Factor 8 Gene Mutation Spectrum of 270 Patients with Haemophilia A: Identification of 36 Novel Mutations

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Abstract

Objective: Haemophilia A (HA) is the most severe X-linked inherited bleeding disorder caused by hemizygous mutations in the F8 gene. The aim of this study is to determine mutation spectrum of F8 gene in a large HA cohort from Turkey, and then to establish a phenotype-genotype correlation.

Materials and Methods: All HA patients (270 patients), analyzed molecularly in Ege University Pediatric Genetics Molecular Laboratory between March 2017 and March 2018, were included in this study. To identify “intron 22 inversion” (Inv22), “intron 1 inversion” (Inv1), “small deletion/insertions” and “point mutations”, molecular analyses of F8 were performed using a sequential application of molecular techniques.

Results: The mutation detection success rate was 95.2%. A positive Inv22 was found in 106 patients (39.3%), Inv1 was found in 4 patients (1.5%), and 106 different disease-causing sequence variants were identified in 137 patients (50.6%). In 10 patients (3.7%), amplification failures involving one or more exonic regions, considered to be large intragenic deletions, were identified. Of 106 different F8 mutations, 36 were novel. The relationship between F8 genotype and inhibitor development was considered significant.

Conclusion: A high mutation detection rate was achieved via the broad molecular techniques performed in this study; including 36 novel mutations. With regard to mutation types, mutation distribution and their impact on clinical severity and inhibitor development were found to be similar to those previously reported in different haemophilia population studies.

Keywords: Haemophilia A, F8 gene, mutation, inhibitors, intron 22 inversion, Turkey

Running title: The mutation spectrum of F8 gene in Haemophilia A
**Introduction**

Haemophilia A (HA) is an X linked disease with a prevalence of approximately 1 in 5000 males, and it is the most severe inherited bleeding disorder. The clinical phenotype of HA is classified as severe (FVIII:C<1%), moderate (FVIII:C 1–5%) and mild (FVIII:C >5%) in accordance with the level of coagulant activity of FVIII (FVIII:C) (1).

The coagulation factor VIII gene \(F_8\) is one of the largest genes in the genome, spanning 186 kb, consisting of 26 exons, and being localized at Xq28 (2, 3). More than 3000 unique mutations have been recorded across both the haemophilia A mutation database (HAMSTeRS) and the human gene mutation database (HGMD) (4, 5, 6). Mutations are classified into three groups: large rearrangements (intron 22 inversion, intron 1 inversion), intragenic deletions or insertions and single nucleotide variants (missense, nonsense and splice site). In severe HA the most common gene defect is an intron 22 inversion, which is responsible for 40–50% of cases. However, taken all phenotypes into consideration point mutations are found in around 47%, making them the most prolific. The other \(F_8\) gene variants such as Intron 1 inversion and large deletions are seen less frequently (7, 8, 9).

The \(F_8\) genotype is reported to be associated with clinical severity, risk for inhibitor formation, and response to immune tolerance therapy. Taking this into account, mutation analysis of the \(F_8\) gene is crucial for prediction of disease severity, choice of appropriate treatment, and optimal genetic counseling (10). The aim of this study is to determine the mutation spectrum of \(F_8\) gene in HA patients and then establish a phenotype-genotype correlation.

**Materials and Methods**

**Participants**

All HA patients (270 patients), analyzed molecularly in Ege University Pediatric Genetics Molecular Laboratory between March 2017 and March 2018, were included in this study. Demographic features, Factor VIII:C levels, and inhibitor status were all obtained from medical records, retrospectively. The clinical severity of the patients was classified into three groups (severe, moderate and mild) in accordance with the Factor VIII:C levels which had been measured previously using standard 1-stage clotting assay. The FVIII inhibitor titers in all HA patients in this study were quantified using the Nijmegen modification of the Bethesda assay (11). An informed consent for all molecular studies was obtained from either patient directly or their guardians. The study was approved by the Erciyes University Ethics Review Committee.

**Molecular Genetic Analysis**

Genomic DNA was extracted from 2 ml EDTA peripheral blood using a Gentra Puregene Blood Kit (QIAGEN), in accordance with the manufacturer's instructions. Using inverse shifting PCR method (12) all patients were first screened for intron 22 inversion (Inv22) with the Hemophilia A Genotyping Kit, Part A (by Multigen Healthcare). Any negative results were then tested for intron 1 inversion (Inv1) via multiplex PCR. In patients found to be negative for both Inv22 and Inv1, a sequencing analysis of all the coding regions and exon-intron boundaries of \(F_8\) gene was then performed. The sequence analysis was performed on Illumina MiSeq or MiniSeq platform using the Hemophilia A Genotyping Kit, Part B (by Multigen Healthcare).

**Variant Analysis**

Sequence variants were interpreted in accordance with the American College of Medical Genetics and Genomics (ACMG) guidelines (13). All identified \(F_8\) gene variants with a frequency of less than 1% in public databases were selected. Databases included NCBI dbSNP build141 ([http://www.ncbi.nlm.nih.gov/SNP/](http://www.ncbi.nlm.nih.gov/SNP/)), 1000 Genomes Project ([http://www.1000genomes.org/](http://www.1000genomes.org/)), Exome Aggregation Consortium (ExAC) ([http://exac.broadinstitute.org/](http://exac.broadinstitute.org/)) and NHLBI Exome Sequencing Project (ESP) Exome Variant Server ([http://evs.gs.washington.edu/EVS/](http://evs.gs.washington.edu/EVS/)). Selected variants were then checked against HAMSTeRS database ([http://www. HAMSTeRS.ac.uk/](http://www.HAMSTeRS.ac.uk/)) and HGMD (5, 6). The impact of novel variants on the protein structure was than classified using several *in silico* prediction tools.
such as MutationTaster, Polyphen-2, and SIFT (14, 15, 16). Conservation of residues across species was evaluated using PhyloP algorithm and GERP (17, 18).

The mutations found in this study were classified as either high risk (Inv22, Inv1, large deletions, point mutations including nonsense, frameshift) or low risk F8 genotype (missense variants, inframe deletion/insertions and splice mutations). As outlined in the RODIN study, which suggested an association between the F8 genotype and its impact on inhibitor development, the mutations identified in this study were also classified within the same parameters (10, 19).

**Statistical Analysis**

SPSS (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.) was used for all analyses. Comparisons were made using Fisher exact test and a P value of less then .05 was considered significant.

**Results**

**Mutation Spectrum and Novel Mutations**

On two hundred and seventy HA patients (age at the diagnosis: 7.9±5.27 months) from unrelated families, molecular analysis was performed. Of the patients, 269 was boy. In 106 HA patients (39.3%), Inv22 mutation was found. Following the second step, Inv1 was found in 4 HA patients (1.5%). The remaining Inv22 and Inv1 negative patients were then analyzed for F8 sequence variations and a disease-causing variant was found in 137 (50.6%). In patients with no F8 gene mutation, sequence views were reevaluated using IGV (20). In 10 patients (3.7%), amplification failures involving one or more exonic regions, considered as large intragenic deletions, were identified. A sequence analysis of the patients in whom these deletions were found, were then resequenced, and the same results were confirmed: (del ex1, del ex2-9, del ex7-13 (in two unrelated families), del ex9, del ex11-12, del ex12, del ex14, del ex15-22, del ex26)). After the all molecular analysis steps, no mutations were found in 13 HA patients (4.8%). Across the whole study group, considering Inv22, Inv1 and F8 sequence analysis, the mutation detection success rate was 95.2%.

A hundred and six different likely pathogenic and pathogenic variants were identified within 137 families. Of the variants, 56 (52.9%) were missense, 18 (16.9%) nonsense, 25 (23.7%) frameshift, 6 (5.6%) splice site with 1 (0.9%) inframe deletion. Among 106 mutations, 36 mutations in 42 families were novel (33.9%). Of the 36 novel mutations, 16 (44.5%) were frameshift, 15 (41.7%) missense, and 5 (13.8%) nonsense. The novel mutations identified in this study, including their distribution in exonic and domain levels, are given in Table 1. A list of all mutations detected in this study given in a Supplement Data.

Taking clinical severity of the 270 HA patients into consideration 221 (81.9%) were severe, with 49 (18.1%) moderate or mild. The mutation spectrums of both severe and/or mild/moderate HA groups given in Figure 1.

One of the patients in the study group was a 11-month-old girl, admitted to the hospital due to a right occipital fracture and epidural hematoma (21). She was born to consanguineous parents, and her father had severe Hemophilia A. Her coagulation test results have been found compatible with severe Hemophilia A. In this patient, a homozygous variant c.608T>C (L203P) was found. Segregation analysis showed that the father has the same mutation hemizygously, and the mother heterozygously.

Of all HA patients with a causative mutation identified in this study, 67.3% (173 of 257) were classified as high-risk. Among severe HA patients this frequency increase to 78.9% (168 of 213).

The association between mutation risk group and clinical severity was found to be statistically significant (p<0.001).

**Inhibitor Development**
Inhibitor status of all but 2 HA patients (1 in severe and 1 in mild/moderate HA) was evaluated in this study. The frequency of inhibitor positive patients was found to be 14.1% (38 of 268). When only severe HA patients are taken into consideration, this frequency was 16.7% (37 of 220). From among the mild/moderate HA group, only one patient was found to be inhibitor positive (2%; 1 of 48).

In the patients with Inv22, the frequency of inhibitor positivity was 23.6% (25 of 106); significantly higher than those without Inv22 (p=0.001). Two of 4 patients (50%) with Inv1 showed inhibitor positivity.

In the high-risk mutation group, inhibitor positivity was found in 35 of 173 patients (20.2%).

In the low-risk mutation group, inhibitor development was detected in only 3 of 84 patients (3.6%). The association between mutation risk group and inhibitor development is statistically significant (p<0.001).

Discussion

Since the discovery of \( F8 \) gene in 1984, a number of studies evaluating the \( F8 \) gene mutation spectrum of patients have been published. From 846 families with severe and non-severe haemophilia, Oldenburg et. al showed intron 22 inversion to be responsible for 35.7% of patients, point mutations 47.5% and small deletion/insertions 10.2%. Intron 1 inversion, large deletions and splice site mutations were rarely found (22). In our study, the mutation detection rate in \( F8 \) gene was 95.2%. This was achieved through following a protocol that involved up to three procedures; Inv22, Inv1 and \( F8 \) sequencing analysis. The frequencies of mutation type in our study are similar to those found in previous studies (7, 23, 24, 25).

In mild/moderate HA patients, missense mutations are the major mutation type with a frequency of 70-80% (4). In this study, we found the frequency of missense mutations to be highest (77%) among mild/moderate HA patients supporting earlier studies.

Studies from Turkey evaluating \( F8 \) mutation spectrum in HA patients are limited. In 1999, El-Maarri et. al investigated intron 22 inversions using Southern blot analysis in 141 HA patients from Turkey. Intrin 22 inversion mutation was found in 29% of all HA cases, and 42% of the severe HA cases (26). In another study, the mutation detection rate of DNA sequencing in intron 22 inversion negative patients was reported as 61%, with 36 different \( F8 \) gene mutations being detected (27). Within these previous studies from Turkey, patient numbers were limited and a complete molecular diagnosis algorithm was not followed. In terms of patient numbers, this is the largest study from Turkey and also includes the broadest range of molecular testing techniques.

Large deletions are responsible for 3% of severe HA patients. To date about 265 different large deletions (> 50 bp) in \( F8 \) gene have been recorded in the HAMSTeRS database (5). Multiplex ligation-dependent probe amplification (MLPA) is a standard test used for the detection of large deletions in the \( F8 \) gene. MLPA analysis was not available in our laboratory; however, following the reanalysis of \( F8 \) gene due to amplification failures in 10 patients, large deletions were considered. In \( X \) linked diseases it has been shown that, in males, amplification failure in PCR may indicate deletion mutations. However, MLPA analysis should be performed to confirm the hemizygous deletions. Furthermore, due to the existence of the other \( X \) chromosome with wild type \( F8 \) gene MLPA analysis is also necessary for detecting female deletion carriers.

Currently, more than 3000 unique mutations have been recorded in the HAMSTeRS and HGMD (5, 6). In this study, we found 36 different novel mutations in 42 unrelated families. Interestingly, a novel specific missense mutation (c.608T>C) in 5 unrelated families has been reported here for the first time; suggesting a founder effect. We reinvestigated the family history from these patients to evaluate for a common ancestral region. We identified no consanguinity between these families. Three of them were from Aegean region of Turkey, and 2 from the Middle Anatolian Region. However, each family was from a different city.
Approximately 25-30% of patients with severe HA develop inhibitors within 14 exposure days. Inhibitor risk is lower in patients with mild and moderate disease than those with severe disease. However, these patients had still developed inhibitors at an incidence reported as 6.7% by the 50th exposure day (10). In our study in mild/moderate HA patients, the risk of inhibitor development was also significantly low (2%) when compared to the risk of severe HA. It has been reported that there is a firm correlation between genotype and inhibitor development in HA patients. The incidence of inhibitors is greatest in patients with disruptive structural variations such as large multi-exon deletions (67-88%), while being comparatively lower in those with HA due to missense variants (<12%) (10). Data from the recent SIPPET (Survey of Inhibitors in Plasma-Product Exposed Toddlers) study showed the risk of inhibitor formation to be the highest in those with variants predicted as being null. No correlation between inhibitor risk and type of product used was observed (28). These results support HA genotype being a strong driver of inhibitor formation, with variants resulting in little or no protein synthesis putting patients most at risk. Consistent with this data, inhibitor formation was identified as 23.6% in patients with Inv22, 30% large intragenic deletions and 50% Inv1. However, in patients with missense mutations it was found to be 2.6%. By dividing the mutations into two groups; high-risk and low-risk we were able to show the risk of inhibitor development being significantly higher in patients with high-risk mutations.

In this study, no causative F8 gene defect could be found in 4.8% of the patients. While MLPA for large deletions/duplications could not be performed in the mutation negative patients, amplification failures in one or more exonic regions in 10 HA patients were found, indicative of large intragenic deletions. Despite large duplications being rare molecular defects causing HA, we considered that the mutation detection rate would increase slightly following an evaluation of mutation negative patients using MLPA. However, this is by no means a guarantee of 100% mutation identification. Several causes can lead to this situation. Firstly, some complex gene rearrangements and intronic mutations cannot be detected using standard molecular tests currently available. Secondly, Type 2 von Willebrand Disease and the combined deficiency of FVIII and FV can also decrease the FVIII activity level, leading to a misdiagnosis of HA. These alternatives should be considered in the differential diagnosis of cases being F8 gene mutation negative but having low levels of FVIII activity. Although, the source of factor concentration (plasma or recombinant) is considered to be an important factor of inhibitor development in patients with HA, the type of factor concentrate was not reviewed, and considered outside the scope of this study.

Study Limitations
In this study, we present a large cohort of Turkish HA patients and their mutation spectrum. Our molecular analysis pipeline includes intron 22 and 1 inversion analysis and DNA sequencing of all exonic regions of F8 gene. Multiplex ligation-dependent probe amplification (MLPA) is a standard test used for the detection of large deletions in the F8 gene, but unfortunately we couldn’t perform MLPA as a part of our protocol. As a X-linked disorder, in HA, hemizygous deletions in one or more exonic regions of F8 gene can be determined by evaluating amplification failure in PCR and showing in NGS. However, NGS analysis needs to be standardized for confirmation and carrier detection.

Conclusion
In conclusion, a high mutation detection rate has been achieved via the broad molecular techniques performed in this study; including 36 novel mutations. Regarding mutation types, mutation distribution and their impact on clinical severity and inhibitor development, results were found to be similar to those reported in previous studies conducted in different haemophilia populations.

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References


**Figure 1.** Frequencies of different types of F8 DNA variants detected in all HA (A), severe HA (B) and mild/moderate HA (C) patients.

<table>
<thead>
<tr>
<th>Table 1. Detailed description of novel mutations detected in our patients.</th>
</tr>
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<tbody>
<tr>
<td><strong>cDNA and Protein level</strong></td>
</tr>
<tr>
<td>c.233del (p.F78fs*14)</td>
</tr>
<tr>
<td>c.304A&gt;C (p.T102P)</td>
</tr>
<tr>
<td>c.374G&gt;A (p.W125*)</td>
</tr>
<tr>
<td>c.529T&gt;A (p.Y177N)</td>
</tr>
<tr>
<td>c.608T&gt;C (p.L203P)</td>
</tr>
<tr>
<td>c.812C&gt;A (p.S271*)</td>
</tr>
<tr>
<td>c.1028T&gt;C (p.V343A)</td>
</tr>
<tr>
<td>c.1405G&gt;A (p.G469R)</td>
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<tr>
<td>c.1406G&gt;A (p.G469E)</td>
</tr>
<tr>
<td>Variant Description</td>
</tr>
<tr>
<td>---------------------</td>
</tr>
<tr>
<td>c.1631A&gt;T (p.D544V)</td>
</tr>
<tr>
<td>c.1715del (p.D544V)</td>
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</tr>
<tr>
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<tr>
<td>c.1941dup (p.V648Sfs*5)</td>
</tr>
<tr>
<td>c.2005T&gt;C (p.S669P)</td>
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<tr>
<td>c.2092T&gt;C (p.F698L)</td>
</tr>
<tr>
<td>c.2227G&gt;T (p.E743*)</td>
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<tr>
<td>c.2464del (p.L822Yfs*23)</td>
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<tr>
<td>c.2696del (p.S899Ifs*6)</td>
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<tr>
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MS: Missense, FS: Frameshift, NS: Nonsense