and 4). EcoRI-digested DNA (lanes 2 and 3) were compared with undigested DNA (lanes 1 and 4) from the same subjects. In the patient, no cleavage was possible owing to mutation of the EcoRI site.
the extent of its hypermutability was estimated as 10 to 20 times greater than the average mutation rate for hemophilia A. In comparison, the missense mutation of the EcoRI site reported in this study changed the amino acid serine at codon 189 (amino acid position 170) to leucine. This amino acid substitution in A1 domain affects a biologically important part of the mature protein, because the sequence in this area lies within a “disulfide loop” where there is marked sequence homology (SGLIG_LL_C) in all three A domains of FVIII. Although it does not affect the thrombin cleavage sites, it alters the conformation of the protein and increases hydrophobicity in this region as demonstrated by protein structure analyses (DNASTAR protein software, DNASTAR, Madison, WI). These changes could have rendered the structure more unstable and enhanced degradation, resulting in moderately severe disease in the patient. The postulation of an enhanced degradation in the mutant FVIII is supported by our finding of a low FVIII:C activity. Furthermore, it is unlikely that the present mutation TCA → TTA represents a neutral polymorphism, since it has not been observed in the other 29 unrelated hemophilic X and 81 normal X chromosomes studied.

The usefulness of PCR gene amplification and direct genomic sequencing had been clearly demonstrated in previous work. In our experience, direct sequencing could be performed using either primary (amplification) primer or an internal primer. To avoid sequencing artifacts such as bands across all four lanes owing to secondary structure of DNA and compression in G-C-rich regions, sequencing should be made in both the sense and antisense strands.

With this mutation, loss of the EcoRI site could be easily demonstrated in PCR-amplified genomic DNA and visualized by fractionation of the EcoRI digest on polyacrylamide gel. This offers a direct and rapid detection of the affected FVIII:C gene without the need of linkage analysis.

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