

Direct and indirect gene diagnosis of hemophilia A pedigrees in the Chinese population

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Abstract. Hemophilia A (HA) is an X-linked recessive hereditary disorder caused by defects in the coagulation factor VIII (*FVIII*) gene. In order to diagnose patients with presymptomatic HA and carriers, the present study conducted direct gene diagnosis for the common abnormalities in *FVIII* and subsequently performed indirect gene diagnosis for the other abnormalities in *FVIII* for Chinese HA pedigrees. Direct gene diagnosis was performed in 10 HA pedigrees using inverse shifting-polymerase chain reaction to detect intron 22 inversion (inv22), intron 22 deletion, intron 22 duplication and inv1 of *FVIII*. Pedigrees with no detected mutations were further analyzed using indirect genetic diagnosis (haplotype linkage analysis), where the genetic markers of *FVIII* included one variable number of tandem repeat, seven short tandem repeats and three restriction fragment length polymorphisms. The results of three pedigrees were taken as examples. Pedigree 1 underwent direct gene diagnosis, which demonstrated that the proband was inv22 distal pattern hemophiliac and the mother was an inv22 distal pattern carrier. The other two pedigrees were subjected to indirect gene diagnosis. In pedigree 2, the detection of DXS52, 13(CA) n, DXS9901(GT) n, intron (int)18, int19 and int22 confirmed the proband's baby brother was normal, the proband's maternal aunt was a carrier and her baby son was normal. Detection of DXS9901(GT)n, int18, int19 and int22 in pedigree 3 demonstrated that the proband's maternal grandmother was not a carrier. As the maternal grandfather

was not affected by the disease, it was deduced that a mutation of *FVIII* occurred in the proband's mother. The combination of direct and indirect gene diagnoses provides reliable evidence for the use of genetic counseling in HA pedigrees, particularly for screening presymptomatic males and female carriers with normal offspring.

Introduction

Hemophilia A is a hemorrhagic disease occurred much more frequently in males than in females, since it is an X-linked recessive hereditary disorder caused by diverse mutations in the coagulation factor VIII (*FVIII*) gene that affects 1 in 5,000 male newborns (1). Patients are primarily infused with *FVIII* products for hemorrhage prophylaxis and there is currently no radical cure. *FVIII* gene mapping to Xq28 is 186 kb long and consists of 26 exons and 25 introns (2). A total of ~20% of HA cases have a genetic etiology of intron 22 inversion (inv22) in the *FVIII* gene (3), which can be detected via direct gene diagnosis. However, the majority of patients with HA harbor different *FVIII* mutations, of which there are >1,000 types, thus direct gene diagnosis is difficult to perform (4). Consequently, linkage analysis using closely-linked genetic markers (extragenic and intragenic) of the *FVIII* gene, has emerged as a principal method for detecting carriers. Genetic diagnosis of HA carriers is one of the most effective ways to reduce the birth numbers of newborns with HA. Currently the most widely used markers are variable number of tandem repeats (VNTRs), short tandem repeats (STRs) and restriction fragment length polymorphisms (RFLP) (5). However, the linkage analysis method has limitations as families lacking heterozygous genetic markers are not suitable for this type of analysis (5). Recombination between the genetic marker and mutation locus may also lead to misdiagnosis (5). Therefore, in the present study, direct diagnosis, using inverse shifting-polymerase chain reaction (IS-PCR) to detect inv22 and inv1, was performed in combination with indirect gene diagnosis methods, using 11 genetic marker loci of VNTR, STR and RFLP for haplotype linkage analysis, for the genetic diagnosis of HA. The aim of the present study is to provide reliable evidence for genetic counseling for HA

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pedigrees, particularly for screening presymptomatic males and female carriers with normal or no offspring, which may facilitate earlier treatments and disease prevention.

Materials and methods

Samples and DNA extraction. The present study was approved by the Ethics Committee of Wenzhou Medical University (Zhejiang, China) and written informed consent was obtained for all participants. Investigations were performed in 10 HA pedigrees recruited by the Family Planning Publicity and Technical Guidance Station of Wenzhou City between January 2008 and December 2014 in Wenzhou, China. All ten patients with HA were males aged 4-12 years old with a history of bleeding; HA diagnoses were verified by confirming each patient exhibited very low levels of Factor VIII. Blood samples were obtained from the ten probands and other 42 members of the pedigrees. Genomic DNA was extracted using the standard phenol chloroform method (6).

Direct gene diagnosis. The present study developed the direct gene diagnosis methodology using IS-PCR to detect *inv22* and *inv1*. In addition, supplementary experiments were performed to detect intron 22 deletion (*del22*) and intron 22 duplication (*dup22*) in order to avoid misdiagnosis for *inv22*. The following primers were used: For *inv22*, the ID, IU, 2U and 3U primers were used; for *del22* and *dup22*, the ED, IU, 2U and 3U primers were used; and for *inv1*, the 1-ID, 1-IU and 1-ED primers were used. The sequences of these 8 primers are listed in Table I (7). The DNA samples were digested using *Bcl*I enzyme. The purified products were then ligated with T4 DNA ligase and purified. Finally, IS-PCR amplification (95°C for 30 sec, 60°C for 60 sec, 72°C for 90 sec; 35 cycles) was performed. The products were run on a 1% agarose gel with ethidium bromide, and the image was reversed. The diagnoses were made based on the different fragment lengths of the IS-PCR products for *FVIII*, which are presented in Table II, and the experiments were repeated three times.

Indirect gene diagnosis. As different pedigrees may have different effective genetic marker loci, the present study used ≤11 genetic marker loci for linkage analysis of HA pedigrees. These sites included one *FVIII* extragenic VNTR site (DXS52), three *FVIII* extragenic STR sites [DXS15(CA)_n, DXS9901(GT)_n and DXS1073(GT)_n], four *FVIII* intragenic STR sites [1(GT)_n, 13(CA)_n, 22(GT)_n(AG)_n and 24(GT)_n] and three *FVIII* intragenic RFLP sites [intron (int)18, int19 and int22]. The distribution of these genetic marker loci inside or outside the *FVIII* gene is shown in Fig. 1. The present study used PCR to amplify the target fragments of VNTR, STR or RFLP loci, and then performed analysis of the polymorphisms. VNTR polymorphisms were reflected by the PCR fragment lengths identified via 1% agarose gel electrophoresis with ethidium bromide, and the image was reversed. STR polymorphisms were reflected by the PCR fragment lengths observed following 6% PAGE with silver staining and capillary electrophoresis. RFLPs were reflected by the length of enzyme-digested PCR fragments identified on 6% PAGE gels with silver staining. Annealing

Table I. Sequences of the primers used for inverse shifting-polymerase chain reaction.

Primer	Sequence (5'-3')
ID	ACATACGGTTTAGTCACAAGT
ED	TCCAGTCACTTAGGCTCAG
IU	CCTTTCAACTCCATCTCCAT
2U	ACGTGTCTTTTGGAGAAGTC
3U	CTCACATTGTGTTCTTGTAGTC
1-IU	GCCGATTGCTTATTTATATC
1-ID	TCTGCAACTGGTACTCATC
1-ED	GCCTTTACAATCCAACACT

temperatures, primer sequences and PCR product fragment sizes of the genetic marker loci are summarized in Table III (8-11). All of the PCR amplification except for DXS52 was carried out according to the following condition for 35 cycles: Denaturation at 95°C for 45 sec; annealing at the temperature indicated in Table III for 45 sec; extension at 72°C for 45 sec. For the DXS52 amplification, PCR was carried out for 26 cycles: 95°C for 40 sec, 60°C for 40 sec, 72°C for 90 sec. The PCR amplification was carried out using high fidelity PCR Mix (Vazyme, Piscataway, NJ, USA). The diagnoses made based on the size of enzyme-digested products are presented in Table IV, and the experiments were repeated three times.

Results

Direct gene diagnosis of HA pedigree 1. DNA samples from 10 probands were analyzed by IS-PCR in order to detect *inv22*. Only one proband (pedigree 1) harbored the *inv22* mutation. Subsequently, supplementary experiments were performed to confirm that it was *inv22* and not *del22* or *dup22*. Electrophoresis of the IS-PCR products for *inv22* revealed that the proband was a distal *inv22* homozygote (Fig. 2A). The proband's mother was a distal *inv22* heterozygote and the father was normal. The results of electrophoresis of the IS-PCR products for *del22* or *dup22* revealed that neither the proband, nor the proband's mother or father had *del22* or *dup22* mutations (Fig. 2B). In addition, IS-PCR was performed in order to detect *inv1* in the other 9 probands; however, *inv1* was not detected in any of these patients.

Indirect gene diagnosis of HA pedigree 2 and 3. In the present study, 11 genetic marker loci were used for linkage analysis of the other nine pedigrees without *inv22* and *inv1*; there were ≥4 genetic marker loci that could provide effective information for each pedigree. As the different families had different effective genetic marker loci, the present study presents two pedigrees as examples to present the results of indirect gene diagnosis analysis in the present study.

Pedigree 2. Detection of the 11 genetic marker loci for pedigree 2, identified six marker loci that were heterozygous and could provide effective linkage information, including one VNTR locus (DXS52), two STR loci [13(CA)_n and

Table II. Diagnoses made based on the different fragment sizes of the inverse shifting-polymerase chain reaction products for the coagulation factor VIII gene.

Diagnosis	Fragment size, bp		
	Inv22 test	Del22 and dup22 test	Inv1 test
Male			
Normal	487	457 and 405	304
Inv-1			224
Inv22-1(distal)	333	559 and 457	
Inv22-2(proximal)	385	559 and 405	
Dup22	487	559, 457 and 405	
Del22-1	333	457	
Del22-2	385	405	
Female			
Normal homozygote	487	457 and 405	304
Inv-1 heterozygote			304 and 224
Inv22-1 heterozygote	487 and 333	559, 457 and 405	
Inv22-2 heterozygote	487 and 385	559, 457 and 405	
Dup22 heterozygote	487	559, 457 and 405	
Del22-1 heterozygote	487 and 333	457 and 405	
Del22-2 heterozygote	487 and 385	457 and 405	

Inv, intron inversion; del, intron deletion; dup, intron duplication.

Table III. Annealing temperature, primer sequences and fragment size for the polymerase chain reaction of genetic markers.

Author, year	Genetic marker	Annealing temperature	Primer sequence and label (5'-3')	Fragment size, bp	(Refs.)
Yan <i>et al</i> , 2011	DXS52	60°C	GGCATGTCATCACTTCTCTCATGTT	700-3,000	(8,9)
Richards <i>et al</i> , 1991			CACCACTGCCCTCACGTCACCT		
Fang <i>et al</i> , 2006	DXS15(CA)n	60°C	AGCACATGGTATAATGAACCTCCACG (FAM)-CAGTGTGAGTAGCATGCTAGCAT TTG	148-168	(10)
Fang <i>et al</i> , 2006	DXS9901(GT)n	62°C	GACCAGTCCTCCCTTCTGTT (FAM)-GTGTGGAGTGAAAGGGACAG	186-214	(10)
Fang <i>et al</i> , 2006	DXS1073(GT)n	60°C	ATGCCCTCTCCGAGTTATTACA (FAM)-ATTGGTGGCCTTTGAAACAC	122-142	(10)
Liang <i>et al</i> , 2009	1(GT)n	55°C	GGTCTCCAATGAAACAGATCC CCTTGACATAAGCATTCTTGG	120-132	(11)
Liang <i>et al</i> , 2009	13(CA)n	45°C	GTTTCTTTGCATTCAACTGTACA TAATGTATCTT	146-172	(11)
Liang <i>et al</i> , 2009	22(GT)n(AG)n	50°C	CCAAATTACAGATTGAATAAGCCTAG GTTTCTTATTAATGCCACATTATAGACT CTC	207-219	(11)
Liang <i>et al</i> , 2009	24(GT)n	58°C	AATAAGACCCTTAGCTGTTTCAT GCCTGGACTACAGAGGGAGAC AAATTTTCACACGCACACCTG	182-198	(11)
	Intron 18	58°C	TTCATTTTCAGTGGACATGTG CCTATGGGATTTGAGATGGT	374	
	Intron 19	55°C	TTGGCGAGCATCTACATGCT CTAATGTGTCCAGAAGCCAT	702	
	Intron 22	60°C	CACGAGCTCTCCATCTGAACATG GGGCTGCAGGGGGGGGGGACAACAG	96	

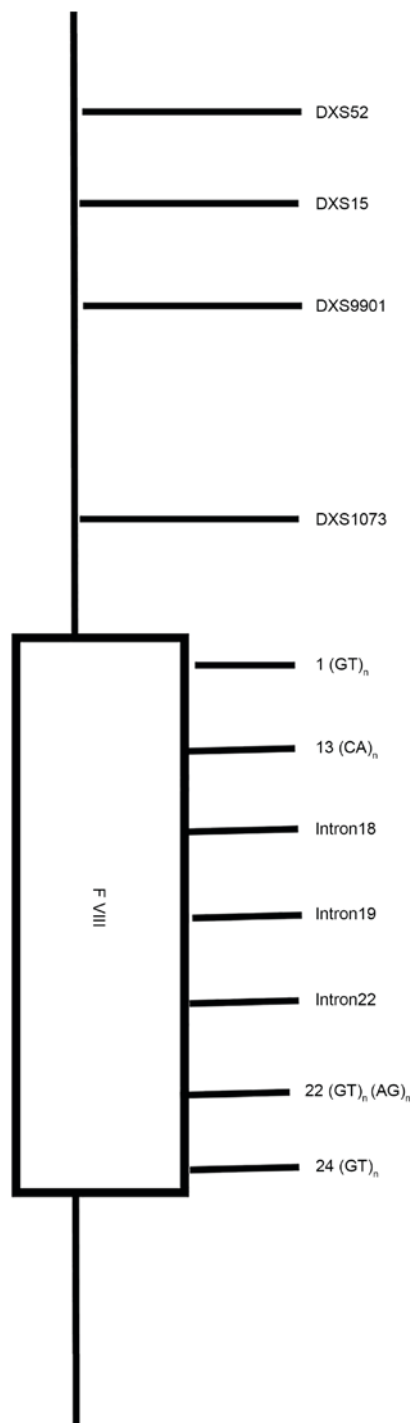


Figure 1. Distribution of the genetic marker loci inside and outside of the *FVIII* gene that were used in the present study. *FVIII*, coagulation factor VIII.

DXS9901(GT)_n] and three RFLP loci (int18, int19 and int22). The genogram and the electrophoretogram of the PCR products of pedigree 2 are presented in Fig. 3.

The results of the DXS52, DXS9901(GT)_n and 13(CA)_n loci (Fig. 3B-D) demonstrated that the band of the proband (III1) was in the same position as the proband's mother's (II3) lower band, indicating that the X chromosome was pathogenic and the mother was a carrier. The band of the proband's baby brother (III2) was in the same position as the mother's upper band, indicating his X chromosome was normal. The pathogenic X chromosome of the proband's maternal aunt (II5) and

Table IV. Diagnoses made based on the size of the enzyme-digested products.

RFLP loci	Enzyme	Allele negative, bp	Allele positive, bp
Intron 18	<i>Bcl</i> I	374	211 and 163
Intron 19	<i>Hind</i> III	467 and 235	467, 154 and 81
Intron 22	<i>Xba</i> I	96	68 and 28

RFLP, restriction fragment length polymorphisms.

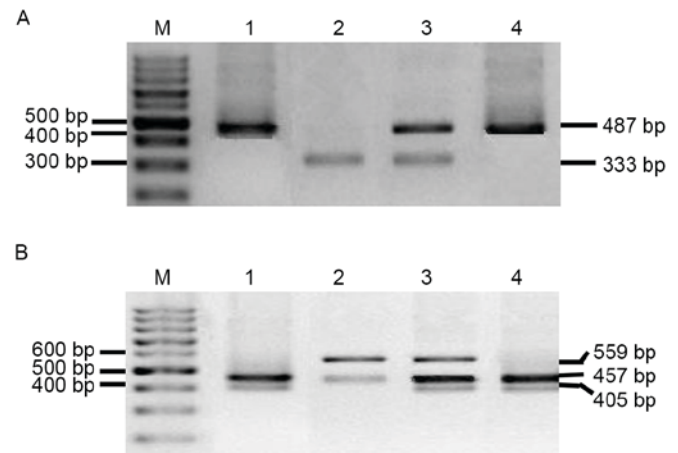


Figure 2. Electrophoresis of the inverse shifting-polymerase chain reaction products of pedigree 1 for the detection of (A) inv22, and (B) del22 or dup22. Lane M, marker; lane 1, normal male control (band size, A: 487 bp; B: 457 and 405 bp; normal); lane 2, proband (band size, A: 333 bp; B: 559 and 457 bp; inv22-1); lane 3, mother (band size, A: 487 and 333 bp; B: 559, 457 and 405 bp; inv22-1 heterozygote); lane 4, father (band size, A: 487 bp; B: 457 and 405 bp; normal). inv22, intron 22 inversion; del22, intron 22 deletion; dup22, intron 22 duplication.

the proband's mother was inherited from the proband's maternal grandmother (I2), and the normal X chromosome was inherited from the proband's maternal grandfather (I1). The proband's maternal grandmother and aunt were carriers, and the proband's maternal grandfather was normal. The band of the baby son of the proband's maternal aunt (III3) was in the same position as the upper band of the proband's maternal aunt (II5), indicating their X chromosome was normal. In addition, the number of STR repeats (detected via capillary electrophoresis) of DXS9901(GT)_n and 13(CA)_n loci in each sample is presented in Fig. 3C and D.

The consistency in the size of the enzyme-digested products of int18 (Fig. 3E), int19 (Fig. 3F) and int22 (Fig. 3G) loci indicated that the X chromosome of the proband (III1), the same as one of the mother's (II3) X chromosomes, was pathogenic and thus, the mother was a carrier. The X chromosome of the proband's baby brother (III2) was not pathogenic, indicating he was normal. One of the X chromosomes of the proband's maternal grandmother (I2) was pathogenic while the X chromosome of the proband's maternal grandfather (I1) was normal, demonstrating that the mother's pathogenic and normal X chromosome came from the proband's maternal grandmother and maternal grandfather, respectively. The

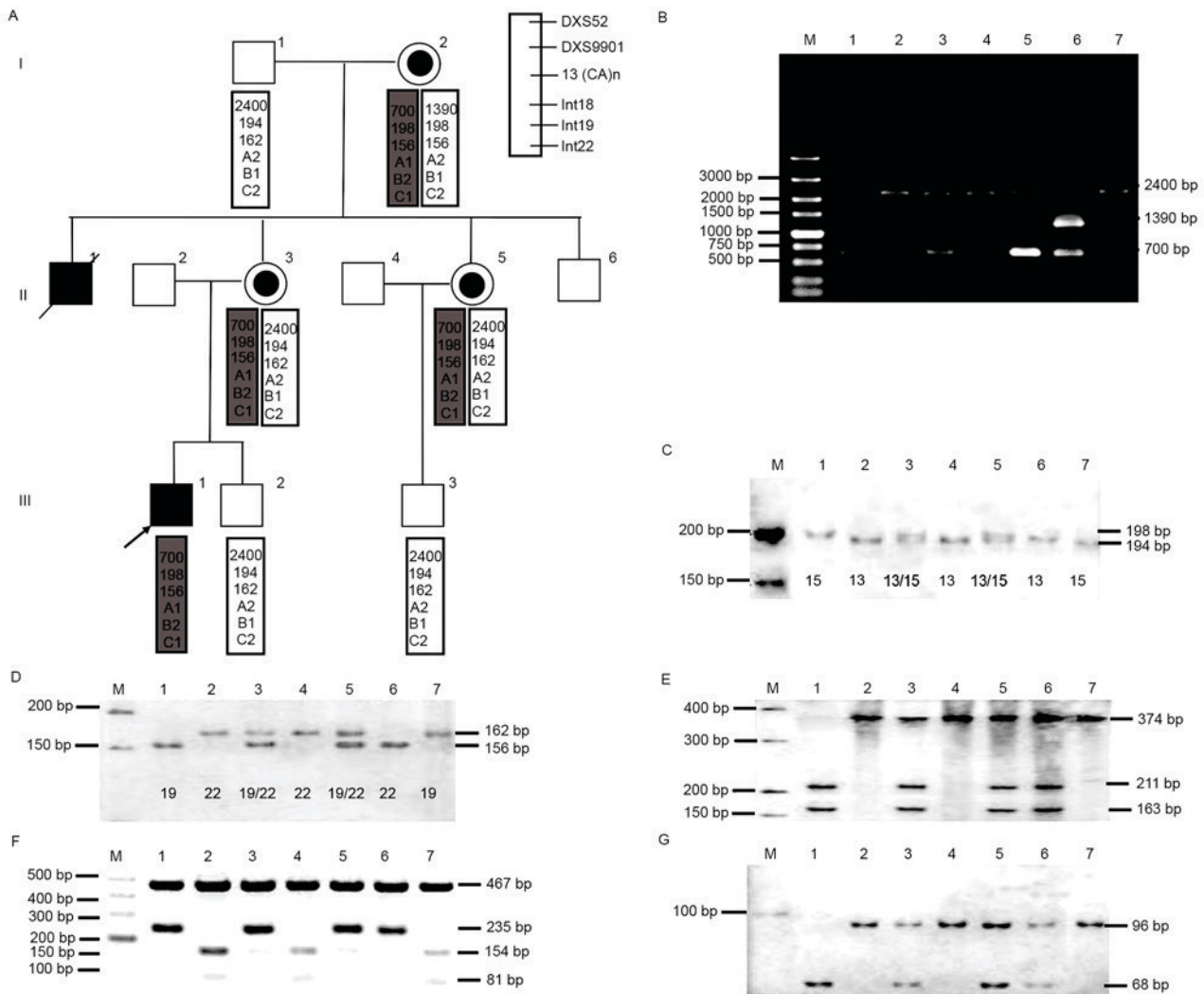


Figure 3. Genogram and electrophoretogram of the PCR products of pedigree 2. (A) The haplotype linkage analysis results of the DXS52, DXS9901(GT)n, 13(CA)n, int18, int19 and int22 genetic marker loci of pedigree 2. According to the mutation haplotype of III1 (proband; indicated by an arrow), the grey bar represents the haplotype with a mutation while the white bar represents the haplotype without a mutation, the numbers within the grey and white bars indicate fragment size (bp) from the PCR. White squares, normal males; black square with an arrow, proband; black square with a forward slash, deceased male patient; black/white circles, female carriers; A1, int18/*BclI* allele positive; A2, int18/*BclI* allele negative; B1, int19/*HindIII* allele positive; B2, int19/*HindIII* allele negative; C1, int22/*XbaI* allele positive; C2, int22/*XbaI* allele negative. Electrophoresis of PCR products for the (B) DXS52, (C) DXS9901 (GT)n, (D) 13(CA)n, (E) int18, (F) int19 and (G) int22 loci. The numbers written on the blots (C and D) represent the number of short tandem repeats and refers to the genotype of each sample. Lane M, marker; lane 1, III1; lane 2, III2; lane 3, II3; lane 4, III3; lane 5, II5; lane 6, I2; lane 7, I1. PCR, polymerase chain reaction; int, intron.

proband's maternal grandmother was a carrier and the proband's maternal grandfather was normal. One of the proband's maternal aunt's (II5) chromosomes was pathogenic and inherited from the proband's maternal grandmother, while the other normal X chromosome was inherited from the proband's maternal grandfather. The proband's maternal aunt was a carrier. Since her son (III3) had no pathogenic X chromosome, he could be diagnosed as normal.

To conclude the results of pedigree 2, the pathogenic X chromosome of the proband was inherited from the proband's mother who was a carrier. The proband's baby brother was normal and therefore, has no risk of severe bleeding in the future. The proband's maternal grandmother was a carrier and the proband's maternal grandfather was normal. The proband's maternal aunt was a carrier, and her baby son was normal and, therefore, also has no risk of severe bleeding in the future.

Pedigree 3. Detection of 11 genetic marker loci for pedigree 3 identified four marker loci that were heterozygous and

could provide effective linkage information, including one STR locus [DXS9901(GT)n] and three RFLP loci (int18, int19 and int22). The genogram and the electrophoretogram of the PCR products of pedigree 3 are presented in Fig. 4.

The results of DXS9901(GT)n locus analysis revealed that the band of the proband (III3) was in the same position as the proband's mother's (II5) lower band, indicating that the X chromosome was pathogenic and the mother was a carrier (Fig. 4B). One of the two bands of the proband's baby sister (III4) was from the mother (the pathogenic X chromosome) and the other band was from the father (II4; the normal X chromosome), indicating that the proband's baby sister was a carrier. There was only one band for the proband's maternal grandmother (I2), which was in the same position as the upper band (the normal X chromosome) of the proband's mother, indicating it was not pathogenic. In addition, the number of STR repeats (detected via capillary electrophoresis) of the DXS9901(GT)n locus was marked in Fig. 4B.

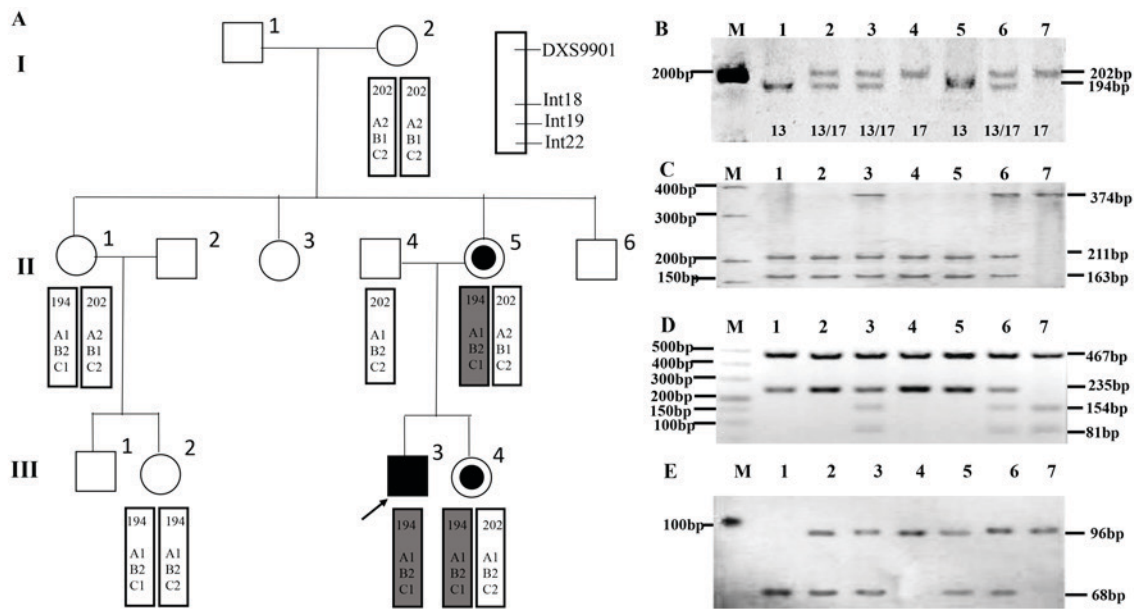


Figure 4. Genogram and electrophoretogram of the PCR products of pedigree 3. (A) The haplotype linkage analysis results of the DXS9901(GT)_n, int18, int19 and int22 genetic marker loci of pedigree 3. According to the mutation haplotype of III3 (proband; indicated by an arrow), the grey bar represents the haplotype with a mutation while the white bar represents the haplotype without a mutation, the numbers within the grey and white bars indicate fragment size (bp) from the PCR. White squares, normal males; black square with an arrow, proband; black/white circles, female carriers; white circles, normal females; A1, int18/Bcl I allele positive; A2, int18/Bcl I allele negative; B1, int19/HindIII allele positive; B2, int19/HindIII allele negative; C1, int22/XbaII allele positive; C2, int22/XbaII allele negative. Electrophoresis of the PCR products of the (B) DXS9901(GT)_n, (C) int18, (D) int19 and (E) int22 loci. The numbers written on the blots (B) represent the number of short tandem repeats and refers to the genotype of each sample. Lane M, Marker; lane 1, III3; lane 2, III4; lane 3, II5; lane 4, II4; lane 5, II2; lane 6, III1; lane 7, I2. PCR, polymerase chain reaction; int, intron.

The results of the int18 (Fig. 4C), int19 (Fig. 4D) or int22 (Fig. 4E) loci analysis demonstrated that the X chromosome of the proband (III3) was the same as one of the mother's (II3), as the size of enzyme-digested products were the same. Therefore, this X chromosome was pathogenic and the mother was a carrier. The pathogenic X chromosome of the proband's baby sister (III4) was inherited from the mother and the normal X chromosome was from the father (II4). Therefore, the baby sister was a carrier. The two X chromosomes of the proband's maternal grandmother (I2) were not pathogenic, therefore, the proband's maternal grandmother was normal.

To conclude the results of pedigree 3, the proband's maternal grandmother was normal and the pathogenic X chromosome of the proband was inherited from the proband's maternal grandfather. As the proband's grandfather was not diagnosed with HA, it is possible that a mutation of the *FVIII* gene occurred in the germ cell of the maternal grandfather, which was passed on to the mother. As with the proband's mother, the baby sister of the proband was also a carrier, and therefore, if she were to have sons they would be at risk of HA. However, the X chromosomes of the proband's maternal uncle and aunts, though inherited from the maternal grandfather, did not have the same mutant as the proband's mother, indicating that the maternal aunts and their female offspring were not carriers. The maternal uncle was normal.

Discussion

A total of ~40% of severe cases of HA have a genetic etiology of inv22 of the *FVIII* gene (12), whereas inv1 is present in only 2-5% of severe cases (13,14). Hence, a direct method should be performed first to detect inversion mutations for patients

with severe HA. Long distance PCR was once advocated to detect inv22; however, its limitations, including difficulties in amplification, a stringent requirement for template quality and poor repeatability, make it less reliable (15). In recent years, investigators began to use inversion PCR (I-PCR), which consists of enzyme digestion, T4 ligase connection and PCR amplification. The advantages of I-PCR include lower quality requirement for templates, shorter DNA fragments to be amplified, good repeatability, an easy protocol and simple interpretation of the results (15). This research adopted the IS-PCR method (7), which was the result of optimization and improvements made to the I-PCR protocol. IS-PCR can detect either inv22 or inv1, and homozygous patients or heterozygous carriers, it can distinguish inv22 distal inversion with proximal inversion, and it can eliminate the possibility of misdiagnosing del22 or dup22 as inv22. Previous studies including the use of the IS-PCR method for detecting HA have been infrequent until recently, and IS-PCR may be widely applied in clinical direct gene diagnosis for HA (16).

The *FVIII* gene is associated with significant genetic heterogeneity, a large structure and complex alterations to molecular pathology, thus, current molecular biology methods have not been able to uncover all of the molecular defects (2). This renders the direct gene diagnosis of HA challenging (in all cases except for inv22). Consequently, the use of genetic markers inside or nearby the *FVIII* gene for indirect genetic diagnosis has become one of the main methods for HA gene diagnosis (17).

Indirect genetic diagnosis for HA generally uses genetic markers, including VNTR, STR and RFLP, for linkage analysis. The commonly-used extragenic VNTR or STR loci include DXS52 (18,19), DXS15(CA)_n (18,20), DXS9901(GT)_n

and DXS1073(GT)n (10). Intragenic STR loci include 13 (CA)n (11,21,22) and 22(GT)n(AG)n (21,22). Intragenic RFLP loci include *BclII* (23,24), *HindIII* (23,24), *XbaII* (23) and *BglII* (18) enzyme cutting loci in introns 18, 19, 22 and 25, respectively. Genetic markers used for linkage analysis should have a certain rate of heterozygosity (usually at least 30%) and should provide sufficient polymorphism information in a certain population. In general, in accordance with the heterozygous rate of the local population, genetic markers with a high heterozygous rate should be selected, and the combine use of several genetic markers may improve the efficiency of diagnosis. According to a previous report, the heterozygous rate of the RFLP loci int18, int19 and int22 in the north Indian population was 57, 38 and 43%, respectively (23). The heterozygous rate of the STR loci in the Chinese population have been reported to be 34.6% (11) or 37.9% (22) for intron 1 (GT)n, 60.9% (25) or 61.0% (11,22) for 13(CA)n, 43.6% (11) for 22(GT)n(AG)n and 38.2% (11) for 24(GT)n, respectively. The heterozygous rate of extragenic genetic markers of the STR loci DXS15(CA)n, DXS9901(GT)n and DXS1073(GT)n in the Chinese population have been reported to be 88.24 (10), 82.35 (10) and 62.0% (25), respectively. Therefore, the present study applied these 11 genetic marker loci with high heterozygous rates for indirect genetic diagnosis in Chinese pedigrees. As there is a 3% recombination possibility within the large HA gene during meiosis (10), the present study used multiple heterozygous loci for haplotype linkage analysis in order to avoid incorrect linkage analysis as result of recombination between the genetic marker locus and the mutation locus.

In conclusion, the results of the present study may have academic and clinical implications. Linkage analysis could be used to diagnose affected pedigree members other than the proband, particularly presymptomatic males who would suffer from severe bleeding following trauma. Linkage analysis could also be used to accurately diagnose female carriers from potential female carriers. On this basis, linkage analysis could be applied for prenatal diagnosis in the male infants of female carriers, including all accurately diagnosed female carriers within the pedigree, not just the patient's mother. The results of the present study indicated that the combination of direct and indirect gene diagnosis may provide reliable evidence for the use of genetic counseling for HA pedigrees, particularly for screening presymptomatic males and female carriers with normal or no offspring, which may facilitate earlier treatments and disease prevention.

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