

## ORIGINAL ARTICLE

# Genomic alterations in the F8 gene correlating with severe hemophilia A in Egyptian patients

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## Abstract

**Background:** Hemophilia A (HA) is an inherited X-linked recessive coagulation disorder caused by factor VIII (*F8*) deficiency. *F8* rearrangements involving intron 22 (int22) and intron 1 (int1) account for almost half of severe HA phenotype also a hotspot exon 14 provides numerous mutational patterns. This study aims to identify *F8* gene mutations among Egyptian HA patients.

**Methods:** DNA samples from 60 HA patients were screened for int22 and int1 rearrangements using simplified inverse shifting PCR (IS-PCR) followed by exon 14 sequencing. Also, four uncharacterized patients were studied by targeted exome sequencing.

**Results:** In 33.3% of the studied patients, we identified three int22 rearrangements, three exon 14 mutations (two frameshift; one novel (NM\_000132.3:c.2734\_2735delAA, p.(N912Ffs\*6)), a second reported mutation (NM\_000132.3:c.3091\_3094delA GAA, p.(K1031Lfs\*9)), and one nonsense mutation (NM\_000132.3:c.2440C>T, p.(R814\*)). All identified mutations were detected in patients with severe HA phenotype. Targeted exome sequencing could not detect any known pathogenic variants.

**Conclusion:** Intron 22 rearrangement and exon 14 mutations correlate with most severe hemophilia A Egyptian patients.

## KEYWORDS

FVIII protein, hemophilia A, human *F8* gene, mutation, nonsense

## 1 | INTRODUCTION

Hemophilia A (HA) is the most common inherited recessive X-linked disorder of blood coagulation caused by deficiency of factor VIII (OMIM, 300841) in the coagulation cascade with an estimated incidence about 1 in 5,000 male live births (OMIM, 2020). Hemophilia A is more common

than hemophilia B, representing 80%–85% of the total hemophilia population (Srivastava et al., 2013). In 2019, the World Federation of Hemophilia (WFH), in its global survey in Egyptian population (98,423,595) estimated the number of affected patients with hemophilia as 6,028 (World Federation of Hemophilia report on the Annual Global Survey, 2018).

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Hemophilia A is associated with marked increased risk of bleeding. According to the coagulation activity of FVIII, hemophiliacs are categorized into mild (>5%), moderate (1%–5%), and severe (<1%) phenotypes. In severe HA, bleeding might be spontaneous and with no obvious etiology (Santagostino et al., 2010). Heterozygous females with *F8* clotting activity level lower than 40% are at risk for bleeding that is usually comparable to that seen in males with mild hemophilia. Also, subtle, abnormal bleeding may occur with a baseline *F8* clotting activity between 35% and 60% or higher (Paroskie et al., 2015; Plug et al., 2006).

Human *F8* gene has 26 exons and maps to the long arm of the X chromosome at the Xq28 region. The protein product of *F8* possesses no enzyme activity. A 19 amino acid signal peptide at the N-terminus leads to the passage of *F8* protein through hepatocytes to the blood vessels (Saenko et al., 2002). The matured *F8* protein is composed of 2332 amino acids and comprises three homologous A-domains, two homologous C-domains, and a single B-domain. These domains, arranged as A1A2BA3C1-C2 from the N- to the C-terminus. Upon activation, the inactive *F8* is proteolyzed via thrombin where the B-domain is cleaved leaving the heterotrimeric active *F8* composed of heavy and light chains linked together by a calcium ion (Lenting et al., 1998). The spectrum of *F8* gene mutations is heterogeneous as reported at the CDC HA Mutation Project (<http://www.cdc.gov/ncbddd/hemo-philial/champs.html>, 2016; Payne et al., 2012). Inversion mutations involving intron 22 (Inv22) and intron 1 (Inv1), account for about 45% and 2%–5% of severe phenotype, respectively (Bagnall et al., 2006; Cumming, 2004; Gouw et al., 2012). Inverse shifting PCR (IS-PCR) is used in a rapid and robust manner to detect Inv22 and Inv1 as well as other related DNA rearrangements (Rossetti et al., 2005, 2008). Point mutations are the most prevalent defect in 90%–95% of patients who tested negative for inversion typing, followed by deletions in about 5%–10% (Graw et al., 2005).

Numerous types of previously reported HA gene mutations have been identified in exon 14. Being the largest exon in *F8* gene (3,106 bp), exon 14 provides a mutational hotspot and codes partially for two domains (A2 and A3) as well as the entire B-domain of *F8* protein (Repesse et al., 2007).

Recently, targeted next-generation sequencing (NGS) is utilized to sequence the whole coding region of *F8* (Atik et al., 2020; Bastida et al., 2017; Inaba et al., 2017). HA pedigrees' linkage analysis can also identify HA carriers providing reliable method used in genetic counseling and prenatal diagnosis (Hussein et al., 2008).

In the current study, Egyptian HA patients with variable clinical phenotypes were studied for the identification of *F8* gene mutation spectrum for this serious coagulation disorder, with an ultimate goal of providing proper genetic counseling and prenatal diagnosis.

## 2 | PATIENTS AND METHODS

### 2.1 | Patients

Sixty HA patients from 43 families were recruited from the Hereditary Blood Disorders Clinic, National Research Centre (NRC), and Hematology clinic, Pediatric hospital, Cairo University. Informed written consents were obtained from the patients or their guardians according to the Helsinki Declaration of 1975, as revised in 1983. Following approval of the National Research Centre Review Board, the following variables were obtained; detailed family history, pedigree analysis up to three generations, thorough clinical examination, coagulation, and immunologic assays. Subsequently, patients were classified according to the *F8* activity into mild (>5%), moderate (1%–5%), and severe (<1%) phenotypes.

Blood samples were collected from affected individuals, their mothers, siblings and available family members whenever possible for molecular analyses.

## 3 | METHODS

### 3.1 | Molecular genetic analysis

#### 3.1.1 | Genomic DNA extraction

DNA was extracted from blood samples of 60 patients, their mothers, and available family members using standard salting out method (Miller et al., 1988).

#### 3.1.2 | Detection of *F8* int22 and int1 rearrangements using IS-PCR

IS-PCR was performed as directed by Rossetti et al. (2008). Briefly, genomic DNA digested with Fast Digest *BclI* kit according to the manufacturer (Thermo Fisher Scientific). Digested DNA was isolated via phenol chloroform extraction followed by precipitation with ethanol. Ligation of the digested DNA was carried out using Rapid DNA Ligation kit (Thermo Fisher Scientific). Ligated samples undergo PCR using previously published primers.

#### 3.1.3 | Sequencing of exon 14 of *F8* gene

Samples negative for *F8* int22 and int1 rearrangements were subjected to sequencing of exon 14. Polymerase Chain Reaction (PCR) was performed on the DNA samples of the patients using primers pairs of six fragments in exon 14 (14A, 14B, 14C, 14D, 14 E, and 14F) of the *F8* gene according to

Steve Keeney, 2011 (Keeney, 2010). PCR reaction was carried out in 25  $\mu$ l reaction mixture containing 1.5 mM MgCl<sub>2</sub>, 0.2 mM dNTP, 10 pmoles of each primer, 0.5 U of Taq DNA polymerase, and 200 ng of genomic DNA. PCR conditioning was as follow: initial denaturation for 5 min at 95°C, 30 cycles of 30 s at 94°C, annealing for 30 s at 57°C, extension for 30 s at 72°C, and a final extension for 5 min at 72°C. Amplified fragments were subjected to direct sequencing in both directions using Big Dye Terminator v3.1 Kit and injected to ABI 3500 Genetic Analyzer (Applied Biosystems).

### 3.1.4 | Targeted NGS of *F8*

Four patients (two of mild and two of moderate HA patients) who were negative for both int22 rearrangements and any known pathogenic variants in exon 14 were selected for sequencing of the whole coding region of *F8* and its flanking intronic regions using targeted NGS (Illumina).

### 3.1.5 | Data analysis

*F8* nucleotide numbering is designated according to coding bases from A (nucleotide+1) from the initiation codon for methionine (ATG) at position -171 (*F8*: ref. NM\_000132.3).

While the protein numbering (p.) follows the amino acid sequences that assign the first residue methionine as +1 in factor VIII sequences (FVIII: NP\_000123.1) according to the Human Genome Variation Society guidelines.

Sequence variants were aligned with the corresponding wild-type sequences using BLAST (NCBI) and compared to the HA mutation databases (Human Gene Mutation Database and CDC Haemophilia A Mutation Project database 29).

## 4 | RESULTS

### 4.1 | HA phenotyping

Fifty-two HA patients' age ranged from 2 to 12 years (mean age ~6.7) and eight patients were between 30 and 50 years (mean age ~38). Pedigree analyses revealed positive consanguinity within 50% of studied pedigrees and other affected family members were detected in 37.5%. According to *F8* activity, 35% of HA cases were classified as severe HA, 40% as moderate, and 25% as mild phenotype.

### 4.2 | Inverse-shifting PCR

IS-PCR revealed the presence of three different int22 rearrangements in 9/60 (15%) of all patients and 9/21 (42.8%) of

severe HA patients. Inv22 type 1 was found in six patients, del22 type 1 in one patient, and del22 type 2 in two patients (Figure 1). All nine patients were severe HA patients (P1–P9). None of the studied patients had Inv1 (Table 1).

### 4.3 | Sequencing analysis of exon 14

Sequencing of amplified fragments of exon 14 among 51 HA patients negative for int22 rearrangements and Inv1 could detect molecular alterations in 17/51 as follows:

1. Patient 10 harbored a novel deletion mutation of 2 bp (AA) (NM\_000132.3:c.2734\_2735delAA, p.(N912Ffs\*6)) which creates a frame shift starting at codon N912 and the new reading frame ends in a stop codon 5 positions downstream (Figure 2a). This mutation was submitted in ClinVar with accession number SCV000328623.

In P11, a previously reported deletion mutation c.3091\_3094del AGAA was detected (NM\_000132.3:c.3091\_3094del AGAA, p.(K1031Lfs\*9)) (Figure 2b).

2. Nine patients (P12–P20) exhibited previously reported nonsense mutation (NM\_000132.3:c.2440C>T, p.(R814\*)) (Figure 2c).
3. Moreover, the previously reported natural variant (NM\_000132.3:c.3780C>G, p.(D1260E)) (rs1800291) was detected in six patients (P21–26), which was inherited in a heterozygous state in four of their mothers (Figure 2d).

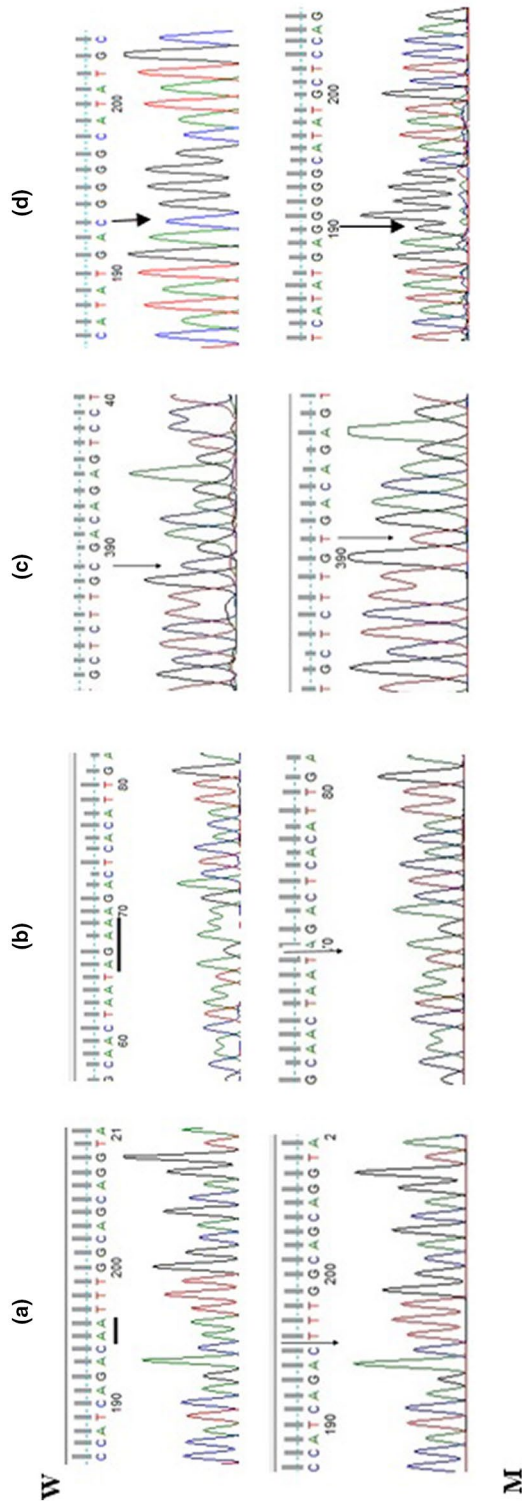
### 4.4 | Targeted NGS of *F8* gene

Targeted NGS of the whole coding regions of *F8* and its flanking introns revealed no known pathogenic mutations in the selected four HA patients who were negative for int22 rearrangements and exon 14 known pathogenic variants. The only detected alteration was (NM\_000132.3:c.3780C>G, p.(D1260E)) (rs1800291) in P21 which was identified earlier by direct Sanger Sequencing of exon 14.

## 5 | DISCUSSION

Based on the clinical presentation of the patients, we initially performed the molecular analysis of the most pathogenic *F8* variants in severe HA patients known as inversion which leads to abnormal protein product.

In our cohort of 60 HA patients we found six mutations including intron 22 rearrangements, nonsense mutations, and



**FIGURE 1** Partial nucleotide sequence of exon 14 in FVIII gene. (a) Frameshift mutation (NM\_000132.3:c.2734\_2735delAA, p.(N912Ffs\*6)) identified in patient No. 10. (b) Frameshift mutation (NM\_000132.3:c.3091\_3094delAGAA, p.(K1031Lfs\*9)) identified in patient No. 11. (c) Nonsense mutation (NM\_000132.3:c.2440C>T, p.(R814\*)) identified in patients No. (P12–P20) (d) Natural variant (NM\_000132.3:c.3780C>G, p.(D1260E)) (rs1800291), (W for wild type and M for mutant)

small deletions. Reviewing the HAMSTeRS, HGMD, and CHAMP database one of these six mutations is novel.

In the current study 21 (35%) of our studied patients, presented with severe clinical phenotype. Nine (42.8%) of these patients harbored three different int22-related rearrangements. Abou-Elew et al. (2011) reported intron 22 rearrangements in 70% of severe cases, combining our results with that of Abou-Elew et al. (2011), intron 22 rearrangements would be responsible for about 21% of all reported Egyptian hemophilia molecular pathology and 53% of severe cases, (Table 2). Both studies fall within the range of reported molecular pathology of severe HA in different populations (Abdulqader et al., 2020; Aquila et al., 2004; Astermark et al., 2005; Coppola et al., 2009; Hill et al., 2005; Vinciguerra et al., 2006). Considering these collective studies, targeted molecular diagnosis for severe HA phenotype in Egyptians should target Inv22 together with del22 to start.

By excluding IVS22 and IVS1 mutations, 11 (52.4%) out of 21 severe HA studied patients, mainly harbored pathogenic mutations within exon 14 corresponding to the B-domain of factor VIII. FVIII B-domain has no homology sequence to any other known genes and participates in the intracellular processing and trafficking of factor VIII. (Aquila et al., 2004; Santacroce et al., 2008). Preferential mutational hotspots are usually detected in FVIII B-domain, in most cases creating a premature stop codon within the reading sequence (Kemball-Cook et al., 1998).

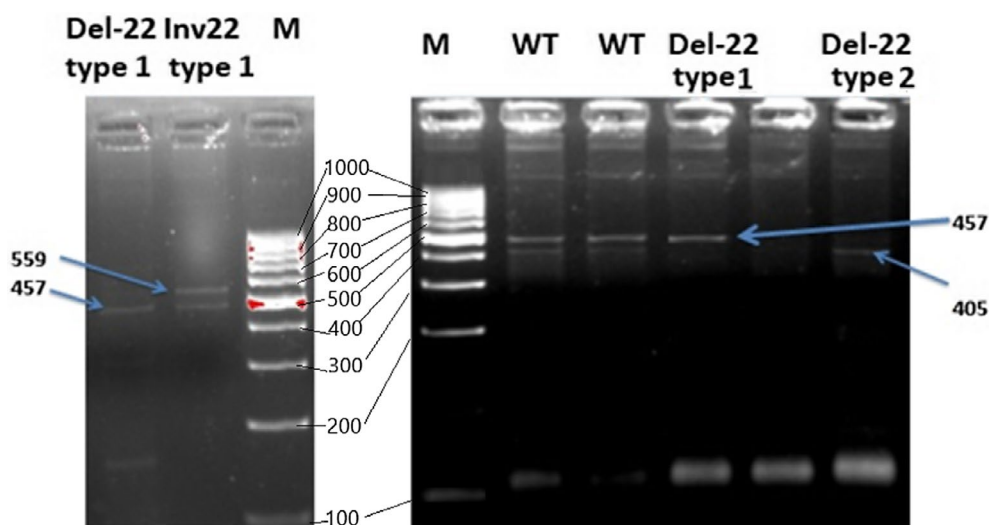
Among the studied patients of B-domain affection, patient No 10 harbored a novel frameshift mutation within exon 14 of *F8* gene, due to a novel deletion (NM\_000132.3:c.2734\_2735delAA, p.(N912Ffs\*6)). Frameshift mutation (NM\_000132.3:c.3091\_3094delAGAA, p.(K1031Lfs\*9)) detected in Patient No 11, was similarly correlated with severe HA phenotype in American and Italian populations (Coppola et al., 2009). Both detected deletion mutations are speculated to affect the protein product through disturbing the architecture of the B-domain binding and thus alter the function with consequent severe phenotype presentation.

Patients No 12–20 also presenting with severe phenotype, harbored the reported nonsense mutation (NM\_000132.3:c.2440C>T, p.(R814\*)), that might point to this mutation as a hotspot in Egyptian severe HA patients or to the presence of common ancestors as per previous reports (Abdulqader et al., 2020; Aquila et al., 2004; Atik et al., 2020; <http://www.factorviii-db.org/index.php>, 2017; <http://factorviidb.org>, 2014; Hill et al., 2005; Santacroce et al., 2008). The (NM\_000132.3:c.2440C>T, p.(R814\*)) nonsense mutation results in altered mRNA decay which would allow for the production of the truncated protein sufficient for dampening the severe clinical outcome.

As Inv22 and Inv1 in addition to exon 14 sequencing could only unravel the molecular pathology in severe HA

**TABLE 1** F8 mutations in Egyptian hemophilia A patients

Patients	Mutations	Nucleotide changes	Amino acid changes	Novelty	Disease severity
P1	Inv22 type1	-----	-----	Reported	Severe
P2	Inv22 type1	-----	-----	Reported	Severe
P3	Inv22 type1	-----	-----	Reported	Severe
P4	Inv22 type1	-----	-----	Reported	Severe
P5	Inv22 type1	-----	-----	Reported	Severe
P6	Inv22 type1	-----	-----	Reported	Severe
P7	Del 22 type1	-----	-----	Reported	Severe
P8	Del 22 type2	-----	-----	Reported	Severe
P9	Del 22 type2	-----	-----	Reported	Severe
P10	Frameshift	c.2734_2735delAA	N912Ffs*6	Novel	Severe
P11	Frameshift	c.3091_3094del AGAA	K1031Lfs*9	Reported	Severe
P12–20	nonsense	c.2440C>T	p.Arg814*	Reported	Severe

**FIGURE 2** Genotyping patterns of different Inversion 22 (Inv 22) rearrangements using inverse shift PCR (IS-PCR). The figure shows genotyping patterns of different Inv22 rearrangements detected by IS-PCR on 1.5% agarose gel. On the left, Del22 type 1 (one band at 457 bp) and Inv22 type 1 (two bands at 559 & 457 bp) are shown. On the right gel, the Del22 type 2 (one band at 405 bp) is shown versus Del22 type 1 (one band at 457 bp) and wild type (WT; two bands at 457 & 405 bp). The sizes of 100 bp DNA marker (M) bands are shown**TABLE 2** Genotype–phenotype findings in two cohorts of Egyptian hemophilia A patients

Genotypes of intron 22-related re-arrangements	Number of patients studied by Abou-Elew et al. (2011)	Number of patients included in our study	Total reported Egyptian HA	HA phenotype
Wild-type allele	20 (66.7%)	51 (85%)	71 (78.8%)	22 mild, 33 moderate, and 16 severe
Intron 22 inversion type 1 (Inv22-1)	6 (20%)	6 (10%)	12 (13.3%)	11 severe and one moderate
Intron 22 inversion type 2 (Inv22-2)	1 (3.3%)	0 (0%)	1 (1.1%)	All severe
Intron 22 deletion type 1 (Del22-1)	1 (3.3%)	1 (1.6%)	2 (2.2%)	All severe
Intron 22 deletion type 2 (Del22-2)	2 (6.7%)	2 (3.3%)	4 (4.4%)	All severe
Total	30 (100%) 7 mild, 10 moderate, and 13 severe	60 (100%) 15 mild, 24 moderate, and 21 severe	90 (100%) 22 mild, 34 moderate, and 34 severe	

cases within our study, targeted NGS of the whole coding regions of *F8* and its flanking introns were performed for two mild and two moderate HA patients.

Failure of detection of any pathogenic mutations through targeted NGS analyses correlates with mild or moderate phenotype. However, suggesting the possibility of either a rare or unique individual variants located deep within the introns of *F8* gene (Atik et al., 2020; Bastida et al., 2017; <http://www.factorviii-db.org/index.php>, 2017; <http://factorviidb.org>, 2014). No further analyses could be performed due to budget limitation.

The natural homozygous variant (NM\_000132.3:c.3780C>G, p.(D1260E)), was detected in six of mild and moderate studied HA patients where we failed to detect the causative mutation. It was also detected in a heterozygous state in four of patient's mothers providing valuable linkage analyses. Viel et al. (2007) provided evidence for significant association between this nonsynonymous variant located in B-domain of *F8* gene and FVIII coagulation activity. FVIII: C level, the association persisted with each C-allele additively increasing the FVIII: C level by 14.3 IU dl<sup>-1</sup> ( $p = 0.016$ ). This variant was also previously detected with 23 entries from different populations (Bastida et al., 2017; Casaña et al., 2008; Fernández-López et al., 2005; Reitter et al., 2010; Repesse et al., 2007; Viel et al., 2007).

Linkage analysis application utilizing such polymorphic sites, helped in successfully providing genetic counseling services for families with informative mothers (Hussein et al., 2008).

In conclusion, the current study concluded that severe HA is associated with major disruptions of the *F8* gene, whereas mild and moderate hemophilia correlates with other alterations that need further molecular analyses. Facing the high management cost, QoL, and limited resources in developing countries, the study is an important step to characterize the molecular pathology underlying HA coupled with linkage analyses, as essential tools for providing patients and their families with proper genetic counseling and prenatal services.

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## CONFLICT OF INTEREST

The authors have no conflict of interest relevant to this article.

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