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# An intronic mutation c.6430-3C>G in the *F8* gene causes splicing efficiency and premature termination in hemophilia A

Zunjing Xia<sup>a</sup>, Jie Lin<sup>a</sup>, Lingping Lu<sup>b</sup>, Chol Kim<sup>c</sup>, Ping Yu<sup>a</sup> and Ming Qi<sup>a,d,e,f</sup>

Hemophilia A is a bleeding disorder caused by coagulation factor VIII protein deficiency or dysfunction, which is classified into severe, moderate, and mild according to factor clotting activity. An overwhelming majority of missense and nonsense mutations occur in exons of *F8* gene, whereas mutations in introns can also be pathogenic. This study aimed to investigate the effect of an intronic mutation, c.6430-3C>G (IVS22-3C>G), on pre-mRNA splicing of the *F8* gene. We applied DNA and cDNA sequencing in a Chinese boy with hemophilia A to search if any pathogenic mutation in the *F8* gene. Functional analysis was performed to investigate the effect of an intronic mutation at the transcriptional level. Human Splicing Finder and PyMol were also used to predict its effect. We found the mutation c.6430-3C>G (IVS22-3C>G) in the *F8* gene in the affected boy, with his mother being a carrier. cDNA from the mother and pSPL3 splicing assay showed that the mutation IVS22-3C>G results in a two-nucleotide AG inclusion at the 3' end of intron 22 and leads to a truncated coagulation factor VIII protein, with partial loss of the C1 domain and complete loss of the C2 domain. The in-silico tool predicted that the mutation induces altered pre-mRNA splicing by using a cryptic acceptor site in intron 22. The IVS22-3C>G mutation was confirmed to affect pre-mRNA

splicing and produce a truncated protein, which reduces the stability of binding between the F8 protein and von Willebrand factor carrier protein due to the loss of an interaction domain. *Blood Coagul Fibrinolysis* 29:381–386 Copyright © 2018 Wolters Kluwer Health, Inc. All rights reserved.

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<sup>a</sup>Department of Cell Biology and Medical Genetics, School of Medicine, Zhejiang University, <sup>b</sup>Hangzhou Inogene Clinical Laboratories Co., Ltd., Hangzhou, China, <sup>c</sup>Department of Medical Genetics, Pyongyang Medical College, Kim Il Sung University, Pyongyang, Democratic People's Republic of Korea, <sup>d</sup>Assisted Reproduction Unit, Department of Obstetrics and Gynecology, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, Key Laboratory of Reproductive Dysfunction Management of Zhejiang Province, <sup>e</sup>DIAN Diagnostics, Hangzhou, China and <sup>f</sup>Department of Pathology and Laboratory Medicine, University of Rochester Medical Center, Rochester, New York, USA

Correspondence to Ming Qi, Department of Cell Biology and Medical Genetics, School of Medicine, Zhejiang University, 866 Yuhangtang Road, Hangzhou 310058, China  
Tel: +86 13666686943; fax: +86 057188208274; e-mail: mingqi@zju.edu.cn

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## Introduction

Hemophilia A (OMIM #306700) is an X-linked recessive bleeding disorder. It is caused by deficiency or dysfunction of the coagulation factor VIII protein, which is encoded by the *F8* gene and affects one to two per 10 000 males [1]. Hemophilia A typically occurs in males, but mutations of the *F8* gene with X-chromosome inactivation of females with hemophilia A have also been reported [2,3]. Patients with severe hemophilia A (factor VIII clotting activity less than 1%) are usually diagnosed before the age of 2 years [4] and often suffer from frequent spontaneous bleeding or excessive pain and swelling from minor injuries, surgery, or even tooth extractions. Patients with moderate hemophilia A (factor VIII clotting activity 1–5%) rarely bleed spontaneously and are diagnosed before 5–6-year old, whereas patients with mild hemophilia A (factor VIII clotting activity 5–40%) are diagnosed later in life [5]. Moderate and mild hemophilia A constitute about 10% and 30–40% of the total, respectively [6]. *F8* mutation carriers do not usually exhibit bleeding symptoms, whereas heterozygous females with a factor VIII clotting activity level lower than 40% may be at risk of mild hemophilia A and slight

bleeding may occur in those with a factor VIII clotting activity level between 35 and 60% or higher [7,8].

The *F8* gene (NM\_000132.3), located in the Xq28 region, spans 26 exons and encodes 2351 amino acids, forming an F8 precursor protein with the following domain structure: A1-A2-B-A3-C1-C2 [9]. The first 19 amino acids (–19) comprise a signal sequence, followed by the A1 domain (residues 1–336), A2 domain (residues 373–710), B domain (residues 741–1648), A3 domain (residues 1690–2019), C1 domain (residues 2020–2172), and C2 domain (residues 2173–2332). In plasma, the mature F8 protein of 2332 amino acids circulates as a heterodimer consisting of a heavy chain (A1-A2-B domains) and a light chain (A3-C1-C2 domains), which associate with each other via noncovalent bonds. It also interacts with von Willebrand factor (vWF) to stabilize this heterodimeric complex [10]. To date, more than 1000 variants of the *F8* gene have been found and collected in the Human Genome Mutation Database (<http://www.hgmd.cf.ac.uk/ac/index.php>) [11], including point mutations (missense, nonsense mutations), splice mutations, insertions, deletions as well as rearrangements [12]. To illustrate the pathogenic effects of these mutations, studies have

suggested the value of investigating their influence on *F8* pre-mRNA. Therefore, cDNA sequencing and minigene assay were conducted to clarify this, whereas the bioinformatic tool Human Splicing Finder (HSF) and a three-dimensional model were also used to clearly elucidate the effects of these mutations.

## Methods

### Patient

The patient was a young boy diagnosed with moderate hemophilia A (factor VIII clotting activity 2.6%) at The Children's Hospital, School of Medicine, Zhejiang University. He has a clinical symptom with prolonged or delayed bleeding or poor wound healing following trauma such as the time when blood drawing and his grandfather suffered from hemophilia A, whereas his mother was an obligate carrier. Sample materials from the patient and his mother were collected with written informed consent using protocols approved by an institutional review board in compliance with all of the principles of the Declaration of Helsinki.

### Mutation screening

Genomic DNA was extracted from peripheral blood leukocytes in accordance with the guidelines of Blood DNA Mini Kit (SIGMEN, Hangzhou, Zhejiang, China). All 26 exons and exon–intron boundaries were determined. Primers for PCR amplification were designed in accordance with sequences collected in the NCBI database (<https://www.ncbi.nlm.nih.gov/>, NC\_000023.11). PCR products were sequenced and the results were analyzed using the software DNAMAN.

### In-vivo analysis

RNA extraction was conducted using RNAiso Plus Reagent (Takara, Otsu, Shiga, Japan). Then, the total RNA was reverse-transcribed into cDNA and amplified using the primers F8-cDNA-Exon22-24-F: GGATCAATCAATGCCTGGAGCA and F8-cDNA-Exon22-24-R: TCCTTCACATACATGCTGGTAAGC. The amplification consisted of 5 min at 95 °C; 35 cycles of 30 s at 95 °C, 30 s at 54 °C, and 40 s at 72 °C; and then finally 10 min at 72 °C. PCR products were validated by Sanger sequencing.

### In-silico analysis

The bioinformatic splicing tool HSF version 3.0 (<http://www.umd.be/HSF3/>) was applied to predict the possible influence of mutation on pre-mRNA splicing using the donor or acceptor splice site signal with the default threshold [13]. Primary structures were compared between the wild-type and mutant sequences using ORF Finder (<https://www.ncbi.nlm.nih.gov/orffinder/>). In addition, a three-dimensional model was established to predict the probable conformational change or discrepancy of molecular interaction associated with the mutation using the PyMol software (South San Francisco, California, USA).

### Minigene construct, transfection, and RT-PCR

An in-vitro strategy was developed to clarify the effects of the splicing mutation. Appropriate primers covering the target region were designed for each intron/exon segment for the mutation, as follows: F8-Trap-Ex23-24-F: GTCActcgagGATGTTGGATGCTGTTGAGAAAC; F8-Trap-Ex23-24-R: GTCAggatccATACCTCAGAA-GAAACAGTCAAGG. We then constructed wild-type and mutant mini genes using pSPL3 plasmid (Invitrogen Corporation, Carlsbad, California, USA). The plasmid and intron 22 mutation PCR products were double-digested using XhoI and BamHI endonucleases at 37 °C for 2 h. The inserts were then ligated into pSPL3 at 16 °C for 2 h using T4 DNA ligase (Invitrogen Corporation). *Escherichia coli* DH5 $\alpha$  competent cells were transformed with both wild-type and mutant minigene constructs. The transformed constructs were then isolated using a Nucleoband Xtra Midi Kit (MN, Düren, Germany). The wild-type, mutant, or empty control vector was transfected into COS7 cells using Polyjet reagent (SignaGen Laboratories, Ijamsville, Maryland, USA). After 48 h, RNA was extracted and reverse transcription-polymerase chain reaction (Primer Script Reverse Transcription Reagent; Takara, Kusatsu, Shiga, Japan) was performed using the vector primers SD6 (5'-TCTGAGTCACCTGGACAACC-3') and SA2 (5'-ATCTCAGTGGTATTTGTGAGC-3'). The PCR products for the wild-type, mutant, and empty vectors were analyzed on 2% TBE agarose gel and sequenced.

## Results

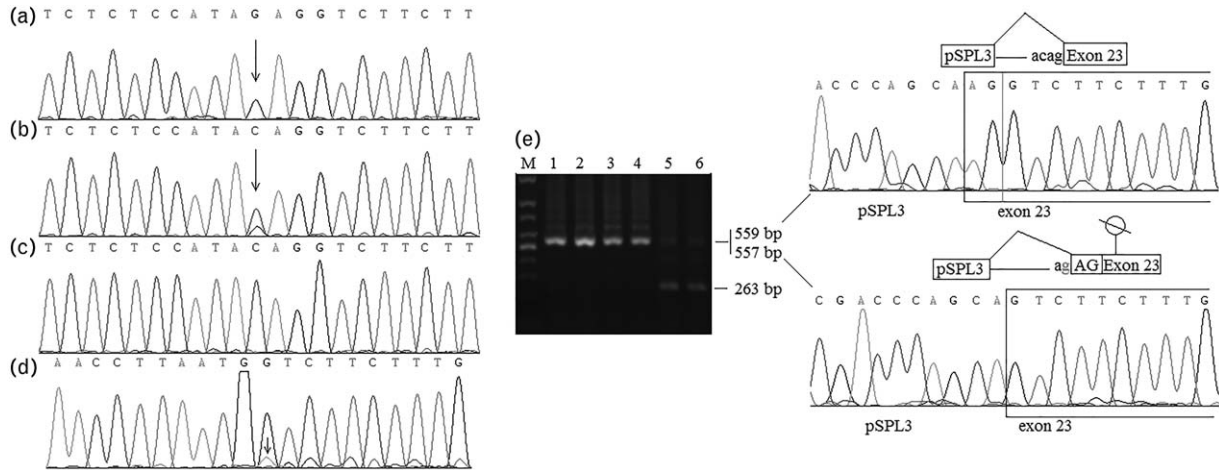
### Mutation analysis

We performed sequencing of the *F8* gene in the patient and identified an intronic variant (c.6430-3C>G or IVS22-3C>G) located in the less conserved 3' splice region of intron 22 (Fig. 1a). This mutation was first reported in 2002 [14], but no experimental data have yet been presented on if and how it affects F8 pre-mRNA splicing. The patient's mother was also shown to be a heterozygous carrier of this mutation (Fig. 1b).

### In-silico splicing analysis

HSF can be used by clinicians, geneticists, and other researchers to identify splicing mutations, providing a better understanding of clinical and biological data. Here, this bioinformatic tool was applied and showed that the score of the mutation c.6430-3C>G decreases significantly, with the wild-type score being 94.07 in comparison with the mutant score of 83.77. Therefore, it is suspected that the mutant sequence may break the original splice site. Meanwhile, the mutation is related to a 53.87% increase in a new acceptor site associated with the atctctcatagAG of a new motif (Table 1). This produces a cryptic acceptor site and leads to a dinucleotide AG inclusion at the last site of intron 22, which may cause a frameshift mutation in the normal coding sequence and produce a truncated F8 protein with a premature

Fig. 1



Identification of *F8* gene mutation and analysis of its effect on mRNA splicing. (a) Sanger sequencing of the hemophilia A patient. (b) Sanger sequencing of the patient's mother. (c) Sanger sequencing of a normal control. (d) cDNA sequencing of the patient's mother. (e) Agarose gel electrophoresis and sequencing diagram of minigene assay. The circle with a slash indicates a premature termination codon in exon 23. Arrows indicate mutations. M, markers; lanes one and 2, recombinant contains mutated genomic DNA fragment, mutant-pSPL3; lanes three and 4, recombinant contains normal genomic DNA sequence, wild-type-pSPL3; lanes five and 6, empty pSPL3 plasmid.

termination codon in exon 23. Exonic splicing enhancer (ESE) and exonic splicing silencer (ESS) were also used for illustrating these results. As Table 1 shows, the motif atac(a>g)gG linked SR protein SRp 40 predicted to break the original ESE element by decreasing -11.21% of wild-type score; meanwhile, this mutation sequence also predicted to create an ESS element linked by heterogeneous nuclear ribonucleoprotein (hnRNP) A1 protein and thus may greatly reduce the efficiency of transcription of the specific mutant allele. Alternatively, it may explain why the level of mutant transcript is markedly lower than that of the normal transcript. In brief, the score decrease of -10.95% indicated that this mutation may break the original splicing site of *F8* gene and predicted to be a splicing mutation. In addition, the score increase of +53.87% manifested the production of a new possible acceptor site, which also forecasted a change of *F8* pre-mRNA splicing in another aspect.

**In-vivo and in-vitro splicing assays**

The variant c.6430-3C>G of the *F8* gene may be associated with a two-nucleotide inclusion at the 3' splice site by creating a cryptic acceptor site, as determined using the splicing prediction software HSF. This mutation may

affect pre-mRNA splicing; so, a test was performed to evaluate its pathogenicity. An in-vivo assay showed a minor peak representing a mutant sequence in the boundary of exon 22 and 23, in addition to a major normal sequence peak, which reflected a two-nucleotide inclusion at intron 22 in the 3' splice site (Fig. 1d). In the minigene assay, the pSPL3 vector worked well by forming a 263-bp fragment in empty plasmid using SD6 and SA2 primers (Fig. 1e). Mutant-pSPL3 and wild-type-pSPL3 recombinants were associated with similar bands of 559 and 557 bp, respectively, indicating the inclusion of the two nucleotides AG at the end of the 3' splice site of intron 22, as confirmed by Sanger sequencing. This intronic mutation led to a premature termination codon in exon 23 of the *F8* protein, p.Val2144Glufs\*12, which is consistent with the results of the in-vivo assay and bioinformatic prediction. Both in-vivo and in-vitro assays demonstrated the same effect on *F8* pre-mRNA splicing.

**Three-dimensional modeling analysis**

The mutation identified in this study leads to a truncated *F8* protein of 2129 amino acids, in comparison with the wild-type sequence of 2332 amino acids, due to an extra two-nucleotide inserted AG into the normal sequence,

**Table 1 In-silico predictions for 3' acceptor splice sites using Human Splicing Finder, exonic splicing enhancer, and exonic splicing silencer**

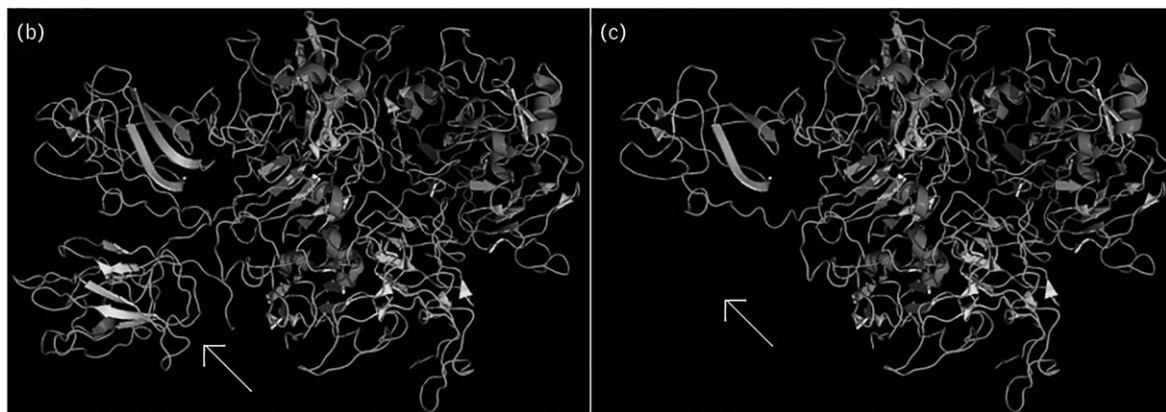
cDNA position	Type	Reference motif	Mutant motif	Score (0-100) WT/MU	Variation (%)	Interpretation
-12	Acceptor	ctctccatagGT	ctctccatagagGT	94.07/83.77	-10.95	WT SS broken
-14	Acceptor	atctctccatagAG	atctctccatagAG	53.72/82.66	+53.87	New acceptor site use
-6	SRp40	atacagG	AtaGagG	91.86/81.56	-11.21	Breaks the ESE
-5	hnRNP A1	tacagG	taGagG	66.43/85.71	+29.03	Creates a new ESS

Consequences were predicted using the HSF prediction algorithm. ESE, exonic splicing enhancer; ESS, exonic splicing silencer; HSF, Human Splicing Finder; SRp40 and hnRNP A1, linked SR protein and hnRNP protein, respectively; SS, splice site; WT/MU, wild-type and mutated sequences.

Fig. 2

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(a) WT 1   MQIELSTCFPL. // . KWQTYRGNSTGTLMVFFGNVDSSG IKHNI FNPP I IARY IRLHP THYSIRSTLRMELMGCDLN 2191
      MU 1   MQIELSTCFPL. // . KWQTYRGNSTGTLM -----ESS ----- LAMWIHLG ----- 2154
      WT 2192 SCSMPLGMESKAISDAQITASSYF INMF ATWSP SKARLHLQGRSNAWRPQVNNPKEWLQVDFQKTMKVTGVTTCGVKSL 2271
      MU
      WT 2272 TSMYVKEFLISSSQDGHQWTLFFQNGKVKVFCGNQDSF TPVVNSLDPPLLTRYLRHPQSWVHQIALRMEVLGCEAQDLY 2351
      MU
  
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Primary and three-dimensional structures of wild-type and mutant F8 proteins. (a) Amino acid sequences of the wild-type and mutant were subjected to a Blast search using ORF Finder, revealing that the mutation produced a truncated F8 protein. (b) Three-dimensional modeling of the F8 protein using PyMol software. (c) Three-dimensional modeling of the truncated F8 protein, which loses part of the C1 domain and all of the C2 domain, in comparison with the wild-type protein, as indicated by an arrow.

which results in a premature termination codon in exon 23 (Fig. 2). This leads to the mutant F8 protein having partial deletion of the C1 domain and total loss of the C2 domain. This partial deletion changes not only the primary structure but also the protein conformation, as well as the protein's interactions with other proteins and small molecules such as von Willebrand factor. This mutation may also change the formation of the F8 heterodimer or the stability of the whole F8 complex by abolishing or altering hydrogen bonds and hydrophobic interactions, not only the brief conformational change of the F8 protein compared with that of the wild-type on the surface, as determined by three-dimensional modeling.

## Discussion

Mutations occurring in less conserved regions are usually overlooked by geneticists or physicians, which should be confirmed by experiments such as *in vivo* or minigene assays. The findings suggest that in-silico splicing tools are a powerful option for researchers to identify splicing variants in a time and cost-effective manner. A series of reports on the use of HSF software or the evaluation of its ability in predicting splice mutations has been published [15–17]. In addition, in-vivo splicing assay is a direct method to prove pathogenicity, whereas minigene assay

can also be an effective validation technique when there is difficulty obtaining materials such as fresh blood or specific organs from patients.

The mutation c.6430-3C>G has been previously reported but it did not provide any experimental evidence except clinical phenotype [14]. In our in-vitro splicing assay, the mutant constructs produced a new acceptor site in intron 22. This was confirmed by the in-vivo splicing assay (Fig. 1d). However, one problem with this approach is that the quantity of mutant sequence is very low compared with wild-type cDNA sequence according to the ratio of peak heights from sequencing diagram (Fig. 1d). There are some possible reasons for this as follows. First, a general decrease of mutant transcript probably occurs via nonsense-mediated mRNA decay as reported in F13a [18], F5 [19], F11 [20], and vWF genes [21]. The second reason for the low level of mutant sequence is that the alteration of regulatory elements such as ESE and ESS, which act as *cis*-acting elements, regulates premature mRNA splicing via the SR protein family and hnRNP family protein, respectively. Alternatively, the motif change from tacagG to taGagG may also act as a new ESS by increasing 29.03% of the wild-type score. These regulatory elements may change the splicing site, as well as the transcriptional efficiency.

The findings from this study suggest that the ESE and ESS elements may play important roles in the pre-mRNA splicing process and transcriptional efficiency. Third, the marked decrease in the level of mutant sequence may result from another unknown mechanism due to different experimental conditions.

It should be noted that deficient interaction between the F8 protein and the carrier protein vWF due to the lack of the sequence encoding the carboxyterminal end binding region (residues 2303–2332) [22,23] in the mutant *F8* allele may play an important role in stabilizing the mutant protein by protecting the F8 protein from proteolytic attack [24]. There is another interacting region at the amino terminal end of the factor VIII light chain [25], but the affinity of binding of these two separate fragments to vWF is markedly lower than that for the intact F8 heterodimer [26]. The truncated protein destroyed the formation of a complex between F8 and vWF and prevented the next step of activation of the F8 protein in the blood coagulation process, which may result in a moderate symptom of the patient with prolonged or delayed bleeding or poor wound healing following trauma such as the time when blood drawing.

Most reported splice mutations causing hemophilia A were identified at mRNA level isolated from patients and predicted splicing efficiencies [27] or splicing effect using in-silico prediction tools of *F8* gene. Mutations c.5587-93C>T and c.5999-277G>A were identified by in-vitro assays using pcDNA 3.1/V5-His-TOPO vector [28]. To our knowledge, this was the first report using pSPL3 plasmids as the vectors in identifying splice mutations of *F8* gene, and it provided another further verification method in genetic, prenatal diagnosis and preimplantation genetic diagnosis of hemophilia A [29]. The pSPL3 exon trapping vector contains two exons SD and SA, and a functional intron, with transcription beginning following the SV40 promoter and ending at the late poly (A) signal [30]. In addition, the exon SD can recognize the nearest and the most probable acceptor site to complete the splicing process, which may imitate a in-vivo splicing environment. However, the entire *F8* gene contains more than 180 000 nucleotides, whereas to our knowledge, the maximum capacity of 5000 nucleotides can be amplified and successfully cloned into the pSPL3 exon trapping vector due to the experimental limitations. Therefore, only a fragment of *F8* gene sequence containing the mutation c.6430-3C>G was cloned into the pSPL3 vector. Actually, the in-vivo assay also illustrated the accuracy of the in-vitro splicing assay. In brief, to some extent, in-vitro splicing assay can be another valid method to auxiliarily diagnosis splicing mutations especially when experiments *in vivo* are difficult to carry out.

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## Conflicts of interest

There are no conflicts of interest.

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