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*Disorders of Hemostasis*

## Identification of 32 novel mutations in the factor VIII gene in Indian patients with hemophilia A

**Seventy-five unrelated hemophilia A patients from India were analyzed for factor VIII gene defects. Intron 22 inversion was identified in 22 patients and intron 1 inversion in 2 patients. In the remaining 51 patients without inversions screening the FVIII gene by denaturing high performance liquid chromatography (DHPLC) revealed 42 different mutations in 44 unrelated subjects. These included 14 missense, 7 nonsense, 9 splice site, 8 deletional, 3 insertional mutations and one indel mutation. Of these, 32 were novel gene alterations. The hotspots included intron 22 inversion, CpG and adenine runs.**

haematologica 2005; 90:283-284
(http://www.haematologica.org/journal/2005/2/283.html)

Hemophilia A (HA) is an X chromosome-linked bleeding disorder, caused by defects in the factor VIII gene (*F8*). Apart from intron 22 and 1 inversions, all other mutations are distributed heterogeneously throughout the gene (*http://europium.csc.mrc.ac.uk*). There is a paucity of data on HA mutations in India. We report here, for the first time, the distribution of causal mutations in the *F8* gene of Indian HA subjects. Seventy-five patients with HA were studied. DNA was extracted from blood and polymerase chain reaction (PCR) was performed to detect inversions of intron 1 and 22.[1] In patients negative for these inversions mutation screening was done by denaturating high performance liquid chromatography (DHPLC).[2] Splice site mutations were predicted by a program from *http://www.fruitfly.org* which predicts potential splice sites in a given sequence. It assigns them a score from 0.4 to 1.0, with values less then 0.4 predicting absence of a splice site. The fragments with abnormal chromatograms were sequenced. We used the nomenclature for nucleotides and amino acids proposed by Dunnen *et al*.[3]

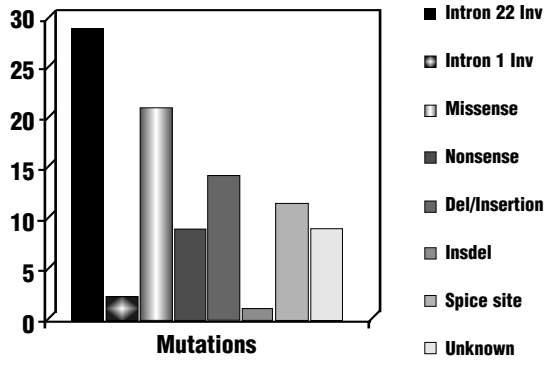


**Figure 1.** Mutation profile in hemophilia A patients.

Of the 75 patients with HA studied, 50 had severe HA (66.6%), 13 had moderate disease (17.3%) and 12 had mild HA (16%) were studied. Twenty-two had intron 22 inversion, 2 had intron 1 inversion, 16 had missense, 7 had nonsense and 9 had splice site mutations. Eight patients had deletions, 4 had insertions. Thirty-two of these were novel mutations, previously unreported in the Hemophilia A Mutation Database (HAMSTeRS). These novel mutations included 8 missense, 5 nonsense and 9 splice junction mutations and 10 deletions and insertions (Figure 1). The hotspots included intron 22 inversion, adenine runs and CpG.

Novel missense mutations were seen throughout the *F8* gene, but not in the B domain (Table 1). This further reinforces the view that single nucleotide substitutions within the B domain are largely unimportant.[4] Novel missense mutations identified may be causative. Moreover, polymorphisms in the coding regions of *F8* gene are rare and these changes were not found in normal individuals or as second site changes in hemophilia A patients.

These mutations replace amino acids that are conserved in humans, pigs, murine and canine species. The nonsense novel mutations were seen in A2, B, A3, C1, and C2 domains. These resulted in truncated proteins and were considered pathogenic. Nine novel splice junction mutations were seen in domains A1, A2 and C1. Six out of these had a score < 0.4, predicting absence of a splice site (Table 1). In 3 patients the program did not predict a splicing error. As no other gene alteration was found in these patients, further studies on the RNA are being undertaken to confirm the causal nature of these defects.

Ten of the 12 novel deletions and insertions caused a frame shift, leading to the introduction of a premature termination codon and thus a truncated protein (Table 2). One small deletion c.5293_5295delCCC was an in-frame mutation leading to deletion of proline at position 1746, which leads to a milder phenotype with 9% FVIII:C. In patient HA50, screening for all fragments except exon 26 showed normal amplification but amplification of exon 26 was unsuccessful despite a number of repetitions and multiplexing, suggesting a deletion of the whole exon in this patient. Large deletions are reported to be the causal defects in 3.9 to 7% of patients with hemophilia A,[5] which is mirrored in our study. Three severe hemophiliacs

**Table 1.** Mutations in hemophilia A in Indians.

| Missense and nonsense mutations | | | | | | |
|---|---|---|---|---|---|---|
| Sample ID | Exon | Nucleotide change | Codon change | Amino acid change | F VIII domain | CpG |
| HA20* | 4 | c.431 A→C | GAT→GCT | Asp125 Ala | A1 | |
| HA10 | 7 | c.822 G→C | TGG→TGC | Trp255Cys | A1 | |
| HA51* | 7 | c.824 A→G | CAT→CGT | His256Arg | A1 | |
| HA28 | 8 | c.1171 C→T | CGC→TGC | Arg372Cys | A1 | CpG |
| HA35* | 8 | c.1203 G→A | TGG→TGA | Trp382End | A2 | |
| HA48* | 9 | c.1332 A→T | AAA→AAT | Lys 425Asn | A2 | |
| HA38* | 10 | c.1592 T→A | TTA→TAA | Leu491 End | A2 | |
| HA22 | 11 | c.1648 C→T | CGC→TGC | Arg531Cys | A2 | CpG |
| HA25 | 11 | c.1648 C→T | CGC→TGC | Arg531Cys | A2 | CpG |
| HA17* | 11 | c.1702 G→A | GGC→AGC | Gly549Ser | A2 | |
| HA18 | 11 | c.1750 C→A | CAG→AAG | Gln565Lys | A2 | |
| HA 2 | 12 | c.1809C→G | AGC→AGG | Ser584Arg | A2 | |
| HA21* | 13 | c.1963 T→C | TAC→CAC | Tyr636His | A2 | |
| HA54* | 14 | c.4662 G→A | TGG→TGA | Trp1535 End | B | |
| HA31* | 15 | c.5284 T→C | TTT→CTT | Phe1743Leu | A3 | |
| HA14 | 18 | c.5953 C→T | CGA→TGA | Arg1966 End | A3 | CpG |
| HA41* | 18 | c.5985 C→A | TAC→TAA | Tyr1976 End | A3 | |
| HA 9* | 21 | c.6269T→A | ATC→AAC | Ile2071Asn | C1 | |
| HA45 | 22 | c.6403 C→T | CGA→TGA | Arg2116 End | C1 | CpG |
| HA16* | 23 | c.6537 C→G | AGC→AGG | Ser2160Arg | C1 | |
| HA53 | 23 | c.6545 G→A | CGC→CAC | Arg2163His | C1 | CpG |
| HA13 | 23 | c.6545 G→A | CGC→CAC | Arg2163His | C1 | CpG |
| HA 6* | 24 | c.6694 C→T | CAA→TAA | Gln2213 End | C2 | |

| Splice site mutations | | | | |
|---|---|---|---|---|
| Sample ID | Nucleotide change | Mutant/ normal score | Amino acid change | F VIII domain |
| HA12* | c.294-2A→T | <0.4/0.46 | | A1 |
| HA29* | c.601+5G→A | <0.97/1.00 | | A1 |
| HA37* | c.1009+1G→A | <0.4/0.77 | | A1 |
| HA 4* | c.1009+3A→G | <0.4/0.77 | | A1 |
| HA 5* | c.1443+5G→C | <0.4/0.99 | | A2 |
| HA24* | 9 | <0.94/0.99 | Leu462Leu | A2 |
| HA7* | c.1443+18T>G | <0.99/0.99 | | A2 |
| HA44* | c.1473-2A>G | <0.4/0.81 | | A2 |
| HA42* | c.6273+1G>T | <0.4/0.98 | | C1 |

*Novel mutations.

**Table 2.** Deletional and insertional mutations.

| Patient ID | F VIII:C | Exon | Nature of insertion/deletion | Domain | Codon | Comments |
|---|---|---|---|---|---|---|
| HA55* | <1% | 13 | c.1990_1991delCA | A2 | 645 | Frame shift |
| HA43* | <1% | 14 | c.3303_3318dup GGGCCCCATTCCACCA | B | 1082-1087 | Frame shift |
| HA1* | <1% | 14 | c.3991_3992delAA | B | 1312 | Frame shift |
| HA47 A runs | <1% | 14 | c.4372_4379delA | B | 1439-1441 | Frame shift |
| HA49 A runs | <1% | 14 | c.4819_4826insA | B | 1588-1590 | Frame shift |
| HA52* A runs | <1% | 14 | c.5137_5140delA | B | 1694-1695 | Frame shift |
| HA23* | 9% | 15 | c.5293_5295delCCC | A3 | 1746 | Inframe |
| HA34* | <1% | 18 | c.5954_5985del32bp | A3 | 1966 to 1976 | Frame shift |
| HA 3* | 2% | 19 | c.6069_6070insC | A3 | 2005 | Frame shift |
| HA36* | <1% | 19 | c.6094delCinsTT | C1 | 2013 | Frame shift |
| HA40* | 1% | 25 | c.6796delG | C2 | 2247 | Frame shift |
| HA50* | <1% | 26 | Del Ex-26 | C2 | – | Large deletion |

*Novel alterations.

developed inhibitors at levels ranging from 50 to 80 Bethesda units (BU). Of these, two had intron 22 inversion and one had a novel Trp1535Stop End nonsense mutation.

In 7 (9.3%) patients, no gene alterations could be identified in the coding region. In 6 of these, no mutation was identified even after sequencing the whole coding region. These patients had FVIII:C levels ranging from 3 to 20% and 5 had a positive family history. von Willebrand's disease type 2N mutations were excluded in all the patients by analyzing the von Willebrand factor (*VWF*) gene (exon 18 and 19) coding for the FVIII-binding domain of VWF.[6] It is possible that two of these had somatic mosaicism, as 5 had a positive family history. Otherwise, there may have be a common founder mutation in the intronic region of the *F8* gene, in analogy to a recent report of a novel exon 13 duplication in 32% of unrelated patients with mild HA.[7] In the present study, the rate of DHPLC detection of mutations in the *F8* gene was 86.2%, which is lower than in previous studies. This may be attributed to the absence of mutations in the coding region as whole gene sequencing failed to identify any mutation in 6 of the 7 patients. It can be concluded that Indians hemophilics have a large number of novel mutations which need to be studied further.

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*Funding: we acknowledge the Department of Biotechnology, Ministry of Health, Govertment of India, for providing us with funds to carry out inversion studies at the Department of Hematology, AIIMS, India.*

*Acknowledgments: we thank Mrs. Ging Eimer and Mr. Suresh Kumar for all their technical support throughout the study.*

*Key words: hemophilia A, mutations F8 gene, Indians.*

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