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Determining common variants in patients with haemophilia A in South Vietnam and screening female carriers in their family members

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ABSTRACT

Aims The aim of this study was to determine common variants in *F8*, including intron 22 inversion (Inv22), intron 1 inversion (Inv1) and point mutations, the transmission of these variants between patients with haemophilia A (HA) and their family members.

Methods Genetic analysis was conducted in 71 patients who were clinically diagnosed with HA and 152 related female members in South Vietnam by a combination of inversion PCR (I-PCR), multiplex PCR and direct sequencing.

Results Variants in *F8*, including Inv22, point mutations (with 37 genotypes) and two novel variants, occupied 60 patients with HA. Among severe patients, the rate of Inv22 was 44%. Missense was the common point mutation of over 50% in patients with moderate HA and mild HA. Inv1 was absent in all patients. *F8* variants were also found in 119 female carriers (FCs) (78.3%) from families related to patients with HA. There were 56 mothers (93.3%) carrying *F8* variants and passing the same variants to their sons.

Conclusions These findings were the first to provide important information about the presence of Inv22 and point mutation in Vietnamese patients with HA, the mothers and their female family members. It demonstrated that genetic diagnosis and counselling for HA carriers were essential factors for future improvements in comprehensive and equitable healthcare polices for patients with HA and FCs in Vietnam.

INTRODUCTION

Haemophilia A (HA) is known as an X-linked recessive bleeding disorder caused by inherited or spontaneous variants in the *F8* gene.[1] Severe HA (concentration of FVIII <0.01 IU/mL)[2] was characterised by frequent spontaneous haemorrhage or abnormal bleeding after an injury or surgical intervention, and it was possibly life-threatening without timely diagnosis. Typically, this disease, identified by coagulation factor assay and clinical evaluations, causes heavy and prolonged bleeding symptoms for men. In female carriers (FCs), bleeding symptoms can manifest unclearly and occur in cases such as operations, tooth extractions, tonsillectomy and primary postpartum haemorrhage.[3–5] It is necessary to assess bleeding risk for HA carriers to give early diagnosis and support medical care. However, measurement of coagulation factor activity levels in patients with mild HA and FC are not simple. Mild HA cases have FVIII activity levels between 0.5 and 0.40 IU/mL, and others have nearly normal levels (over 0.40 IU/mL).[6] In women, concentration of FVIII is changed by factors including age, ABO blood type, menstrual cycle, pregnancy and physiological condition.[4 5] FCs may be asymptomatic but are at risk of transferring the haemophilia mutant gene to their sons. Thus, some studies recommended that genetic analysis should be performed as soon as possible to determine accurate variants in *F8* gene for women in haemophilia family and prevent serious complications for patients with HA.[3 5 6]

In Vietnam, data from a report of National Survey of Haemophilia in 2019 showed that there were over 6000 Vietnamese patients with HA and the morbidity rate has been increasing. At present, bleeding treatment for patients with HA is improved better; however, healthcare services for them and their family members are still limited. This limitation is due to the fact that only male patients are more attentive to be given treatment than female patients. Furthermore, HA diagnosis needs an effective tool for HA carrier screening and diagnosis. In addition, coagulation factor tests are traditionally performed in Vietnam but detect only severe HA cases.

The information about FC is a new concept in Vietnam. There have not been yet any study about genetic diagnostics or genetic counselling to examine the carrier status for Vietnamese patients with HA as well as FC. In high-income countries, quality medical care and treatment policies for patients with HA give advances for increasing life expectancy and reducing mortality rate.[7] The application of appropriate molecular analysis methods will bring many benefits about healthcare, treatment and developing preventive health programmes to decrease the morbidity and mortality rate of haemophilia.[8 9] For instance, the report of Luu *et al* showed that intron 22 inversion (Inv22) was found in 34% of patients with severe HA at North Vietnam but lacks data from other areas of the country.[10]

Therefore, this study was conducted in South Vietnam in order to (1) determine genetic variants in Vietnamese patients with HA from genetic information in the article of Luu *et al*; (2) choose efficiency molecular techniques for HA diagnosis in Vietnam by the combination of inversion PCR (I-PCR), multiplex PCR (M-PCR) and direct sequencing; and (3) provide a useful reference

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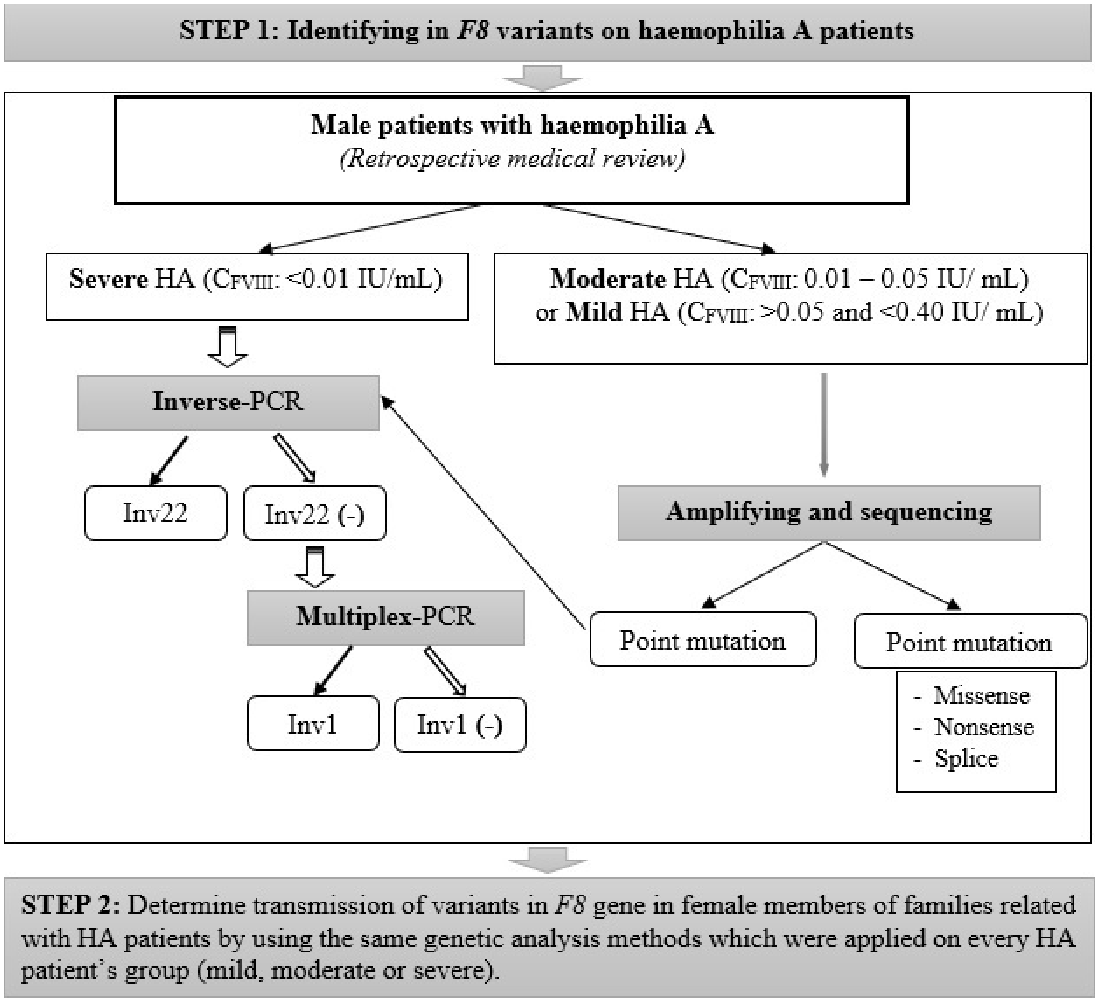

Figure 1 Procedure of identifying variants in F8 in patients with HA and female members of haemophiliac family. HA, haemophilia A.

database of the carrier status to improve genetic counselling and prenatal diagnosis programme for haemophilia FC.

MATERIALS AND METHODS

Sample collection

Procedure of sample collection and genetic analysis were shown in figure 1. From historical patient records of hospitals and medical centres at South of Vietnam, male patients that clinically diagnosed with HA were recruited to the study and divided into severe HA group or moderate and mild group. Genetic analysis was performed in both groups of patients and female members of their families such as mother, aunt, sister, and grandmother if their results were the presence of variants in *F8*. After informed consent, 2 mL peripheral blood of every participant was collected separately in EDTA (1.5 mg/mL) anticoagulant tube for genetic analysis.

Procedure of identifying variants in F8

From peripheral blood, DNA was extracted using QIAamp DNA Blood Mini Kit (Qiagen). The concentration and purity of extracted DNA were determined by using the photometric method on the NanoDrop 2000 machine with the following parameters: DNA concentration of 300–380 ng/mL; Evaluate the purity by the rate of A 260/280 nm=1.8–2.0.

We used the I-PCR kit (Promega Wizard Genomic DNA Purification Kits, USA) to detect Inv22 as protocol described by Rossetti *et al*.[11] If the sample was negative for Inv22, M-PCR would be the next method to evaluate intron 1 inversion (Inv1). We designed the primer pairs of sequence and location as the study of Bagnall *et al*.[12] Primers were checked via SNPCheck3 software to recognise the presence of SNP on primers and run OligoAnalyzer V.3.1 of IDT in order to measure the ΔG value of the secondary structure. All values of length, % Guanine-Cytosine (GC), Melting Temperature (Tm), and specificity of primers are calculated by the Primer-BLAST tool on National Center for Biotechnology Information (NCBI).

Negative samples for Inv22 and Inv1 were subjected to sequencing all exon and exon–intron boundaries of *F8* to screen point mutations by M-PCR. PCR was performed on the DNA samples of the patients by using primer pairs of 23 fragments of *F8*. Amplified fragments were subjected to direct sequencing in two directions by Big Dye Terminator V.3.1 kit of Applied Biosystems and read DNA sequences by ABI 3500 Genetic Analyzer. The sequencing results were analysed by CLC Main Workbench V.5.

RESULTS

Identification of variants in groups of patients with HA

A total of 71 patients who were diagnosed with HA (50 severe, 15 moderate and 6 mild) and 152 female members (from HA families) participated in this study. As described in figure 2, based on molecular techniques, the study found

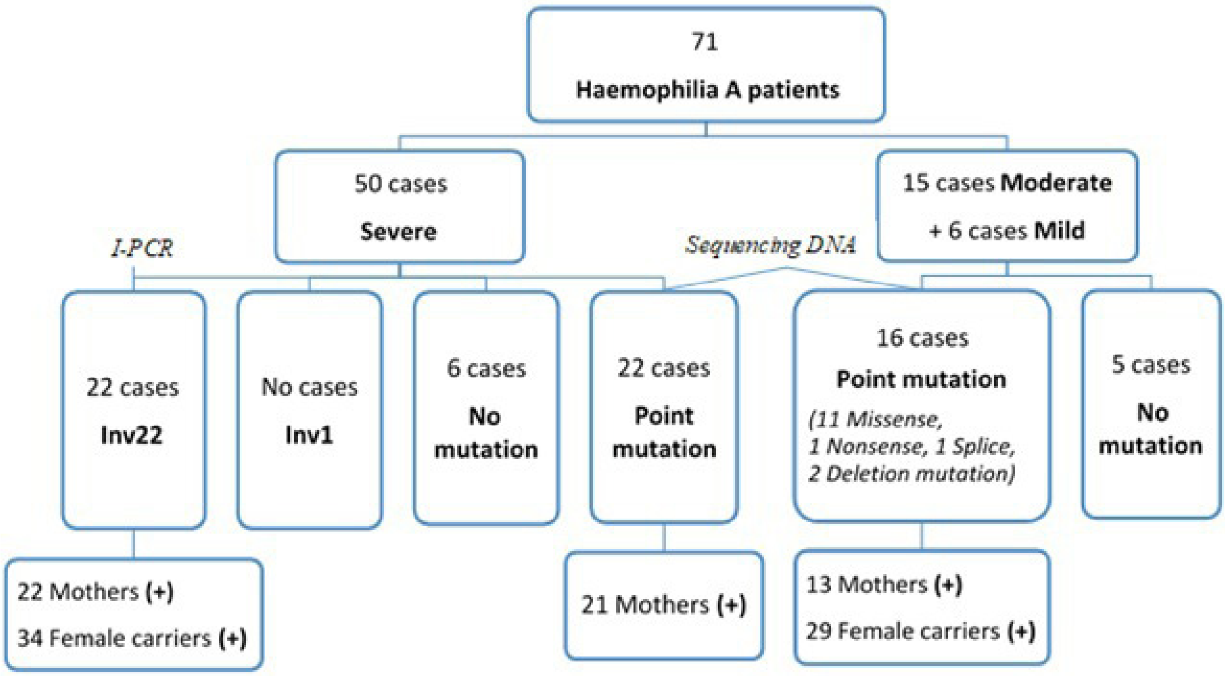

Figure 2 Summary report of identified variants in F8 in patients with HA and heterozygous carriers. HA, haemophilia A.

60/71 patients with genetic variant *F8*, accounting for 84.5%. Eleven patients (15.5%) had no variants. In the group with 50 patients with severe HA, most variants in *F8* were Inv22 (44%). The point mutation had 22 identified cases (11 frameshifts, 7 missense, 3 nonsense and 1 splice site) and was found in exon 14 higher than other exons. Otherwise, in the groups of 16 patients with moderate and mild HA, missense mutation was a major cause of almost moderate (8/12) or mild (3/4) cases. Details of the summary of point mutations for each group are shown in tables 1 and 2. None of the patients with HA had Inv1.

With regard to point mutation particularly, the study was the first to record two new variants (as represented in tables 1 and 2), including a nonsense variant in exon 13 (13c.2025T>A) and a false mutation p.Ser1484Gly at exon 14 that have not been published in variant databases.

Assessment of variants in FC

From the results of genetic analysis in patients with HA, this study also determined the carrier status of 152 female members and relevant variants in the HA family. The proportion of those that had the heterozygous genotype in *F8* variants was 119/152 (78.3%). Remarkably, in mutated patients, 93.3% of the mothers were obligate carriers with the same mutation of their sons. On the other hand, in the group of patients with severe HA, Inv22 not only occurred in patients and their mothers but also in female members (shown in figure 2).

DISCUSSION

Among low-health resource countries such as Vietnam, early diagnosis of the variants in *F8* (including In22) was the necessary solution to have appropriate prophylaxis treatment and to improve the quality of life for patients with HA.[13] The present study was successful in both identifying popular variants in the *F8* gene that caused disease in patients with HA and analysing genetic information for FC in HA families in Vietnam.

First, similar to the report of Luu *et al*,[10] the results of our study demonstrated that Inv22 was the main variant of Vietnamese patients with severe HA (44%). Besides, effective combination of molecular techniques indicated that I-PCR was the simple and convenient method, which should be considered as a priority choice

Table 1 Type and nucleotide of variants on *F8* in the group of patients with severe haemophilia A that was detected by sequencing method (n=22)

ID	Variant type	Variant classification	Exon, domain
Hem01	Deletion	Ex14: c.4121_4124delTAGA/p.Ile1374thrfs*49	14, B
Hem03	Insertion	Ex14: c.2777_2778 insC/p.Ser927Lysfs*7	14, B
Hem04	Splice site	IVS5: c.670+1G>T	Intron 5
Hem10	Deletion	Ex14: c.3965delA/p.Gln1322Argfs*13	14, B
Hem15	Missense	Ex23: c.6545G>A/ p.Arg2182His	23, C1
Hem16	Duplication	Ex14: c.4825dupA/p.Thr1609Asnfs*4vvv	14, B
Hem21	Duplication	Ex14: c.4379dupA/p.Asn1460Lysfs*2	14, B
Hem23	Nonsense	Ex13: c.2025T>A/p.Tyr675Stop	13, A2
Hem25	Duplication	Ex14: c.3870dupA/p.Gly1291Argfs*29	14, B
Hem27	Deletion	Ex8: c.1141delG/p. Asp381Met*fs	8, a1
Hem30	Insertion	Ex8: c.1096_1097insG/p.Asp366Gly*fr	8, a1
Hem34	Deletion	Ex14: c.4512delG/p.Leu1504Phefs*63	14, B
Hem35	Missense	Ex3: c.266G>A/p.Gly89Asp	3, A1
Hem39	Nonsense	Ex7: c.812C>A/p.Ser271Stop	7, A1
Hem40	Duplication	Ex14: c.3637dupA/p.Ile1213Asnfs*28	14, B
Hem46	Missense	Ex12: c.1891A>G/p.Asn631Asp	12, A2
Hem50	Nonsense	Ex14: c.4027G>T/p.Glu1343Stop	14, B
Hem52	Deletion	Ex14: c.3637delA/p.Ile1213Phefs*5	14, B
Hem53	Missense	Ex8: c.1178T>A/p.Ile593Asn	8, A2
Hem56	Missense	Ex1: c.143G>A/p.Arg48Lys	1, A1
Hem60	Missense	Ex14: c.4156C>T/p.Gln1386Stop	14, B
Hem64	Missense	Ex4: c.446C>T/p.Pro149Leu	4, A1

Table 2 Type and nucleotide of variants on *F8* causing moderate (n=12) and mild (n=4) haemophilia A

ID	Severity level	Variant type	Variant classification	Exon, domain
Hem12	Moderate	Missense	3′-UTR exon 26: c.8899G>A	3′-UTR exon 26
Hem14	Moderate	Deletion	Ex13: c.2009_2011delTCT/p.Val670del	13, A2
Hem42	Moderate	Missense	Ex8: c.1172G>A/p.Arg391His	8, A1
Hem43	Moderate	Missense	Ex14: c.4450A>G/p.Ser1484Gly	14, B
Hem48	Moderate	Missense	Ex2: c.223G>T/p.Asp75Tyr	2, A1
Hem54	Moderate	Missense	Ex8: c.1063G>A/p.Arg336Cys	8, A1
Hem59	Moderate	Missense	Ex13: c.1963T>C/p.Tyr655His	13, A2
Hem61	Moderate	Missense	Ex12: c.1801A>C/p.Asn601His	12, A2
Hem63	Moderate	Splice site	IVS14: c.5220–1G>C	Intron 14
Hem67	Moderate	Nonsense	Ex24: c.6694C>T/p.Glu2232Stop	24, C2
Hem68	Moderate	Nonsense	Ex24: c.6682C>T/p.Arg2228Stop	24, C2
Hem70	Moderate	Missense	Ex25: c.6870G>T/p.Trp2290Cys	25, C2
Hem24	Mild	Missense	Ex3: c.386A>T/p.Glu129Val	3
Hem37	Mild	Missense	Ex14: c.5093T>C/p.Ile1698Thr	14, B
Hem49	Mild	Missense	Ex14: c.5093T>C/p.Ile1698Thr	14, B
Hem65	Mild	Deletion	Ex14: c.4976delC /p.Pro1659Hisfs*4	14

in testing Inv22 on these patients and consisted of recommendations from the study of Abdulqader *et al.*[14] However, Inv22 occurred in different proportions of every population, depending on detection technique. For example, the frequency of Inv22 among studied patients was consistent with some other reports from China (44.7% by long-distance PCR (LD-PCR)),[15] Mexico (45% by LD-PCR),[16] Iraq (46.7% by inverse shifting PCR (IS-PCR))[14] and Egypt (42.8% by IS-PCR),[17] but was lower than that from Arab (55% by M-PCR)[18] and higher than that from UK (38% by PCR +Real-Time PCR (RT-PCR)),[19] Palestine (36.6% by S-PCR)[13] and Egypt (33% by LD-PCR).[20] In Inv22-negative cases, Multiplex PCR (M-PCR) was the next test to analyse Inv1, but this variant was not to be found in our current study. As mentioned previously, the prevalence rate of Inv1 in severe HA cases was below 5%,[13 18 19] which consisted of the absence as described in the present study and other populations.[17 21] Surprisingly, our study did not identify any variant in the *F8* gene of 11 patients (6 severe, 3 moderate and 2 mild) accounting for 15.5%. This rate was higher than the report from Germany (2%).[22] This was likely due to the pathogenic variant located deep inside the introns, while the extracted and amplified sequence in this study was DNA. In order to determine these variants, analysis of the patient's mRNA was necessary. In addition, the undetectable variants could be other proteins involved in folding, transporting, secreting and weakening *F8* such as thrombin and von Willebrand factor (vWF).[23 24] These were a promising research direction for us in the future by coordinating many other methods to determine mutant locations in the intron region. In 60 male patients with haemophilia having the *F8* variant, we identified two novel mutations, including c.2025T>A (p.Tyr675Stop) and c.4450A>G (p.Ser1484Gly), which have not been published on any database of genetic mutation *F8*. The variant c.2025T>A as shown in figure 3A, which was caused by stopping transcription at exon 13 encoding of A2 region, made *F8* protein to be truncated and correlated with the severe phenotype of patients with Hem23.

Second, based on discussion for accessing the carrier in the article of Luu *et al*,[10] our study screened women in haemophiliac families to analyse the carrier status as a significant strategy in order to support genetic information and prenatal diagnosis. As a result, there was 78.3% of women in these families who were the heterozygous carriers. Most mutated cases (56/60) inherited the disease gene from their mothers, except for four patients

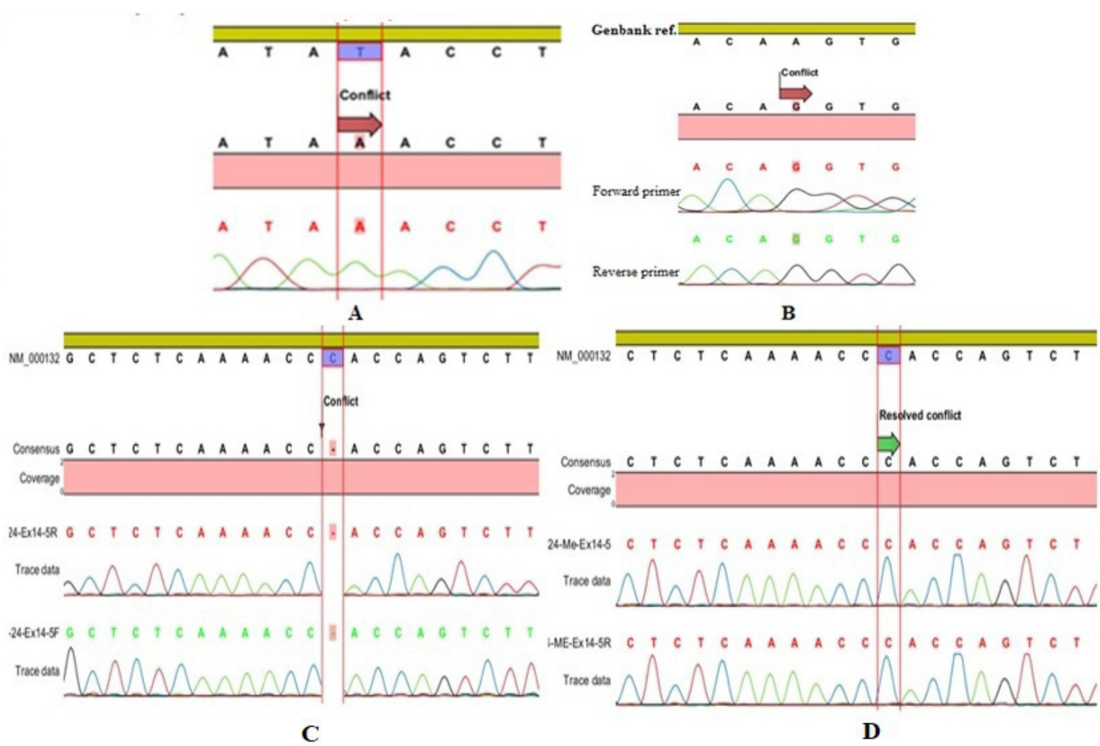

Figure 3 Sequencing results of new variants in F8. (A) Hem23 patient carried a novel variant that a single-nucleotide substitution from T to A at position c.2025 changes TAT trio encode tyrosine to TAA. It was caused by stopping transcript at exon 13 encode of A2 region. (B) Hem43 patient carried a new point mutation at exon 14 (c.4450A>G) that codon AGT changed to GGT, expression p.Ser1484Gly. (C) A frameshift variant in Hem65, c.4976delC on exon 14 of F8 gene. (D) Variant in Hem65's mother.

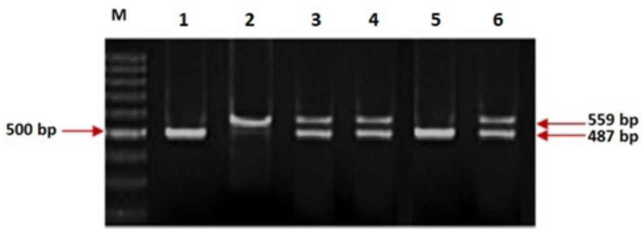

Figure 4 PCR result of intro 22 inversion variant. M, PCR marker; 1, patient no Inv22 variant (band at 487 bp); 2, Hem17 patient with Inv22 (band at 559 bp); 3, Hem17's mother; 4, Hem17's first aunt; 5, Hem17's second aunt; 6, first aunt's daughter.

with HA that were autologous mutant. For instance, the mother of the patient with Hem01 was a benign person that carried the heterozygous genotype of variant c.4121_4124delTAGA on exon 14, which deflected the translation frame and completely changed the amino acid sequence from region B onwards. This mutated gene was transmitted from the mother to the patient with Hem01 and caused a severe phenotype in the patient with Hem01. However, his sister had a completely normal genotype. Specially, the patient with Hem65 carried a variant at position c.4976 delC on exon 14 (deviation from codon 1659) of the *F8* gene, but his mother had no mutation at this position (figure 3C,D). It was due to the fact that a de novo mutation occurred during gametogenesis of the patient's mother. De novo mutations account for about one-third of the cases with point mutations and small segment insertion/deletion mutations in severe, moderate and mild HA cases.[25] Accordingly, it was seriously various because of carriers as the first generation that continued to inherit this mutation for the following generations.

Finally, regarding these findings, molecular genetic diagnosis for patients with severe HA showed that Inv22 was a damage variant that not only occurred on patients but also on female members such as mothers, aunts and aunts' daughters (as presented in figure 2 and figure 4). In case of all carriers included in this study, when they married or got pregnant, they should discuss with the consulting doctor about prenatal diagnosis methods to detect the abnornal fetus and to be supported with treatment during pregnancy in order to improve the quality of life of the baby in the future. In accordance with previous findings, accurate genetic carrier diagnosis was the helpful test to ensure the need for adopting early and was a screening strategy for newborne infants of families at risk in low-income countries.[13 18] In brief, the current study was the first to demonstrate transmission of the Inv22 variant in the pedigree of Vietnamese haemophiliac families. At present, from the findings of this study and those of Luu *et al*, some antenatal counselling and screening strategies for HA are conducted by our colleagues in Vietnam.

CONCLUSIONS

Our study found that Inv22 was the major genetic variant of patients with severe HA in South Vietnam, which was consistent with a previous report in North Vietnam and other populations in the world. Besides, our findings were the first to successfully apply technical procedures to identify Vietnamese HA FCs, transmission of Inv22 and point mutation that occurred in both patients with severe HA and their family members. Furthermore, the study's results provided the necessary databases to establish a screening programme or genetic counselling programme for these carriers.

Take home messages

- ⇒ Genetic characteristics in female Vietnamese haemophilia carriers have not been analysed yet.
- ⇒ Intron 22 inversion (Inv22) is the major genetic variant of patients with severe haemophilia A (HA) in South Vietnam.
- ⇒ Transmission of Inv22 and point mutation occurred in both patients with severe HA and their family members.
- ⇒ It is necessary to establish a screening programme or a genetic counselling programme for female carriers.

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Competing interests None declared.

Patient consent for publication Not applicable.

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Data availability statement Data are available in a public, open access repository. All data relevant to the study are included in the article. Data obtained from our work in clinical laboratory department at University Medical Center, University of Medicine and Pharmacy at Ho Chi Minh City, Vietnam.

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