RESEARCH ARTICLE
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Spectrum of Factor VIII Gene Variants in 78 Patients with Hemophilia A in Guangxi Province, China, Including Nine Novel Variants：A Descriptive Study

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ABSTRACT

Introduction: Hemophilia A is an X-linked hereditary bleeding disorder caused by variants in the coagulation factor VIII (*F8*) gene, with a current estimated prevalence of 17.1 per 100,000 males.

Methods: Long-distance polymerase chain reaction was used to detect intron inversions, and next-generation sequencing gene panels were used to identify small sequence variants.

Results: Fifty-two different *F8* variants were identified in 78 patients from unrelated families, including single-nucleotide alterations (missense, nonsense), frameshifts (small deletions/insertions), splicing-site changes, complex variations, and large rearrangements (Inv22 or Inv1). The nine variants reported here for the first time consisted of two missense variants, two nonsense variants, four frameshifts, and a splicing alteration.

Conclusion: The Factor VIII Gene mutation spectrum of patients with hemophilia A from Guangxi Province was established, and genotype–phenotype correlations were explored. This study will contribute data to the present *F8* mutation database and help systematically draw the mutation spectrum of the hemophilia A gene in southern China.

Keywords: Hemophilia A, variant spectrum, *F8*, Factor VIII, next-generation sequencing

INTRODUCTION

Hemophilia A (HA) is the second common coagulation factor deficiency after von Willebrand disease. This X-linked recessive disorder is associated with variants in the gene encoding coagulation factor VIII (F8) and is caused by deficiency of coagulation factor VIII (FVIII). The overall prevalence of HA of all severities is 17.1 cases per 100,000 males[1], with no racial or regional differences. Patients present with bleeding as the main symptom, especially in large joints, such as the ankles, elbows, and knees.

F8 is located at the end of the long arm of X chromosome, with an estimated length of 186 kb. It contains 26 exons and encodes the essential coagulation protein FVIII[2,3]. At present, more than 3052 unique variants leading to HA have been reported. Variant maps show pathogenic variants distributed throughout the *F8* gene, with 66.2% point variants (https://F8-db.eahad.org/). According to the plasma procoagulant level of FVIII activity (FVIII: C), HA can be classified into three clinical phenotypes: severe (FVIII: C <1%), moderate (FVIII: C 1%–5%), and mild (FVIII: C >5%–<40%)[4].

In southern China, two cases of *F8* gene variant spectrum have been reported from a large cohort of a single center[5,6]. This study aimed to explore the *F8* gene variant in Guangxi Province to supplement existing data and establish a genetic basis for systematically mapping the variant spectrum of hemophilia A gene in southern China. In this study, we sought to establish a variant spectrum using long-distance polymerase chain reaction and next-generation sequencing.

MATERIALS AND METHODS

Participants

All patients included in this study were from Guangxi Province, a multi-ethnic region located in southern China. A total of 78 patients who were diagnosed with HA at the Department of Pediatrics of the First Affiliated Hospital of Guangxi Medical University between August 2016 and May 2023 were included in this study. All patients included in the study met the following criteria: (1) they met the diagnostic criteria for hemophilia A; (2) they received a genetic diagnosis and were found to have variants associated with hemophilia A. Hemophilia was defined according to the World Federation of Hemophilia guidelines for the management of hemophilia7, and clinical staging based on FVIII activity was performed in accordance with international standards. Informed consent was obtained from all participants or their legal guardians following the institutional ethics guidelines. This study was approved by the Ethics Committee of Guangxi Medical University.

DNA extraction

Peripheral blood (4 mL) was collected for gene sequencing and FVIII:C assay. Genomic DNA from the patients and their parents was extracted from peripheral blood samples using the Qiagen FlexiGene DNA Extraction Kit (Qiagen, Germany), and DNA content was determined using a Nanodrop 2000 ultra-micro spectrophotometer (Thermo Fisher Scientific, USA).

F8 gene variant analysis

Inversion detection

The patients and their parents were tested for inversion of the *F8* intron 22 by long-distance polymerase chain reaction (LD-PCR) amplification in a 25-μL reaction system. The PCR products were electrophoresed on a 0.6% agarose gel configured with 1× Tris-borate-EDTA(TBE) and stained after electrophoresis. The results were observed using a gel imager, and multiplex PCR was performed with Inv22-negative samples to determine whether they contained Inv1.

Sequence Variant Detection

Second-generation sequencing of *F8* was performed using panel technology in patients with undetected intron inversion. The online design tool Agilent SureDesign (Agilent, USA) was used to

design a targeted capture probe for the exon and ± 10-bp flanking introns of the target gene and to customize a special target gene capture kit. DNA sequencing was performed using a NovaSeq sequencer (Illumina, USA). The sequencing results were compared with the human reference genome and validated using BWA (v0.7.15). Common polymorphisms were excluded by referring to the 1000-Genome database and the single-nucleotide polymorphism database. The gene sequencing procedures described above were performed by the Kangso Medical Laboratory (Beijing, China).

Molecular genetic analysis

Novel variants were further analyzed for their effects on the factor VIII protein using in silico predictive programs, including Sorting Intolerant From Tolerant (SIFT)[8], Polymorphism Phenotyping (PolyPhen2)[9], And Mutation Taster[10], and classified according to ACMG recommended standards.

RESULTS

A total of 78 patients with HA were included in this study; 52 different variants, including the common variants Inv22 and Inv1, were detected in *F8* (Table 1 and Table 4), along with novel variants (Table 2) and six mutated loci recurring in patients from unrelated families (Table 3). The variants spanned five introns and 16 exons, with exon 14 being the most frequently mutated. The variant types included missense and nonsense variants, large rearrangements, frameshifts, complex variants, and gross deletions. The results were compared with data from available databases, including dbSNP, Factor VIII Variant Database (https://*F8*-db.eahad.org/), and HGMD Professional 2023.1, and we identified 44 known and 9 novel variants.

Assessment of inversions showed 25 cases (32.1%) of intron inversions, including five cases (6.4%) of Inv1 and 20 cases (25.6%) of Inv22. In addition, we used next-generation sequencing and found 27 cases (34.6%) of missense variants; 11 cases (14.1%) of nonsense variants; 13 cases (16.7%) of frame-shift variants, including 5 cases (6.4%) of insertion, 7 cases (9.0%) of deletion, and 1 case (1.3%) of deletion/insertions; and 2 cases of complex variants (2.6%).

The clinical severity of all patients included in the study was determined according to international standards. Overall, 45 patients (57.7%) were classified as showing severe disease; 26 (33.3%) as moderate disease; and only seven (9%) as mild disease. The variant spectrum corresponding to each clinical type is shown in Fig. 1. As the most common variant type in HA, missense variants accounted for 85.7% of mild cases and 46.2% of moderate cases. Intron inversions accounted for 55.6% of the severe type, and Inv22 accounted for 80% of the inversions, showing a clear variant type-clinical typing correlation.

To further assess the impact of novel variants on the FVIII protein, we performed predictive analyses using multiple bioinformatics software for missense variants and Mutation Taster for other types of variants. The results showed that all variants except for p.(Asp1107Glu) were predicted to have damaging effects.

DISCUSSION

All the patients in this study were male. Patients were clinically staged based on FVIII:C levels in accordance with international standards. A total of 45 patients were classified as showing severe disease. Since intron inversions led to truncation of the wild-type *F8* transcription unit and inversion of intron 1 or 22 toward the telomere of the long arm of the X chromosome disrupts the protein structure majorly[11], intron inversions are closely associated with severe HA. Inv22 and Inv1 account for 40%–50% and 0.5%–5% of patients with severe HA, respectively[12,13]. In this study, the proportion of Inv1 cases was higher than that reported generally in the literature, probably due to the limitation of our sample size and differences in the level of development among countries[14,15]. A previous study on the

mutational spectrum of 1296 Italian patients reported a significant predominance of missense variants, which represented 80% of the variants in patients with mild HA and 68% of the variants in patients with moderate HA. Moreover, patients with frameshift variants mostly showed moderate or severe disease[16]. The proportion of patients with missense variants being classified as showing moderate disease was lesser in our study than in the study involving Italian patients; however, our study showed the same general distribution trend.

The relationship between the pathogenicity of p.(Arg245Gln) identified in this study and its clinical manifestations has not been specifically described in the literature, Patient 32, who had the p.(Arg245Gln) variation, exhibited a near normal FVIII:C level of 44.5% . This 4-year-old boy was diagnosed with hemophilia A (subclinical type) because although both VWF:RCo and VWF:Ag were within normal limits, he exhibited recurrent epistaxis and mild clinical symptoms. Analysis using SIFT, Polyphen2, and MutationTaser showed that the variant did not cause deleterious effects on the FVIII product function. Its co-site variant p.(Arg245Trp) has also been reported previously, with an FVIII:C level of 46% (normal range)[17]. This variant site is in A1 domain, which is generally considered to be associated with post-cleavage activation of FVIII and binding of FX [18-20]. We speculate that because the amino acids affected by this variant are farther away from these important sites and are at the periphery of the overall structure of FVIII, their effects on protein function are not major. Clinical typing of variants located close to p.(Arg245) also provides evidence for our speculation[17,21-23]. In fact, referring to the allele frequency of dbSNP, this variant is more common in East Asians, and it is possible that this locus is a very rare polymorphic locus.

Previous research on the structure and function of coagulation proteins has shown that some sites in protein molecules are not necessary to maintain structural stability, and certain sites may produce gain-of-function mutations. The p.Arg245 mutation site in this study shows relevant research potential, and our research team has currently carried out further exploration of its molecular mechanism.

We also found that six variants, i.e., p.(Arg1985*), p.(Gln628*), p.(Asn1460Lysfs*1), p.(Asp1107Glu), p.(Arg2228Gln), and p.(Ser603Ile), were recurrent in unlinked patients. The results of clinical staging were similar between patients with the same variants, except for two patients (Patient 43 and Patient 44) with p.(Arg1985*) (Table 2). These patients had FVIII:C ratios of 14.4% and 0.5%. Patient 43 was diagnosed with HA at the age of 1 year due to swelling of the right upper forehead after a fall, and Patient 44 was also diagnosed at 1 year of age due to subcutaneous hemorrhage followed by bleeding from the elbow joint. In the *F8* database, patients with nonsense variant p.(Arg1985*) were classified as severe type, whereas missense variants at the same site were mostly classified as mild/medium type. Another patient, i.e., Patient 2, with a missense variant p.(Arg1985Pro) at the same locus showed a mild clinical phenotype and was diagnosed at the age of 12 years for a left anterior iliopsoas hematoma and had a subsequent intravertebral hemorrhage. Blood group antigens, vWF levels, and age are important determinants of FVIII levels in the normal population, but the impact of these factors on HA patients has been debated. [24-27]. A major FVIII:C difference between children with the same variant (as in Patient 43/ Patient 44) suggests to some extent that baseline FVIII:C values in HA patients are influenced by factors other than genotype, and identifying this determinant may help improve diagnosis and treatment strategies for patients.

The study is limited by the small sample size. Studies with larger sample size, investigating the molecular structure and function of the pathogenic mechanism of HA, may further aid in the prenatal diagnosis and treatment of HA.

CONCLUSIONS

Our study presents the variant spectrum of patients with HA from Guangxi Province, China. Although we were unable to definitively explain the pathogenic mechanisms of these variants, the evidence of pathogenicity provided by this study has implications for genetic testing and diagnosis of patients. The identification of novel variants will enrich the variant database of *F8*. Moreover, the study provides a data supplement and genetic basis for systematically drawing the variant spectrum of hemophilia A gene in Guangxi Province.

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There are no financial conflicts of interest to disclose.

Conflict of interest

The authors have indicated they have no conflicts of interests regarding the contents of this paper.

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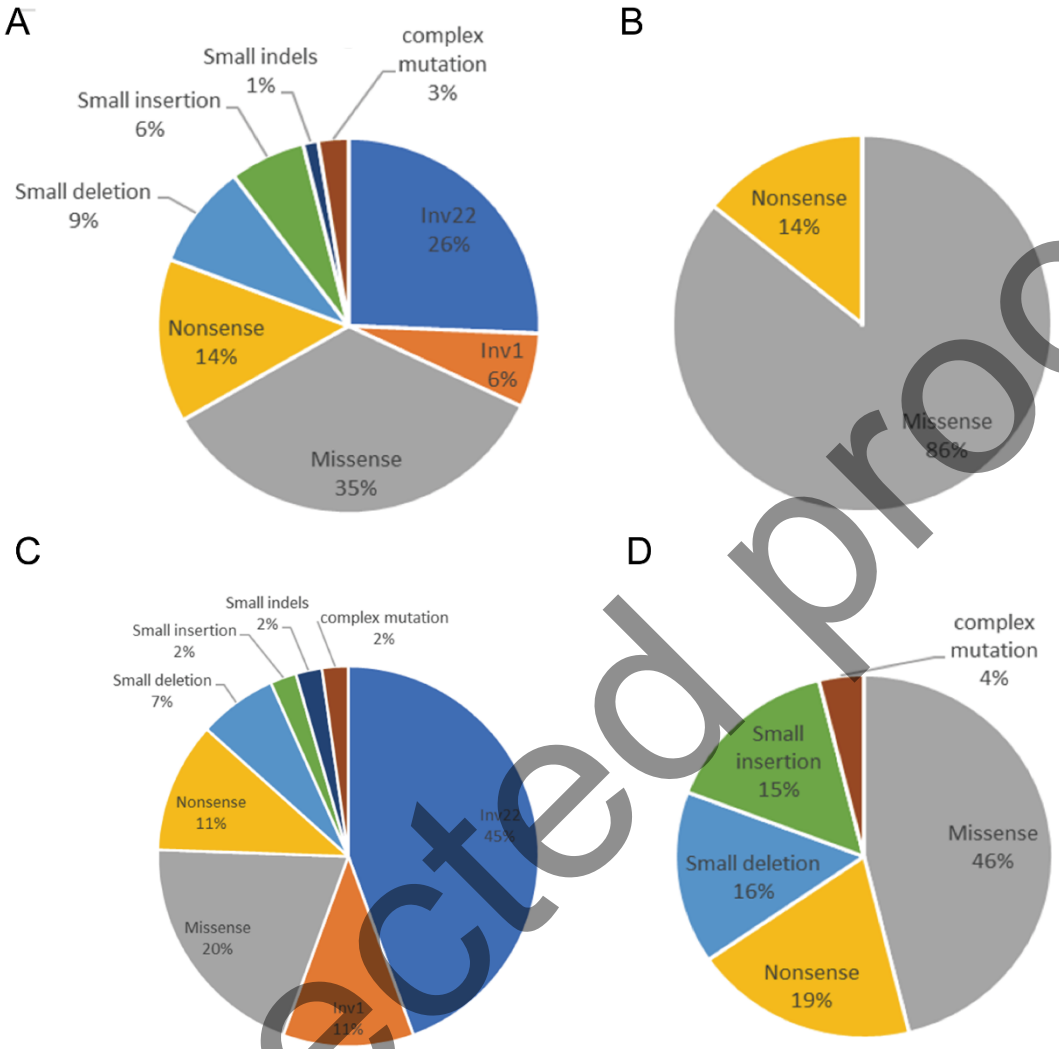

Figure 1. Frequencies of different types of F8 DNA mutations found in all patients (A); The mutation spectrum of mild HA (B); severe HA (C); and moderate HA (D) HA: Hemophilia A.

Table 1. Detailed description of the mutations detected in patients

No.	Exon	F8 domain	Nucleotide Change (NM_000132)	Amino acid change	Phenotype	Mutation type
1	22	C1	c.6371A>G	p.(Tyr2124Cys)	Mild	MS
2	18	A3	c.5954G>C	p.(Arg1985P)	Mild	MS
3	14	A2	c.2167G>A	p.(Ala723Thr)	Mild	MS
4	9	A2	c.1333G>C	p.(Val445Leu)	Mild	MS

5	24	C2	c.6679G>A	p.(Ala2227Thr)	Mild	MS
6	23	C1	c.6521A>G	p.(His2174Arg)	Moderate	MS
7	9	A2	c.1324T>C	p.(Tyr442 His)	Moderate	Complex mutation
	14	B	c.5000G>A	p.(Arg1667Gln)		
9	22	C1	c.6406G>A	p.(Gly2136Arg)	Moderate	MS
10	14	A2	c.2162A>G	p.(Met721Val)	Moderate	MS
11	14	A2	c.2129G>A	p.(Gly 710Glu)	Moderate	MS
12	14	A2	c.2132G>A	p.(Cys711Tyr)	Moderate	MS
13	8	A2	c.1238A>G	p.(Asp413 Gly)	Moderate	MS
14	11	A2	c.1573G>T	p.(Gly 525*)	Moderate	NS
15	23	C1	c.6496C>T	p.(Arg2166*)	Moderate	NS
16	14	B	c.3605delA	p.(His 1202Leufs*16)	Moderate	FS
17	15	A3	c.5257-5270del13	p.(Val1753Leufs*19)	Moderate	FS
18	14	B	c.4380delA	p.(Asn1460Ifs*5)	Moderate	FS
19	13	A2	c.2021G>A	p.(Gly 674Glu)	Severe	MS
20	17	A3	c.5602T>C	p.(Ser1868Pro)	Severe	MS
21	14	A3	c.5219G>T	p.(Arg1740 Met)	Severe	Complex mutation
	14, intor14	A3	*c.5219+2_5219+12del	____		
22	7	A1	c.901C>T	p.(Arg301Cys)	Severe	MS
23	17	A3	c.5593G>A	p.(Asp 1865Asn)	Severe	MS
24	6	A1	c.760A>G	P.(Asn254 Asp)	Severe	MS
25	16	A3	c.5416T>C	p.(Ser 1806Pro)	Severe	MS

26	14	B	c.3341C>A	p.(Ser1114*)	Severe	NS
27	12	A2	c.1804C>T	p.(Arg602*)	Severe	NS
28	13	A2	c.1990C>T	p.(Gln664*)	Severe	NS
29	9	A2	c.1336C>T	p.(Arg446*)	Severe	NS
30	14	B	c.3637delA	p.(Ile1213Phefs*5)	Severe	FS
31	8	A1	c.1024delT	p.(Tyr342 Met fs*4)	Severe	FS
32	6	A1	c.734G>A	p.(Arg245Gln)	mild	MS
33	15	A3	c.5306G>A	p.(Gly1769Glu)	Moderate	MS

MS: missense, NS: nonsense, FS: frameshift, amino acid change: HGVS AA no.

Protein domains：Factor VIII Gene *(F8)*Variant Database

Table 2. Novel mutations and analysis of its pathogenicity

atient No.	xon	omain	Nucleotide change (NM_000132)	Amino acid change	Phenotype	clinical manifestations	variant classification	Mutation type
4、35	4		c.3321T>G	p.(Asp1107Glu)	moderate	Joint bleeding	Uncertain Significance （PM2）	MS
6	2	1	c.6402T>G	p.(Tyr2134*)	moderate	Recurrent cerebral hemorrhage	Likely pathogenic (PVS1、PM2、PP3)	NS
7		1	c.1032delA	p.(Val345*)	moderate	Joint bleeding	Likely Pathogenic (PSV1、PM2、PM4)	FS
8	4		c.3769insA	p.(Gly1257Argfs*4)	moderate	Joint bleeding	Likely Pathogenic (PSV1、PM2、PM4)	FS
1	4	3	c.5219+2_5219+12del	——	severe	Cerebral hemorrhag	Pathogenic (PVS1、PM2)	Complex mutation
9		1	c.884T>G	p.(Phe295Cys)	severe	Scalp hematoma	Pathogenic (PS1、PM1、PM2、PM5、PP3)	MS

0	6	3	c.5570-5571insT	p.(Ser1858Leufs*2)	moderate	Joint bleeding	Pathogenic(PVS1、PM2 PS1、PM4)	FS
1	4		c.3567del	p.(Ser1189Argfs*29)	severe	Ecchymosis、epistaxis	Pathogenic(PVS1、PM2、PM4)	FS
2	5	2	c.6871-6874delinsCA	p.(Thr2291Hisfs*?)	severe	Ecchymosis	Pathogenic(PVS1、PM2、PM4)	FS

MS: missense, NS: nonsense, FS: frameshift, amino acid change: HGVS AA no.

Protein domains：Factor VIII Gene *(F8)*Variant Database

Table 3. Mutations recurrent in unrelated patients

No.	Nucleotide change	Amino acid change (HGVS)	Exon	F8 domain	Patient No.	Phenotype	Mutation type
1	c.5953C>T	p.(Arg1985*)	18	A3	43	mild	NS
					44	severe	
2	c.1882C>T	p.(Gln628*)	12	A2	45	moderate	NS
					46		
3	c.4380-4381insA	p.(Asn1460Lysfs*1)	14	B	47	moderate	FS
					48		
	c.4371-4372insA				49	severe	
4	c.3321T>G	p.(Asp1107Glu)	14	B	34	moderate	MS
					35		
5	c.1808G>T	p.(Ser603Ile)	12	A2	50	moderate	MS
					51	severe	
6	c.6683G>A	p.(Arg2228Gln)	24	C2	52	moderate	MS
					53		

MS: missense, NS: nonsense, FS: frameshift, amino acid change: HGVS AA no, ▲: novel variant

Protein domains：Factor VIII Gene *(F8)*Variant Database

Table 4. Detailed description of the intron inversions detected in patients

Patients No.	Intron	Mutation type	Phenotype
54-74	22	Inv22	Severe
75-78	1	Inv1	Severe

Inv 22: inversion of exons 22, Inv 1: inversion of exons 22