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

Çin'in Guangxi Eyaletinde Hemofili A Tanılı 78 Hastada Faktör VIII Gen Varyantlarının Spektrumu ve Dokuz Yeni Varyant: Tanımlayıcı Bir Çalışma

Jiali Jiang[1], Jinxia Jiang[2], Yuping Li[1], Lifang Huang[2], Hongying Wei[1]

[1]*The Second Affiliated Hospital of Guangxi Medical University, Department of Pediatrics, Nanning, P.R. China*
[2]*Maternal and Child Health Hospital of Guangxi Zhuang Autonomous Region, Department of Pediatric Hematology and Oncology, Nanning, Guangxi, P.R. China*

Abstract

Objective: Hemophilia A (HA) 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 male individuals. This study aimed to establish a gene variant spectrum in China using long-distance polymerase chain reaction (PCR) and next-generation sequencing.

Materials and Methods: Long-distance PCR 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 include two missense variants, two nonsense variants, four frameshifts, and one splicing alteration.

Conclusion: The *F8* gene mutation spectrum of patients with HA from Guangxi Province was established and genotype-phenotype correlations were explored. This study contributes data to the existing *F8* mutation database and helps systematically identify the mutation spectrum of the gene for HA in southern China.

Keywords: Hemophilia A, Variant spectrum, *F8*, Factor VIII, Next-generation sequencing

Öz

Amaç: Hemofili A (HA), pıhtılaşma faktörü VIII (*F8*) genindeki varyantlardan kaynaklanan X'e bağlı kalıtsal bir kanama bozukluğudur ve erkeklerde tahmini prevalansı 100.000'de 17,1'dir. Bu çalışma, Çin'de *F8* gen varyant spektrumunu uzun mesafe polimeraz zincir reaksiyonu (PCR) ve yeni nesil dizileme yöntemleri kullanarak ortaya koymayı amaçlamıştır.

Gereç ve Yöntemler: İntron inversiyonlarını saptamak için uzun mesafe PCR, küçük dizi varyantlarını belirlemek için ise yeni nesil dizileme gen panelleri kullanılmıştır.

Bulgular: Akraba olmayan ailelerden 78 hastada toplam 52 farklı *F8* varyantı tespit edilmiştir. Bu varyantlar arasında tek nükleotid değişimleri (missense, non-sense), çerçeve kaymaları (küçük delesyon/insersiyonlar), ekzon-intron birleşim (splicing) değişiklikleri, kompleks varyasyonlar ve büyük yeniden düzenlenmeler (inv22 veya inv1) yer almaktadır. Bu çalışmada ilk kez bildirilen dokuz varyant; iki missense, iki non-sense, dört çerçeve kayması ve bir splicing değişikliğinden oluşmaktadır.

Sonuç: Guangxi Eyaleti'ndeki HA hastalarının *F8* gen mutasyon spektrumu belirlenmiş ve genotip–fenotip ilişkileri incelenmiştir. Bu çalışma, mevcut *F8* mutasyon veritabanına veri sağlamaktadır ve Güney Çin'de HA hastalarında *F8* geninin mutasyon spektrumunun sistematik olarak tanımlanmasına katkıda bulunmaktadır.

Anahtar Sözcükler: Hemofili A, Varyant spektrumu, *F8*, Faktör VIII, Yeni nesil dizileme

Address for Correspondence/Yazışma Adresi: Hongying Wei, M.D., The Second Affiliated Hospital of Guangxi Medical University, Department of Pediatrics, Nanning, P.R. China
E-mail: whylhr@qq.com **ORCID:** orcid.org/0009-0008-1694-6024

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Introduction

Hemophilia A (HA) is the second most common coagulation factor deficiency after von Willebrand disease. This X-linked recessive disorder is associated with variants in the factor VIII (*F8*) gene encoding coagulation factor VIII (FVIII) and is caused by deficiency of FVIII. The overall prevalence of HA of all severity levels is 17.1 cases per 100,000 male individuals [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 the X chromosome, with an approximate length of 186 kb. It contains 26 exons and encodes the essential coagulation protein FVIII [2,3]. At present, at least 3,052 unique variants leading to HA have been reported. Variant maps show pathogenic variants distributed throughout the *F8* gene, with 66.2% point variants (see 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 of <1%), moderate (FVIII:C of 1% to 5%), and mild (FVIII:C of >5% to <40%) [4].

In southern China, two cases of *F8* gene variants were reported from a large cohort of a single center [5,6]. The present study aimed to explore the *F8* gene variant spectrum in Guangxi Province to supplement existing data and establish a genetic basis for systematically mapping the variant spectrum of this gene in southern China. We sought to establish a variant spectrum using long-distance polymerase chain reaction (PCR) and next-generation sequencing.

Materials and Methods

Participants

All patients included in this study were from Guangxi Province, a multiethnic region located in southern China. A total of 78 patients who were diagnosed with HA in 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 included patients met the following criteria: 1) they met the diagnostic criteria for HA; 2) they received a genetic diagnosis and were found to have variants associated with HA. Hemophilia was defined according to the World Federation of Hemophilia Guidelines for the Management of Hemophilia [7] 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 in line with the relevant guidelines for institutional ethics. The study was approved by the Ethics Committee of the Second Affiliated Hospital of Guangxi Medical University [approval number: 2024-KYL (013), date: April 05, 2024)].

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, Hilden, Germany), and DNA content was determined using a Nanodrop 2000 ultra-micro spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

F8 Gene Variant Analysis

Inversion Detection

The patients and their parents were tested for inversion of *F8* intron 22 by long-distance PCR amplification in a 25-µL reaction system. The PCR products were electrophoresed on a 0.6% agarose gel configured with 1x Tris-borate-EDTA buffer 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 for whom intron inversion was not detected. The online design tool Agilent SureDesign (Agilent, Santa Clara, CA, 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, San Diego, CA, USA). The sequencing results were compared with the human reference genome and validated using BWA (v0.7.15; https://github.com/lh3/bwa). Common polymorphisms were excluded by referring to the 1000 Genomes Project (Coriell Institute, Camden, NJ, USA) and the dbSNP database (National Institutes of Health, Bethesda, MD, USA). The gene sequencing procedures described above were performed by Kangso Medical Laboratory (Beijing, China).

Molecular Genetic Analysis

Novel variants were further analyzed for their effects on the FVIII protein using in silico predictive programs, including the Sorting Intolerant from Tolerant (SIFT) [8], Polymorphism Phenotyping (PolyPhen2) [9], and MutationTaster [10] programs. They were classified according to the relevant standards of the American College of Medical Genetics and Genomics [11].

Results

A total of 78 patients with HA were included in this study and 52 different variants were detected in *F8* (Table 1), including the common variants inv22 and inv1 (Table 2), novel variants (Table 3), and six mutated loci recurring in patients from

Table 1. Detailed description of the *F8* mutations detected in the patients.

No.	Exon	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.(Tyr442His)	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.(Gly710Glu)	Moderate	MS
12	14	A2	c.2132G>A	p.(Cys711Tyr)	Moderate	MS
13	8	A2	c.1238A>G	p.(Asp413Gly)	Moderate	MS
14	11	A2	c.1573G>T	p.(Gly525*)	Moderate	NS
15	23	C1	c.6496C>T	p.(Arg2166*)	Moderate	NS
16	14	B	c.3605delA	p.(His1202Leufs*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.(Gly674Glu)	Severe	MS
20	17	A3	c.5602T>C	p.(Ser1868Pro)	Severe	MS
21	14	A3	c.5219G>T	p.(Arg1740Met)	Severe	Complex mutation
	14, intron14	A3	*c.5219+2_5219+12del	-		
22	7	A1	c.901C>T	p.(Arg301Cys)	Severe	MS
23	17	A3	c.5593G>A	p.(Asp1865Asn)	Severe	MS
24	6	A1	c.760A>G	p.(Asn254Asp)	Severe	MS
25	16	A3	c.5416T>C	p.(Ser1806Pro)	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.(Tyr342Metfs*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: non-sense; FS: frameshift; Amino acid change: based on HGVS nomenclature; Domain: based on the Factor VIII Variant Database.

Table 2. Detailed description of the intron inversions detected in the patients.

Patient no.	Intron	Mutation type	Phenotype
54-74	22	inv22	Severe
75-78	1	inv1	Severe

inv: Inversion.

Table 3. Novel mutations and analysis of pathogenicity.

Patient no.	Exon	Domain	Nucleotide change (NM_000132)	Amino acid change	Phenotype	Clinical manifestations	Variant classification	Mutation type
34, 35	14	B	c.3321T>G	p.(Asp1107Glu)	Moderate	Joint bleeding	Uncertain significance (PM2)	MS
36	22	C1	c.6402T>G	p.(Tyr2134*)	Moderate	Recurrent cerebral hemorrhage	Likely pathogenic (PVS1, PM2, PP3)	NS
37	8	A1	c.1032delA	p.(Val345*)	Moderate	Joint bleeding	Likely pathogenic (PSV1, PM2, PM4)	FS
38	14	B	c.3769insA	p.(Gly1257Argfs*4)	Moderate	Joint bleeding	Likely pathogenic (PSV1, PM2, PM4)	FS
21	14	A3	c.5219+2_5219+12del	-	Severe	Cerebral hemorrhage	Pathogenic (PVS1, PM2)	Complex mutation
39	7	A1	c.884T>G	p.(Phe295Cys)	Severe	Scalp hematoma	Pathogenic (PS1, PM1, PM2, PM5, PP3)	MS
40	16	A3	c.5570-5571insT	p.(Ser1858Leufs*2)	Moderate	Joint bleeding	Pathogenic (PVS1, PM2, PS1, PM4)	FS
41	14	B	c.3567del	p.(Ser1189Argfs*29)	Severe	Ecchymosis, epistaxis	Pathogenic (PVS1, PM2, PM4)	FS
42	25	C2	c.6871-6874delinsCA	p.(Thr2291Hisfs*?)	Severe	Ecchymosis	Pathogenic (PVS1, PM2, PM4)	FS

MS: Missense; NS: non-sense; FS: frameshift; Amino acid change: based on HGVS nomenclature; Domain: based on the Factor VIII Variant Database.

Table 4. Mutations recurring in unrelated patients.

No.	Nucleotide change	Amino acid change	Exon	Domain	Patient nos.	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: non-sense; FS: frameshift; Amino acid change: based on HGVS nomenclature; Domain: based on the Factor VIII Variant Database.

unrelated families (Table 4). 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 (National Institutes of Health), the Factor VIII Variant Database (https://F8-db.eahad.org/), and HGMD Professional 2023.1 (Qiagen), and we identified 44 known and 9 novel variants.

Assessment of the inversions showed 25 cases (32.1%) of intron inversion, including five cases (6.4%) of inv1 and 20 cases (25.6%) of inv22 (Table 2). In addition, we performed 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/insertion; and 2 cases of complex variants (2.6%).

The clinical severity of all cases included in this study was determined according to international standards. Overall, 45 patients (57.7%) were classified as having severe disease, 26 (33.3%) as having moderate disease, and only 7 (9%) as having mild disease. The variant spectrum corresponding to each clinical type is shown in Figure 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 severe cases 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 programs for missense variants and MutationTaster for other types of variants. The results showed that all variants except p.(Asp1107Glu) were predicted to have damaging effects.

Discussion

All 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 having severe disease. Since intron inversions lead to truncation of the wild-type *F8* transcription unit and the inversion of intron 1 or 22 toward the telomere of the long arm of the X chromosome significantly disrupts the protein structure [12], intron inversions are closely associated with severe HA. The inv22 and inv1 inversions account for 40%-50% and 0.5%-5% of cases of severe HA, respectively [13,14]. In this study, the proportion of inv1 cases was higher than that reported in the general literature, probably due to the limitation of our sample size and differences in the levels of development among countries [15,16]. A previous study on the mutational spectrum of 1,296 Italian patients reported a significant predominance of missense variants, which represented 80% of the variants in patients with

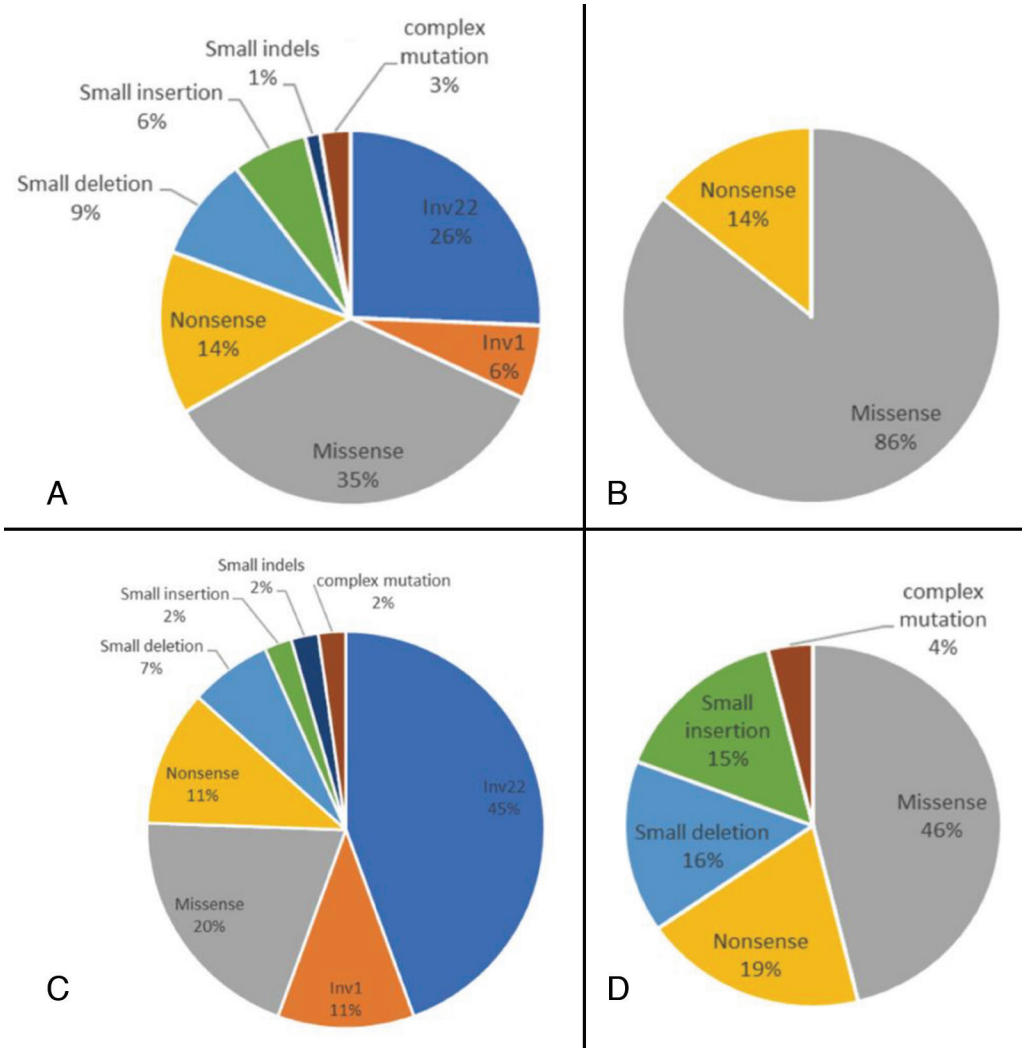

Figure 1. Frequencies of different types of *F8* DNA mutations found in all analyzed patients with hemophilia A (A) and the mutation spectra for mild cases (B), severe cases (C), and moderate cases.

F8: Factor VIII.

mild HA and 68% of the variants in patients with moderate HA. Moreover, patients with frameshift variants mostly showed moderate or severe disease [17]. The proportion of patients with missense variants being classified as having moderate disease was lower in our study compared to the previous study of 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 in this study, exhibited a near-normal FVIII:C level of 44.5%. This 4-year-old boy was diagnosed with HA (subclinical type) because although both VWF:RCo and VWF:Ag were within normal limits, he experienced recurrent epistaxis and mild clinical symptoms. Analysis using SIFT, PolyPhen2, and MutationTaster showed that the variant did not cause deleterious effects on FVIII function. Its co-site variant p.(Arg245Trp) was also reported previously, with a FVIII:C level of 46% (i.e., normal range) [18]. This variant site is in the A1 domain, which is generally considered to be associated with post-cleavage activation of FVIII and binding of factor X [19,20,21]. 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) provides further evidence for our speculation [18,22,23,24]. Considering the allele frequency as described by dbSNP, this variant is more common in East Asians and it is possible that this locus is a very rare polymorphic locus. Overall, 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 described in this study warrants further investigation and our research team is currently conducting further exploration of its molecular mechanisms.

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 unrelated 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 3). These patients had FVIII:C rates 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 having severe disease, whereas missense variants at the same site were mostly classified as mild/medium. Patient 2, who had missense variant p.(Arg1985Pro) at the same locus, showed a mild clinical phenotype and was diagnosed at the age of 12 years due to left anterior iliopsoas hematoma with subsequent intravertebral hemorrhage. Blood group antigens, von Willebrand factor levels, and age are important determinants of FVIII levels in the normal population, but the impact of these factors in HA has been debated [25,26,27,28]. Major differences in FVIII:C between children with the same variant, as in Patient 43 versus Patient 44, suggest to some extent that baseline FVIII:C values in HA patients are influenced by factors other than genotype. Identifying these factors may help improve diagnosis and treatment strategies for patients.

The present study is limited by its small sample size. Studies with larger sample sizes investigating the molecular structure and function of the pathogenic mechanisms of HA may further aid in the prenatal diagnosis and treatment of this disorder.

Conclusion

Our study has presented 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 the genetic testing and diagnosis of patients. Our identification of novel variants enriches the variant database of *F8*. Moreover, this study provides further data and a genetic basis for systematically determining the variant spectrum of the *F8* gene in Guangxi Province.

Ethics

Ethics Committee Approval: The study was approved by the Ethics Committee of the Second Affiliated Hospital of Guangxi Medical University [approval number: 2024-KYL (013), date: April 05, 2024)].

Informed Consent: Informed consent was obtained from all participants or their legal guardians in line with the relevant guidelines for institutional ethics.

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Footnotes

Authorship Contributions

Surgical and Medical Practices: Jin.J., Y.L.; Concept: H.W.; Design: H.W.; Data Collection or Processing: Jin.J., L.H.; Analysis or Interpretation: Jia.J;. Literature Search: Jia.J.; Writing: Jia.J.

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