Iranian Journal of Blood & Cancer

Iran J Blood Cancer, 2024, 16(4), 1-8
https://ijbc.ir

Case Report

F8 Gene Splice Donor Mutation (c.1271+1G>A) in Individual with Mild Hemophilia A in Indonesia: A Case Study

Ni Gusti Ayu Galuh Candra Kirana[1], Suprianto[1], Indra Lesmana[2,3], Usi Sukorini[4], Niken Satuti Nur Handayani[2,3*]

[1]*Faculty of Biology, Universitas Gadjah Mada, Yogyakarta, Indonesia.*
[2]*Department of Tropical Biology, Faculty of Biology, Universitas Gadjah Mada, Yogyakarta, Indonesia.*
[3]*Laboratory of Genetics and Breeding, Faculty of Biology, Universitas Gadjah Mada, Yogyakarta, Indonesia.*
[4]*Department of Clinical Pathology and Laboratory Medicine, Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia.*

Citation Kirana NGAGC, Suprianto, Lesmana I, Sukorini U, Handayani NSN. F8 Gene Splice Donor Mutation (c.1271+1G>A) in Individual with Mild Hemophilia A in Indonesia: A Case Study. Iran J Blood Cancer. 2024 Dec 30;16(4): 1-8.

doi 0

Article info:

Received: 26 Oct 2024
Accepted: 24 Dec 2024
Published: 30 Dec 2024

Abstract

Introduction: Hemophilia A is a bleeding disorder caused by a deficiency of coagulation factor VIII. Hemophilia A is an X-linked recessive disorder. Depending on the level of blood coagulation factor VIII, hemophilia severity is classified as mild (5-40%), moderate (1-5%), or severe (<1%). The absence of hemophilia A mutation studies in Indonesia makes this topic important to study.

Methods: This study detected and classified *F8* gene mutations. A member of the Indonesian Hemophilia Society Association for the Special Region of Yogyakarta provided saliva for DNA testing. Long-read sequencing data were performed using the next-generation sequencing (NGS) technique via the Oxford Nanopore Technologies plc (ONT) PromethION 24 platform. The mutation was confirmed using Sanger sequencing, after amplifying intron 8 of the F8 gene with the PCR technique. The *F8* gene intron 8 nucleotide sequence was aligned using the alignment tool on the Benchling website.

Results: The results of this study showed that there was a splice donor site mutation in intron 8 of the *F8* gene (c.1271+1G>A) in one patient. This mutation can cause the occurrence of cryptic splice donor sites. Cryptic splice donor site prediction was carried out using the splice donor prediction tool available on the NNSPLICE website. The appearance of cryptic splice donor sites can lead to the formation of out-of-frame proteins.

Conclusions: The *F8* gene mutation causing hemophilia A was detected using long-read sequencing and the next-generation sequencing (NGS) technique. The type of mutation identified is a splice donor site mutation, specifically the variant c.1271+1G>A, in sample code HM13.

Keywords:

Hemophilia A
F8 gene
Intron 8
Donor splice mutation
c.1271+1G

[DOI: 10.61186/ijbc.16.4.1]

---

* Corresponding Author:
Niken Satuti Nur Handayani
Affiliation: Faculty of Biology, Universitas Gadjah Mada, Yogyakarta, Indonesia.
E-mail: **niken_satuti@ugm.ac.id**

1. INTRODUCTION

Hemophilia is a medical condition characterized by a deficiency of coagulation factors, leading to abnormal bleeding (1). Hemophilia is categorized into three types: hemophilia A, which is characterized by a deficiency of factor VIII; hemophilia B, which is characterized by a deficiency of factor IX; and hemophilia C, which is characterized by a deficiency of factor XI (2,3). Hemophilia A and B are inherited in a recessive manner on the X chromosome, whereas hemophilia C is inherited in an autosomal recessive pattern (4,5).

Hemophilia A and hemophilia B share the same severity classifications based on the levels of factors VIII and IX: severe (<1%), moderate (1%–5%), and mild (>5%–40%) [5,6]. Meanwhile, hemophilia C is classified as severe when factor XI levels are below 20%, moderate when they range from 20% to 40%, and mild when they range from 40% to 60% (7). Hemophilia is characterized by recurrent bleeding in the joints and muscles, as well as persistent bleeding following an injury or surgical procedure (8).

The presence of a mutation in the *F8* gene is responsible for the development of hemophilia A (9). The *F8* gene mutation affects the functionality of the factor VIII (FVIII) protein, which serves as a cofactor for factor IX (FIX) (10). The FVIII protein, in conjunction with the FIX protein, forms a tenase complex that is involved in the activation of factor X during thrombin production (10). The *F8* gene is located on chromosome Xq28 and spans 186 kilobases. It consists of 26 exons and 25 introns. The introns of the *F8* gene range in length from 0.2 kilobases to 32.4 kilobases (11). Intron 1 and intron 22 are regions of the DNA sequence that are particularly susceptible to mutations responsible for the development of hemophilia A (12).

Intron 22 inversion mutations account for approximately 44% of hemophilia A cases, while intron 1 inversions account for about 3% of hemophilia A cases in Malaysia (12). Other mutations related to hemophilia A have been studied in Turkey, revealing 52.9% missense mutations, 23.7% nonsense mutations, 5.6% frameshift mutations, and 0.9% deletions (13). Similarly, studies on families with hemophilia A in Japan revealed 4% large deletions, 6% small deletions, 4% large insertions, and 6% small insertions (14). Mutations at the splicing site account for 3% of cases, while 2% of hemophilia A cases do not have mutations in the *F8* gene (15,16).

Studies on hemophilia A mutations have not been reported in Indonesia. However, based on a 2021 survey by the World Hemophilia Federation, it is estimated that there are approximately 2,939 people with hemophilia in Indonesia, 2,425 of whom suffer from hemophilia A (17). This underscores the importance of identifying *F8* gene mutations in individuals with hemophilia A, to detect female carriers and predict the potential emergence of hemophilia in offspring (18). Mutation identification was performed using long-read sequencing with the next-generation sequencing (NGS) method, and mutation validation was conducted using Sanger sequencing.

2. MATERIALS AND METHODS

2.1. Patients and samples

The samples studied consisted of male individuals who were members of the Indonesian Hemophilia Society Association (HMHI) in Yogyakarta. The inclusion criteria were patients diagnosed with hemophilia A, aged 7–22 years, who were registered as members of the Hemophilia Society Association in the Special Region of Yogyakarta and were willing to participate in the study. The exclusion criteria were patients diagnosed with hemophilia A who refused to participate in the study.

2.2. DNA extraction

DNA extraction, genome analysis, and molecular analysis were conducted at the Genetics and Breeding Laboratory of the Faculty of Biology, Universitas Gadjah Mada. DNA amplification results were sequenced at the Integrated Research and Testing Laboratory, Universitas Gadjah Mada. Prior to collecting saliva samples, participants completed an informed consent form. The saliva collection process involved using a saliva collection kit from NEST, China. DNA samples were extracted using the Genomic DNA Mini Kit, following the techniques outlined in the kit protocol. Quantitative DNA analysis was performed using a NanoDrop Spectrophotometer. Electrophoresis was performed for qualitative testing, using a 1.5% concentration of agarose gel.

2.3. Long-read Sequencing

Long-read sequencing of the *F8* gene (ChrX: 154835792-155022723 GRCh38) was performed using the NGS method via the Oxford Nanopore Technologies plc (ONT) PromethION 24 platform. Sequencing was performed using EPI2ME software. The Vazyme Equalbit 1x dsDNA HS Assay Kit was used for DNA quantification. DNA quantification results were measured using the Qubit 2.0.

2.4. Amplification and Sequencing

Confirmation of mutations from the NGS results was performed using Sanger sequencing. Genomic DNA was amplified using intron 8 forward primer TGAAATGGATGTGGTCAGGTT and reverse primer AAAGGTCCCAAGATTCCTGA, yielding an amplicon length of 581 bp. DNA amplification was performed using the polymerase chain reaction (PCR) method with a SimplyAMPTM thermal cycler. The PCR mix was prepared with a total volume of 25 µl for each reaction. Samples were amplified for 35 cycles with PCR conditions adjusted to the primer Tm. The amplification stages consisted of pre-denaturation at 95°C for 5 minutes, denaturation at 95°C for 30 seconds, annealing at 56°C for 30 seconds, extension at 72°C for 30 seconds, and post-extension at 72°C for 5 minutes. Next, the amplification results were sequenced using the Sanger method at the DNA Sequencing Services (UGM Integrated Research and Testing Laboratory).

2.5. Data analysis

The NGS results were visualized using the Integrative Genomics Viewer (IGV) desktop application. Furthermore, the NGS mutation results were confirmed using Sanger sequencing, and the Sanger sequencing results were analyzed using the nucleotide alignment analysis tool available on Benchling (https://www.benchling.com/) to obtain sequence consensus and align the research subject's *F8* intron 8 gene sequence with the reference sequence (NM_000132.4). Prediction of splicing variants was carried out using the splice site prediction tool available on the NNSPLICE website.

3. RESULTS

3.1. Description of Research Subject

The research subjects were 11 male individuals with hemophilia A, aged 7 to 22 years. The symptoms experienced included pain in the joints, bruising on the body, and swelling in the knees, elbows, and toes. The treatment administered to the eleven individuals was on-demand, involving the administration of factor VIII concentrate via intravenous injection. The 11 individuals had varying levels of factor VIII based on the results of laboratory tests conducted when they were first diagnosed with hemophilia A (**Table 1**).

Normal levels of factor VIII range from 50% to 150%. Factor VIII levels of 1% to 5% indicate moderate hemophilia, affecting 7 individuals, while factor VIII levels greater than 5% to 40% indicate mild hemophilia, affecting 5 individuals.

Table 1. Factor VIII levels are determined based on the results of each individual's medical records.

Number	Initials	Factor VIII Levels (%)
1	HM1	3
2	HM5	1
3	HM8	27
4	HM9	1
5	HM10	34
6	HM11	1,9
7	HM13	8,3
8	HM14	1,1
9	HM15	1,9
10	HM16	4
11	HM17	27,6

3.2. Molecular Analysis

The *F8* gene mutation was explored using long-read sequencing with the NGS method. The sample used for mutation analysis was sample HM13, which had a DNA concentration of 272 ng/µl and a DNA purity of 1.86. Qualitative DNA testing was performed using the Polymerase Chain Reaction (PCR) method, and amplification of intron 8 was carried out for all samples. The amplification results were visualized using 1.5% agarose gel electrophoresis in TAE IX buffer (**Figure 1**).

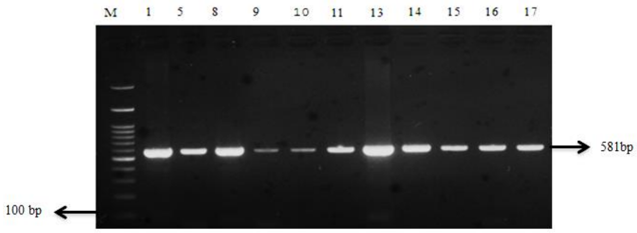


Figure 1. Visualization of the electrophoresis results for intron 8 of the F8 gene.

3.3. Nucleotide Alignment of Intron 8 of the F8 Gene in Eleven Individuals with Hemophilia A

DNA sequencing is a technique used to determine the sequence of nucleotide bases, which can be employed to identify mutations by comparing the target sequence with the reference sequence (19). The mutation was detected using the NGS technique and validated through Sanger sequencing of the *F8* gene intron 8. Nucleotide alignment was performed on the eleven samples (**Figure 2**). The results showed that no mutations in intron 8 of the F8 gene were found in ten hemophilia A patients, except for one sample

[DOI: 10.61186/ijbc.16.4.1]

with the code HM13, which exhibited an *F8* gene mutation with the variant c.1271+1G>A in intron 8.

No mutations in the *F8* intron 8 gene were found in 10 individuals, it is possible that mutations occurred in other parts of the *F8* gene. The mutation variations in hemophilia A differ in each individual, causing varying clinical conditions and levels of severity (20). The CHAMP (Centers for Disease Control and Prevention Hemophilia Mutation Project) database in 2020 recorded 3756 cases of hemophilia A mutations in the world, 1,745 cases of missense mutation variants, 416 cases of nonsense, 908 cases of frameshift, 320 cases of splice site, 320 cases of synonymous as many as 39 cases, deletion of more than 50 bp as many as 210 cases, deletion of less than 50 bp as many as 86 cases, mutations in the promoter as many as 14 cases, changes in the 3' UTR region as many as 13 cases and in the 5' UTR region as many as 5 cases. Most mutation variants are reported to occur in one patient in the world (21).

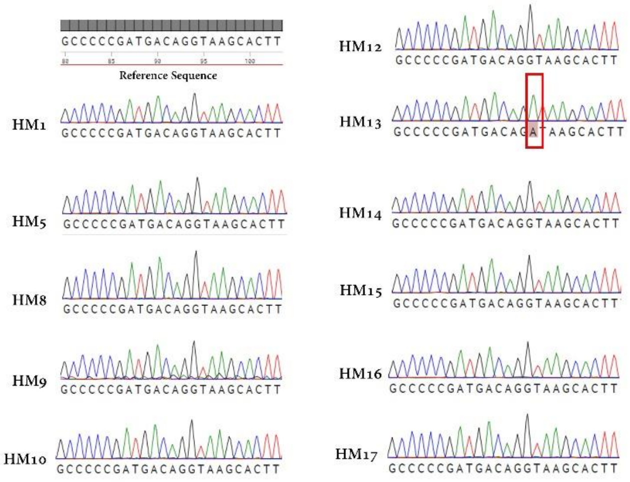


Figure 2. Nucleotide alignment of the F8 gene intron 8 compared to the reference sequence NM_000132.4.

In addition to intron mutations in the *F8* gene, mutations in the exon region of the *F8* gene also contribute to the clinical manifestations of patients with hemophilia A (22). Akkarapatumwong et al reported that mutations occurred in 6 hemophilia A patients (4 severe and 2 moderate) (23). Mutations were only found in exon 14. In addition, hemophilia A mutations studied in 6 hemophilia A patients in Thailand also showed that no mutations were found in introns, mutations occurred in exons 1, 11, 17, 18, 25, and 26 (24). Hemophilia A mutations were studied in 223 patients in China (186 severe, 14 moderate, and 16 mild) resulting in mutations occurring in Exons 1, 2, 4, 5, 6, 7, 8, 19, 11, 13, 14, 15, 16, 18, 19, 21, 22, 23, 24, 26, introns 9, 12, 13, 14, 21, 24 (25). Hemophilia A mutations were studied in 92 hemophilia A patients in Pakistan. Mutations occurred in exons 14 and 26 and occurred in introns 7, 18, and 25 (26).

Hemophilia A can occur due to mutations in other genes that correlate with the FVIII protein. Uen et al stated that in around 2% of hemophilia A cases, no mutations are found in the *F8* gene, but mutations may occur in other genes responsible for coding proteins that interact with the FVIII protein; in this case, mutations occur in the vWF gene (16). The vWF protein plays a role in protecting the FVIII protein, so mutations that occur at the vWF and FVIII protein binding sites can cause phenotypic changes called pseudohemophilia (20). Atik et al reported that no *F8* gene mutations were found in 13 out of 270 hemophilia A patients. This is possible because von Willebrand disease can reduce factor VIII levels, causing a misdiagnosis of hemophilia A (13).

Johnsen et al explained that no mutations were found in 48 hemophilia A patients. This could be due to epigenetic influences related to hypomethylation, which plays a role in the development of inhibitors (27). Hypomethylation causes T cell activation and triggers the production of pro-inflammatory cytokines, thereby increasing the immune response against FVIII protein. This becomes a major factor in the development of inhibitors against FVIII (28). The influence of non-coding RNAs also plays an important role in the development of pathologies of genetic diseases that are not caused by gene mutations (29).

In this research, a mutation was detected in only one individual with hemophilia A, identified by the code HM13. This boy was diagnosed with mild hemophilia A. There was no record of his biological parents having hemophilia, but a male cousin on his mother's side had been diagnosed with hemophilia A (**Figure 3**). The symptoms experienced by the boy included bleeding in the joints, leading to swelling in the knees and elbows during heavy activities. The bleeding occurred during activities such as playing sports, sitting on hard surfaces, eating foods with a hard texture, and he experienced significant bleeding during ear surgery and circumcision.

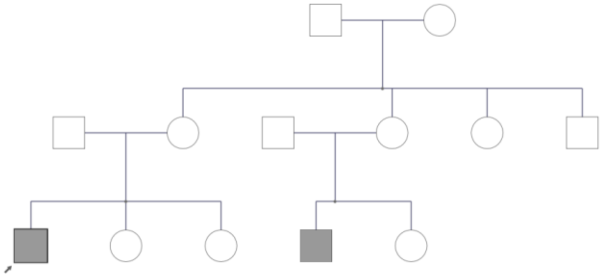


Figure 3. Pedigree of the HM13 Patient.

[DOI: 10.61186/ijbc.16.4.1]

The mutation occurs in the first nucleotide of intron 8 (**Figure 4**) which is the splice donor site region (30). The splice donor site is an intron region with an exon-intron boundary at the 5' (31). Splice donor sites have conservative motifs called sequence invariants (32). The invariant sequence of the 5' intron is in the first two bases of the GT motif (33). The GT motif is recognized by the spliceosome complex, an enzyme involved in splicing (34).

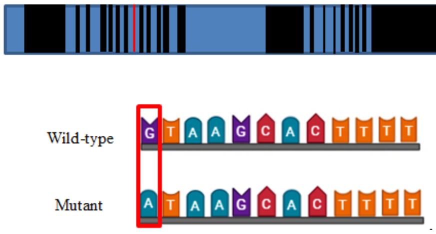


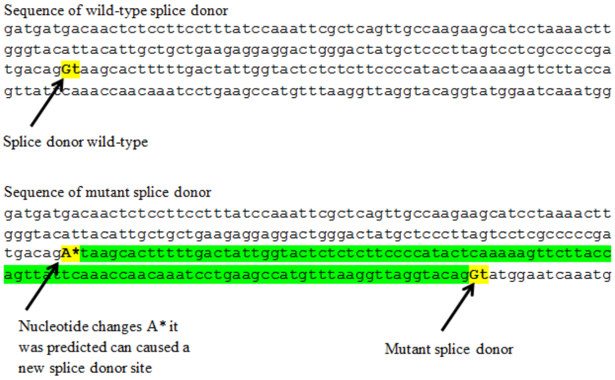
Figure 4. The splice donor mutation in the F8 gene intron 8 c.1271+1G>A.

The c.1271+1G>A mutation impacts the splicing process (35). Splicing is the process of removing introns from pre-mRNA and joining exons to form mRNA (36). The process of cutting the intron at the 5' end is recognized by the U1 spliceosome complex through base pairing interactions between U1 and the 5' end. Mutations in the 5' intron disrupt the efficiency of the splicing process by weakening the base pairing bond, leading to impaired splicing efficiency (37). Mutations in the 5' intron interfere with the recognition of the splicing site by the spliceosome (38). These intron mutations can create new splice donor sites, resulting in cryptic exons. Cryptic exons arise from alternative splicing events, where exons from the intron region are included due to mutations in the splicing site (39).

Mutations in the splicing site lead to the formation of a cryptic splice site, which is an abnormal splice site occurring outside the original splicing site. This can generate variations in the mRNA transcript and result in different gene products (30, 40). The c.1271+1G>A mutation leads to the creation of a cryptic splice site, causing a null mutation. A null mutation renders the FVIII protein non-functional due to alterations in the splice donor site and imperfections in the pre-mRNA splicing process of intron 8 (41).

The cryptic splice site was predicted using the NNSPLICE website. The *F8* gene exon 8 and intron 8 sequence were used for this prediction. The results showed six candidate sequences leading to the cryptic donor site. A score of 0-1 is the threshold for predicting sequences with a cryptic donor site; the closer the score is to 1, the better the sequence is as a donor site prediction. The best score obtained was 0.98, indicating that the c.1271+1G>A mutation produces a cryptic donor site (**Table 1**). The cryptic donor site appears 106 base pairs downstream from the wild-type splice donor (**Figure 5**). A similar case was reported in the study by Castaman et al., where a mutation in intron 9 (c.1443+1G>C) caused the emergence of a new splice donor site, located 28 base pairs downstream from the original splice donor site. This mutation resulted in the insertion of 28 base pairs from intron 9 into the patient's mRNA due to the activation of a cryptic splice site in intron 9 (42).

Mutations at the same nucleotide as the c.1271+1G>T variant cause the skipping of some nucleotides in exon 8, resulting in the creation of a cryptic splice site within exon 8 (41). Exon skipping is an alternative splicing mechanism that leads to the loss of exons in the mRNA, which impacts the protein's domains and structure (43, 44). The c.1271+1G>T mutation triggers a frameshift, producing an out-of-frame protein (41). Out-of-frame proteins occur due to shifts in the codon reading frame caused by mutations at splice sites, resulting in the incorporation of different amino acids than originally intended (45).



Sequence of wild-type splice donor

```
gatgatgacaactctccttcctttatccaaattcgctcagttgccaagaagcatcctaaaactt
gggtacattacattgctgctgaagaggaggactgggactatgctcccttagtcctcgcccccga
tgacagGtaagcacttttttgactattggtactctctcttccccatactcaaaaagttcttacca
gttattcaaaccaacaaatcctgaagccatgtttaaggttaggtacaggtatggaatcaaatgg
```

Sequence of mutant splice donor

```
gatgatgacaactctccttcctttatccaaattcgctcagttgccaagaagcatcctaaaactt
gggtacattacattgctgctgaagaggaggactgggactatgctcccttagtcctcgcccccga
tgacagA*taagcacttttttgactattggtactctctcttccccatactcaaaaagttcttacc
agttattcaaaccaacaaatcctgaagccatgtttaaggttaggtacagGtatggaatcaaatg
```

Figure 5. Comparison of splice donor wild type and splice donor mutant.

Research related to hemophilia A was carried out on multi-ethnic populations in Malaysia, where mutations were found in the intron. The splice donor site mutation c.787+1G>T occurs in the first nucleotide of intron 6, which causes hemophilia A with severe severity and occurred in 2 patients. The splice donor site mutation c.5586+2T>G occurs in the second nucleotide of intron 16, which causes hemophilia A with severe severity in 1 patient. The mutation c.6429+2T>A occurs in the second nucleotide of intron 22, which causes hemophilia A with moderate severity in one patient. The splice site acceptor mutation c.5998-1G>A occurs in the last nucleotide of intron 18, causing severe hemophilia A in 1 patient (12). The A to G transition mutation in the 1.4 kb downstream

[DOI: 10.61186/ijbc.16.4.1]

Table 2. Prediction of mutant splice donor site in intron 8.

Start Number Sequence	End Number Sequence	Score	Exon	Intron
118	132	0.33	tggtcag	GTttgatg
188	202	0.22	aacttgg	GTacatta
277	291	0.04	actattg	GTactctc
251	365	0.85	gtttaaag	GTtaggta
362	376	0.98	ggtacag	GTatggaa
383	397	0.09	ggcaagg	GTattaac

intron 1 region occurred in 2 patients in the UK, causing the emergence of a new donor splice site due to the presence of a splice acceptor site 191 bp upstream of the mutation. This emergence of a new donor splice site causes the addition of an exon between exon 1 and exon 2, which is 191 bp long, thus affecting the formation of normal FVIII protein (46). Mutations in the intron region were also reported by Castaman et al. In their study, the mutation in intron 18, c.5999-277G>A, produces a new splice donor site, thereby causing the part of the intron that should be deleted to become part of the mRNA (42). A 55 bp fragment, which is an intron, remains in the patient's mRNA, resulting in the mutation giving rise to a cryptic exon.

The c.1271+1G>T mutation leads to the development of hemophilia symptoms in individuals who carry the hemophilia gene, resulting in a moderate level of severity. This mutation arises from the inactivation of the X chromosome and a mutation in the HCFC1 gene (47). X chromosome inactivation is a process in which one of the X chromosomes in females is randomly rendered inactive in order to preserve equilibrium in gene numbers (48). The nucleotide sequence c.1271+1G>A mutation occurring at the splicing location results in the formation of inhibitors in individuals classified in the severe category (49). The c.1271+1G>A mutation at the splicing location leads to the formation of inhibitors in individuals classified as severe (50). Inhibitors counteract the procoagulant activity of the FVIII protein by impeding its function through steric hindrance. This occurs when antibodies obstruct the active site of the FVIII protein, preventing it from carrying out its normal function (51).

4. CONCLUSIONS

The mutation identified in this study is a splice-donor mutation in intron 8 with the variant c.1271+1G>A in a patient coded as HM13.

Acknowledgements

The author would like to express gratitude to the patients and members of the Indonesian Hemophilia Society, Yogyakarta Special Region, for their participation in this study, as well as to Dr. Pudjo Hagung Widjajanto, Ph.D., Sp.A(K), and Dr. Agus Wibowo for their valuable discussions. We would also like to express our gratitude to Yayasan Satriabudi Dharma Setia (YSDS) for their support in DNA sequencing for this study.

Ethical statement

This research obtained approval from the Medical Health Research Ethics Committee (MHREC) of the Faculty of Medicine, Public Health, and Nursing at Universitas Gadjah Mada (Ref. No.: KE/FK/1418/EC/2023).

Disclosure Statement

The authors declare no conflict of interest.

Funding

No funding was received for this study.

References

1. Choi SJ, Jang KJ, Lim JA, Kim HS. Human coagulation factor VIII domain-specific recombinant polypeptide expression. Blood Res. 2015;50(2):103-8.

2. Shih MY, Wang J Der, Yin J De, Tsan YT, Chan WC. Differences in major bleeding events between Patients with severe hemophilia A and hemophilia B: A Nationwide, population-based cohort study. Clin Appl Thromb. 2019;25:1-7.

3. Jayakrishnan T, Shah D, Mewawalla P. Hemophilia C: A case report with updates on diagnosis and management of a rare bleeding disorder. J Hematol. 2019;8(3):144–7.

4. Alkarrash MS, Badawi R, Sallah H, Shashaa MN, Argilo J, Alkhoury R. Hemophilia A and C in a female: The first case report in literature. Annals of Medicine and Surgery. 2021; 68:1-3.

5. Goodeve AC. Hemophilia B: Molecular Pathogenesis and mutation analysis. J Thromb Haemost. 2015;13(7):1184–95.

6. Miller CH. The clinical genetics of hemophilia B (Factor IX Deficiency). Appl Clin Genet. 2021;14: 445-454.

[DOI: 10.61186/ijbc.16.4.1]

7. Mandal S, Gami S, Shah S. A case report on an extremely rare disease: Factor XI deficiency. Cureus. 2020;12(10):1-4.

8. Berntorp E, Fischer K, Hart DP, Mancuso ME, Stephensen D, Shapiro AD, et al. Haemophilia. Nat Rev Dis Prim. 2021;7(45):1-19.

9. Zarrilli F, Coppola A, Schiavulli M, Cimino E, Elce A, Rescigno G, et al. Haemophilia a: The consequences of de novo mutations. Two case reports. Blood Transfus. 2018;16(4):392-3.

10. Shen BW, Spiegel PC, Chang CH, Huh JW, Lee JS, Kim J, et al. The tertiary structure and domain organization of coagulation factor VIII. Blood. 2008;111(3):1240-1247.

11. Bowen DJ. Haemophilia A and haemophilia B: Molecular insights. J Clin Pathol: Mol Pathol. 2002;55(2):127-144.

12. Zahari M, Sulaiman SA, Othman Z, Ayob Y, Karim FA, Jamal R. Mutational profiles of F8 and F9 in a cohort of haemophilia A and haemophilia B patients in the multi-ethnic Malaysian population. Mediterr J Hematol Infect Dis. 2018;10(1):1-13.

13. Atik T, Işık E, Onay H, Akgün B, Shamsali M, Kavaklo K, et al. Factor 8 gene mutation spectrum of 270 patients with hemophilia A: Identification of 36 novel mutations. Turkish Journal of Haematology. 2020;37(3):145-153.

14. Shinozawa K, Amano K, Hagiwara T, Bingo M, Chikasawa Y, Inaba H, et al. Genetic analysis of carrier status in female members of Japanese hemophilia families. Journal of Thrombosis and Haemostasis. 2021;19(6):1493-1505.

15. Elisa Bach J, Wolf B, Oldenburg J, Muller CR, Rost S. Identification of deep intronic variants in 15 haemophilia A patients by next generation sequencing of the whole factor VIII gene. Thromb Haemost. 2015;114(4):757-67.

16. Uen C, Oldenburg J, Schroder J, Brackmann H J, Schwaab R, Schneppenheim R, et al. Hamophilie A patienten ohne mutation im FVIII-Gen [2% haemophilia A patients without mutation in the FVIII gene]. Hamostaseologie. 2003;1-5.

17. World Federation of Hemophilia. World federation of hemophilia report on the annual global survey 2021. World Fed Hemoph. 2021;1-100.

18. Wang J, Gu J, Chen H, Wu Q, Xiong L, Qiao B, et al. A novel deletion mutation of the F8 gene for hemophilia A. Diagnostics. 2022;21;12(11):2876.

19. Sinclair A. Genetic 101 : Detecting mutation in human genes. CMAJ. 2002;167(3):275-279.

20. Graw, J., Brackmann, H. H., Oldenburg, J., Schneppenheim, R., Spannagl, M., and Schwaab, R. 2005. Haemophilia A: from Mutation Analysis to new Therapies. Nature Reviews Genetics. 6(6):488-501.

21. Pshenichnikova, O., Salomashkina, V., Poznyakova, J., Selivanova, D., Chernetskaya, D., Yakovleva, E., Dimitrieva, O., Likhacheva, E., Perina, F., Zozulya, N., & Surin, V. (2023). Spectrum of Causative Mutations in Patients with Hemophilia A in Russia. Genes, 14(2), 260.

22. Santacroce, R., Acquila, M., Belvini, D., Castaldo, G., Garagiola, I., Giacomelli, S. H., Lombardi, A. M., Minuti, B., Riccardi, F., Salviato, R., Tagliabue, L., Grandone, E., Margaglione, M., & AICE-Genetics Study Group (2008). Identification of 217 unreported mutations in the F8 gene in a group of 1,410 unselected Italian patients with hemophilia A. Journal of human genetics, 53(3), 275–284.

23. Akkarapatumwong, V., Intorasoot, S., Oranwiroon, S., Thano-Otarakul, P., Pung-Amritt, P., Veerakul, G., Mahasandana, C., Panyim, S., & Yenchitsomanus, P. (2000). Frameshift mutations with severe and moderate clinical phenotypes in Thai hemophilia A patients. Human mutation. 16(6):1-4.

24. Akkarapatumwong, V., Oranwiroon, S., Pung-amritt, P., Treesucon, A., Thanootarakul, P., Veerakul, G., Mahasandana, C., Panyim, S. and Yenchitsomanus, P. (1999). Mutations of the factor VIII gene in Thai hemophilia A patients. Human mutation. 15: 1-6.

25. Guo, Z., Yang, L., Qin, X., Liu, X., & Zhang, Y. (2018). Spectrum of Molecular Defects in 216 Chinese Families With Hemophilia A: Identification of Noninversion Mutation Hot Spots and42 Novel Mutations. journal of the International Academy of Clinical and Applied Thrombosis/Hemostasis, 24(1): 70-78.

26. Khanum, F., Collins, P. W., Harris, R. L., & Bowen, D. J. (2014). Characterization of F8 defects in haemophilia A in Pakistan: investigation of correlation between mutation type and the in vitro thrombin generation assay. Haemophilia : the official journal of the World Federation of Hemophilia, 20(2), 287–293.

27. Johnsen, J. M., Fletcher, S. N., Huston, H., Roberge, S., Martin, B. K., Kircher, M., Josephson, N. C., Shendure, J., Ruuska, S., Koerper, M. A., Morales, J., Pierce, G. F., Aschman, D. J., & Konkle, B. A. (2017). Novel approach to genetic analysis and results in 3000 hemophilia patients enrolled in the My Life, Our Future initiative. Blood advances, 1(13), 824–834.

28. Liu, W., Lyu, C., Wang, W., Xue, F., Chen, L., Li, H., Chi, Y., Ma, Y., Wu, R., Fang, Y., Zhang, L., & Yang, R. (2022). Risk Factors For Inhibitors In Hemophilia A based on RNA-seq and DNA Methylation. Research And Practice In Thrombosis And Haemostasis. 6(6), e12794.

29. Jankowska, K. I., McGill, J., Pezeshkpoor, B., Oldenburg, J., Sauna, Z. E., & Atreya, C. D. (2020). Further Evidence That MicroRNAs Can Play a Role in Hemophilia A Disease Manifestation: F8 Gene Downregulation by miR-19b-3p and miR-186-5p. Frontiers In Cell And Developmental Biology. 8, 669.

30. Cutler JA, Mitchell MJ, Smith MP, Savidge GF. The identification and classification of 41 novel mutations in the factor VIII gene (F8C). Hum Mutat. 2002;19(3):274–8.

31. Jackson LJ. A reappraisal of non-consensus mRNA splice sites. Nucleic Acids Res. 1991;19(14):3795-8.

32. Caminsky N, Mucaki EJ, Rogan PK. Interpretation of mRNA splicing mutations in genetic disease: Review of the literature and guidelines for information-theoretical analysis. F1000 Research. 2014;3(282):1-30.

33. Anna A, Monika G. Splicing mutations in human genetic disorders: Examples, detection, and confirmation. J Appl Genet. 2018;59(3):253-68.

34. Nguyen H, Das U, Wang B, Xie J. The matrices and constraints of GT/AG splice sites of more than 1000 Species/Lineages. Gene. 2018;660:92-101.

35. Zimmermann MA, Gehrig A, Oldenburg J, Muller CR., Rost S. Analysis of F8 mRNA in haemophilia A patients with silent

mutations or presumptive splice site mutations. Haemophilia. 2013;19(2):310-317.

36. Baralle FE, Singh RN, Stamm S. RNA structure and splicing regulation. Physiol Behav. 2017;176(3):139–148.

37. Rogalska ME, Vivori C, Valcárcel J. Regulation of pre-mRNA splicing: roles in physiology and disease, and therapeutic prospects. Nat Rev Genet. 2023;24(4):251–69.

38. Balestra D, Maestri I, Branchini A, Ferrarese M, Bernardi F, Pinotti M. An altered splicing registry explains the differential ExSpeU1-mediated rescue of splicing mutations causing haemophilia A. Front Genet. 2019;10:1-9.

39. Aldalaqan S, Dalgliesh C, Luzzi S, Siachisumo C, Reynard LN, Ehrmann I, et al. Cryptic splicing: Common pathological mechanisms involved in male infertility and neuronal diseases. Cell Cycle. 2022;21(3):219-27.

40. Kapustin Y, Chan E, Sarkar R, Wong F, Vorechovsky I, Winston RM, et al. Cryptic splice sites and split genes. Nucleic Acids Res. 2011;39(14):5837-44.

41. Lannoy N, Abinet I, Bosmans A, Lambert C, Vermylen C, Hermans C. Computational and molecular approaches for predicting unreported causal missense mutations in Belgian patients with haemophilia A. Haemophilia. 2012;18(3):1-9.

42. Castaman G, Giacomelli SH, Mancuso ME, Sanna S, Santagostino E, Rodeghiero F. F8 mRNA studies in Haemophilia A patients with different splice site mutations. Haemophilia. 2010;16(5):786-90.

43. Wang J, Ye Z, Huang TH, Shi H, Jin VX. Computational methods and correlation of exon-skipping events with splicing, transcription, and epigenetic factors. Methods Mol Biol. 2017;1513:163–70.

44. Lee Y, Gamazon ER, Rebman E, Lee Y, Lee S, Dolan ME, et al. Variants affecting exon skipping contribute to complex traits. PLoS Genet. 2012;8(10):1-12.

45. Marian AJ. Clinical interpretation and management of genetic variants. JACC Basic to Transl Sci. 2020;5(10):1029-42.

46. Bagnall, R. D., Waseem, N. H., Green, P. M., Colvin, B., Lee, C., & Giannelli, F. (1999). Creation of a novel donor splice site in intron 1 of the factor VIII gene leads to activation of a 191 bp cryptic exon in two haemophilia A patients. British journal of haematology, 107(4), 766–771.

47. Dardik R, Janczar S, Lalezari S, Avishai E, Levy-Mendelovich S, Barg AA, et al. Four decades of carrier detection and prenatal diagnosis in hemophilia A: historical overview, state of the art and future directions. Int J Mol Sci. 2023;24(14):1-13.

48. Kawashima S, Hattori A, Suzuki E, Matsubara K, Toki M, Kosaki R, et al. Methylation status of genes escaping from X-chromosome inactivation in patients with X-chromosome rearrangements. Clin Epigenetics. 2021;13(1):1-11.

49. Miller CH, Benson J, Ellingsen D, Driggers J, Payne A, Kelly FM, et al. F8 and F9 mutations in US haemophilia patients: correlation with history of inhibitor and race/ethnicity. Haemophilia. 2012;18(3):375-82.

50. Doncel SS, Alejandra G, Cortes JM, Rico CA, Javier F, Cadavid M, et al. Haemophilia A : A review of clinical manifestations, treatment, mutations, and the development of inhibitors pathways FVIIIa. 2023;15(1):130-150.

51. Luo L, Zheng Q, Chen Z, Huang M, Fu L, Hu J, et al. Hemophilia a patients with inhibitors: Mechanistic insights and novel therapeutic implications. Front Immunol. 2022;13:1-15.

[DOI: 10.61186/ijbc.16.4.1]