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Genetic analysis of a hemophilia B family with a novel F9 gene mutation

A STROBE-compliant article

Xue Lv, MA[a], Tao Li, MA[b,c], Hao Li, MA[d], Hong-yan Liu, MD[e], Zhen Wang, MA[f], Zhi-ping Guo, MD[g,h,*]

Abstract

At present, there are no effective methods for the treatment of hemophilia B, and it has high mortality and disability. Therefore, it is very important for the carriers to carry out genetic counseling and make prenatal diagnosis. In this study, we made gene and prenatal diagnoses in a family with a novel *F9* gene mutation, and report a novel *F9* gene mutation.

All exon sequences and flanking sequences of *F9* gene were analyzed by Sanger sequencing in the proband; and then according to the *F9* gene mutation in the proband, the *F9* gene sequencing was performed on the family members. Based on the above results, the pathogenic mutation in *F9* gene was finally identified, which was used for prenatal diagnosis.

Sanger sequencing revealed c.1232G>C [p.Ser411Thr] mutation in *F9* gene in the proband. c.1232G>C heterozygous mutation was also found in the proband's mother and grandmother, but male family members without hemophilia B had no this mutation. The analyses of amniotic fluid samples indicated positive sex-determining region on Y chromosome (*SRY*), and no c.1232G>C [p.Ser411Thr] mutation in *F9* gene.

We identified a pathogenic mutation in *F9* gene in the family, made a prenatal diagnosis for the female carrier and reported a novel *F9* gene mutation.

Abbreviations: HB = hemophilia B, MLPA = multiplex ligation dependent probe amplification, SPD = serine protease domain, SRY = sex-determining region on Y chromosome.

Keywords: *F9* gene, genetic diagnosis, hemophilia B, novel mutation, prenatal diagnosis

1. Introduction

Hemophilia B (HB) (OMIM #306900) is an X-linked recessive hereditary disease. Most of the patients with HB are male and its incidence is about 1/30,000 in live male infants.[1] The patients with HB have repeated bleeding due to prolonged coagulation time from their childhood. Sometimes, HB may lead to disability or endanger lives. At present, there are no effective methods for the treatment of HB, and it has high mortality and disability. Therefore, it is very important for the carriers to carry out genetic counseling and make prenatal diagnosis.

The pathogenic gene of HB is *F9* gene because *F9* gene mutation affects factor IX formation. The *F9* gene is located at Xq27.1 of the long arm of X chromosome.[2] Since *F9* gene is relatively short in length and relatively simple in structure, we often use Sanger sequencing to carry out HB gene diagnosis, obtaining accurate and economical results and providing reliable experimental data for clinical genetic counseling. In this study, we used Sanger sequencing technique to perform gene and prenatal diagnoses for the HB family, and reported this novel *F9* gene mutation.

2. Subjects and methods

All study methods were approved by the Ethics Committee of Henan Provincial People's Hospital. All the subjects enrolled into the study gave their informed consent.

2.1. Subjects

In April 2014, a family with suspected HB went to Henan Provincial People's Hospital for genetic consultation and asked to carry out HB gene diagnosis and prenatal diagnosis (Fig. 1). In the family, the patient (III1) was a 3-year-old boy. The boy had hemorrhagic spots on his skin when he was born. He went to see doctors due to gingival bleeding and was diagnosed with severe HB based on the blood tests including activated partial thrombin time (APTT) of 66.5 s (reference values 25–45 s), factor IX activity (IX:C) < 1% (reference values 67.7%–128.5%), factor VIII activity (VIII:C) of 116.8% (reference values 67.7%–128.7%). At present, the patient receives factor IX for preventive and symptomatic treatment without special clinical symptoms. His

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XL and TL contributed equally to this study.

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[a] Department of Health Management, Henan Provincial People's Hospital, People's Hospital of Zhengzhou University, [b] Medicine laboratory, Fuwai Central China Cardiovascular Hospital, the Heart Center of Henan Provincial People's Hospital, [c] Henan Provincial People's Hospital, Zhengzhou, [d] Department of Plastic Surgery, [e] Institute of Medical Genetics, [f] Department of Hematology, Henan Provincial People's Hospital, People's Hospital of Zhengzhou University, [g] Henan Luoyang Orthopaedics Institute, [h] Fuwai Central China Cardiovascular Hospital, Zhengzhou, China.

[*] Correspondence: Zhi-ping Guo, No. 82, Qiming South Road, Luohe Hui District, Luoyang 450018, China (e-mail: litao0510@163.com).

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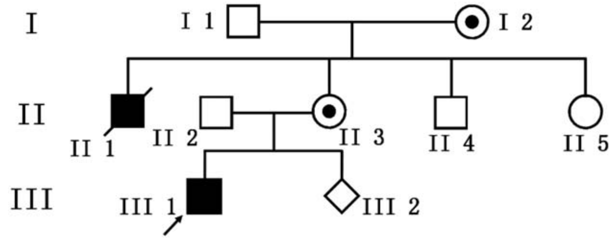

Figure 1. Genealogical tree of the HB family. Notes: The arrow indicates the proband. HB: Hemophilia B; □: Normal male; ⊙: Female HB carrier; ■̸: Dead male HB patient; ○: Normal female; ■: Male HB patient and ◇: Fetus.

mother (II3) was 29 years old and at tenth week of gestation. A total of 100 healthy individuals including 50 males and 50 females, new staffs of our hospital, had no HB family history and no blood relationship with this HB family.

2.2. Specimen collection and DNA extraction

Peripheral blood (3–5 ml) was collected and mixed with EDTA-K_2 for anticoagulation in the family members. Amniotic membrane puncture was performed on the pregnant woman to obtain fetal exfoliated cells under ultrasound guidance. Meanwhile, the peripheral blood samples (3–5 ml) was also collected and mixed with EDTA-K_2 for anticoagulation in the 100 healthy individuals including 50 males and 50 females. Total DNA of the peripheral blood and fetal exfoliated cells was extracted using Qiagen genomic DNA extraction kit (Qiagen, Hilden, Germany). The DNA concentrations of normal control and samples were determined using NANODROP 2000 instrument (Thermo, Waltham, MA).

2.3. Analysis of F9 gene mutation in the family by Sanger sequencing

Primers of coding and flanking regions of *F9* gene were designed by Songon Biotech Co. Ltd (Shanghai, China) according to the *F9* gene sequence.[3] PCR amplification was performed for coding sequences and flanking sequences of *F9* gene in the patient, and PCR amplification products were sequenced by Songon Biotech Co. Ltd (Shanghai, China). And then, according to the mutation locus of *F9* gene in the patients, the sequences at the mutation locus in *F9* genes from other members of the family and 100 healthy individuals

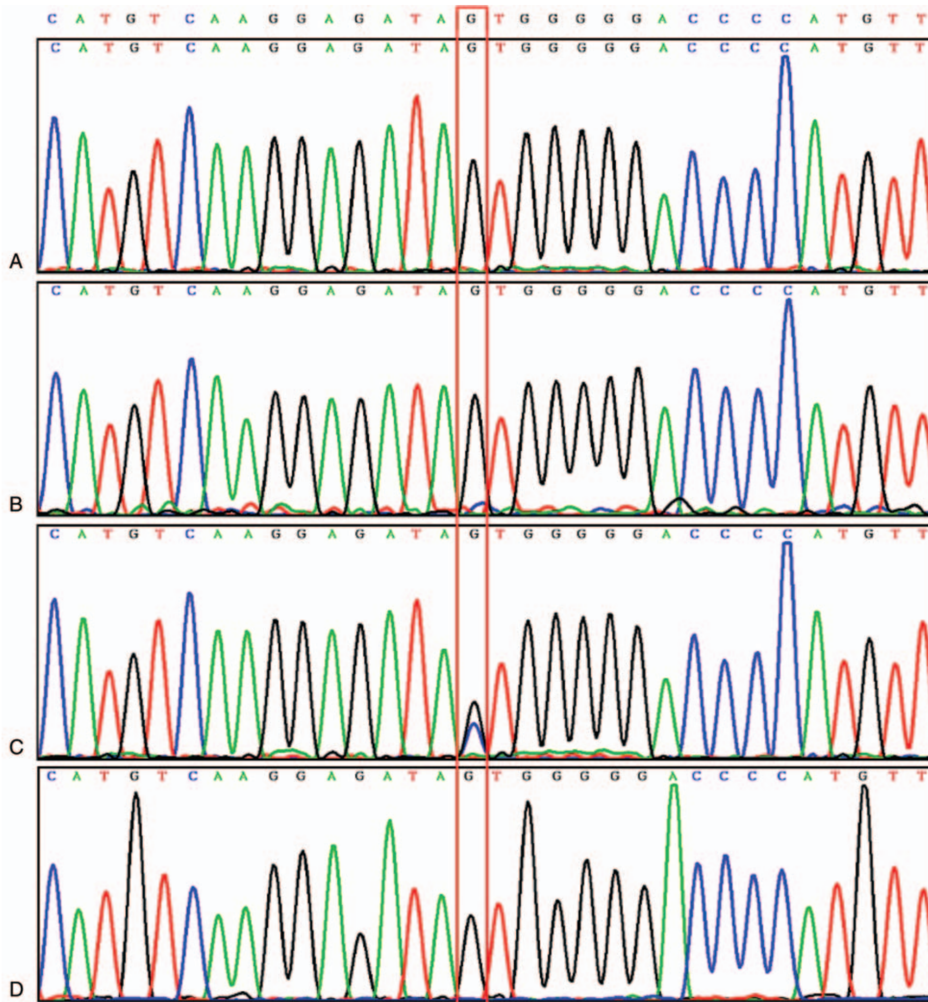

Figure 2. Results of Sanger sequencing. Notes: (A) No c.1232 mutation in *F9* gene of 100 healthy individuals. (B) No c.1232 mutation in *F9* gene of the patient's grandfather (I1). (C) c.1232G>C heterozygous mutation in *F9* gene of the patient's grandmother (I2). (D) No c.1232 mutation in *F9* gene of the patient's father (II2). (E) c.1232G>C heterozygous mutation in *F9* gene of the patient's mother (II3). (F) No c.1232 mutation in *F9* gene of the patient's uncle (II4). (G) No c.1232 mutation in *F9* gene of the patient's aunt (II5). (H) c.1232G>C [p.Ser411Thr] mutation in *F9* gene of the patient.

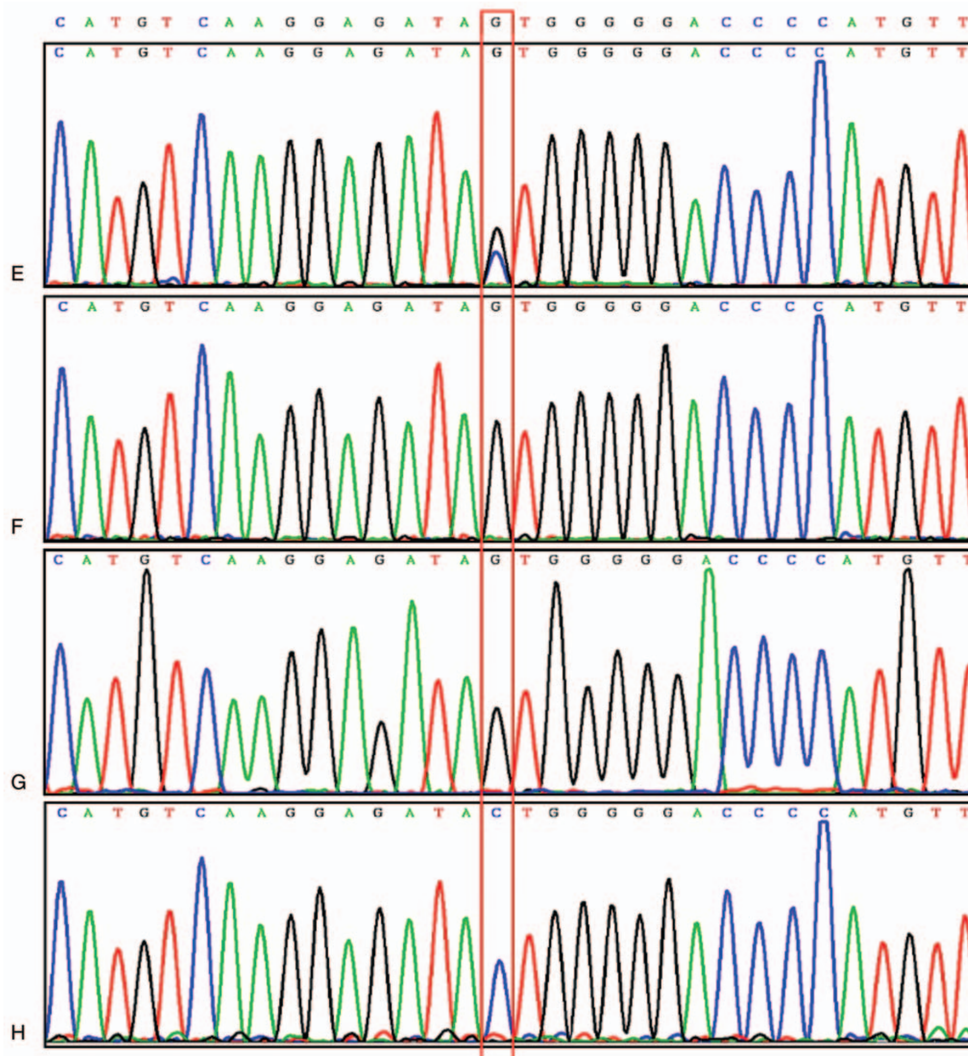

Figure 2. (Continued).

including 50 males and 50 females were analyzed by Sanger sequencing. Base on above results, databanks and the guide for interpretation of clinical significance of sequence variation[4] made by the American College of Medical Genetics and Genomics in 2015, the pathogenic mutation of *F9* gene in this family was identified.

2.4. Detection of maternal cell contamination in amniotic fluid

To make sure the accuracy of results, DNA (5 ng) from the pregnant woman and amniotic fluid were amplified using PowerPlex 21 HS genotyping system (Promega, Fitchburg, WI) according to the manufacturer's instructions[5] to analyze if there was the maternal cell contamination in amniotic fluid.

2.5. Fetal sex diagnosis

After establishment of normal male and female control groups, as well as blank control group; the amplification of fetal sex-determining region on Y chromosome (SRY) gene was performed using 5′-GAATATTCCCGCTCTCCGGA-3′ and 5′-GCTGGTGCTCCATTCTTG AG-3′ primers in triplicate.[6]

2.6. Analysis of F9 gene mutation in the fetus by Sanger sequencing

The sequences at the mutation locus in the fetal *F9* gene were analyzed by Sanger sequencing, and then PCR product was sequenced by Songon Biotech Co. Ltd (Shanghai, China) in triplicate.

3. Results

3.1. Results of Sanger sequencing analysis

In the proband (III1), c.1232G>C [p.Ser411Thr] mutation was detected in *F9* gene. This mutation changes the 411th amino acid codon in *F9* gene from serine codon AGT to threonine codon ACT. c.1232G>C heterozygous mutation (G/C) was found in the proband's mother (II3) and grandmother (I2); but in the proband's uncle (II4), aunt (II5), grandfather (I1), and father (II2), as well as 100 healthy individuals, no mutations (G/C) were found at this locus (Fig. 2). PolyPhen-2 database (http://genetics.bwh.harvard.edu/pph2) displays that the c. 1232G > C mutation in the *F9* gene is a pathogenic mutation located in the active

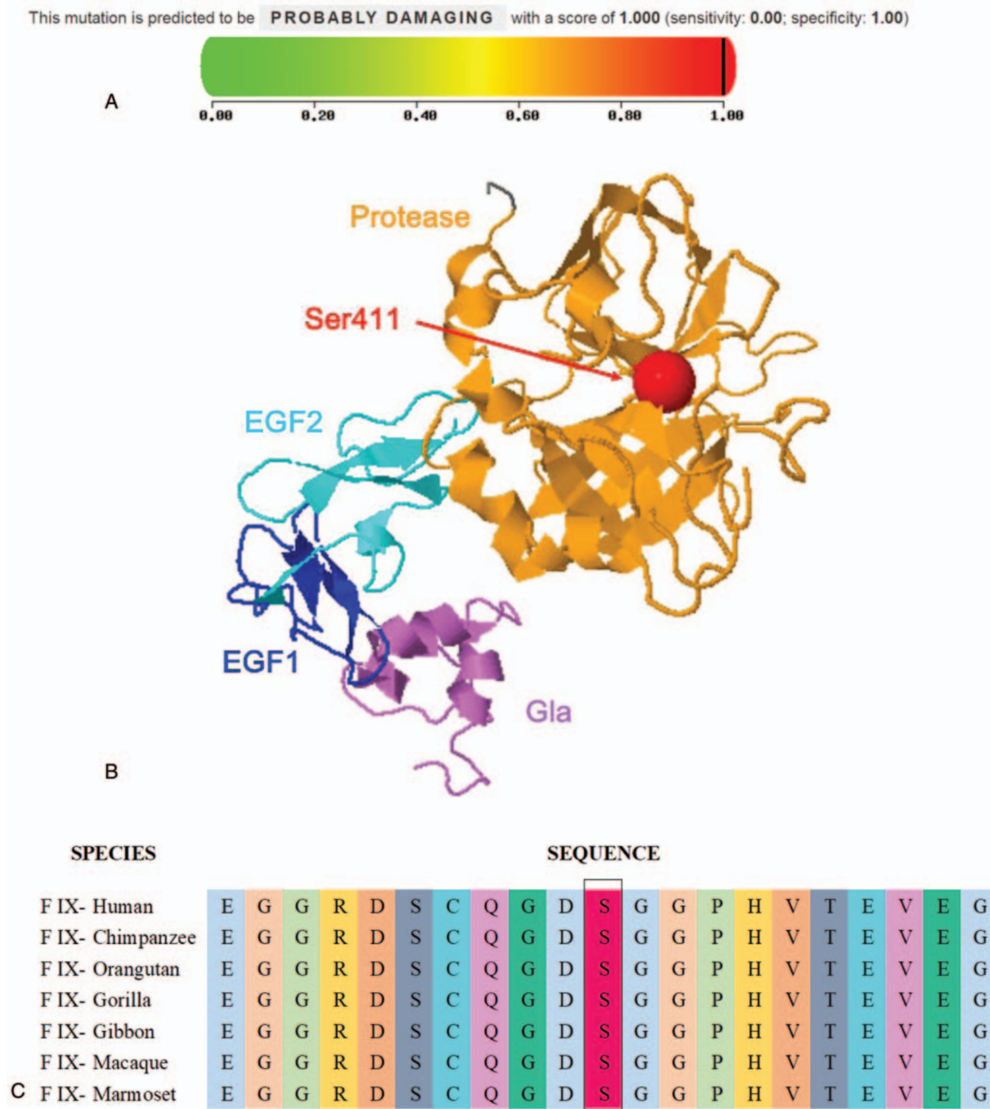

Figure 3. Prediction of mutant pathogenicity by PolyPhen-2. Notes: (A) It shows that the mutation is a pathogenic mutation due to its pathogenicity score of 1.000. (B) It shows the spatial structure of factor IX, and red arrow indicates the mutation site located in the active region of protease. (C) It shows the analysis of conserved amino acid sequence; p.411Ser is highly conserved and is indicated within the black frame.

region of factor IX protease. This mutation site and its adjacent region are highly conserved (Fig. 3).

3.2. Detection of maternal cell contamination in amniotic fluid

PowerPlex 21 HS system (Promega, Fitchburg, WI) indicated that there was no maternal type in the fetal DNA typing, the fetus was male (sex chromosomes: X,Y), and the pregnant woman as well as the fetus were in line with the maternal and son relationship. This suggested that there was no maternal cell contamination in the amniotic fluid specimens which might be used in future assays (Fig. 4).

3.3. Detection of SRY gene

Agarose electrophoresis showed *SRY* gene bands amplified from the amniotic fluid samples, demonstrating that *SRY* gene was positive in the amniotic fluid samples, which was consistent with the results detected by PowerPlex 21 HS system (Promega, Fitchburg, WI).

3.4. Sanger sequencing analysis in the fetal sample

There was no mutation at the c.1232 locus in the fetal *F9* gene, which was performed in triplicate and these results were consistent in the three-time tests (Fig. 5). Based on above results, it was concluded that the fetus was male without HB.

4. Discussion

HB is a serious clinical hereditary hemorrhagic disease. Its pathogenic gene is *F9* gene because factor IX encoded by *F9* gene has coagulant function and *F9* gene mutation affects factor IX synthesis. It has been found that there are many types of *F9* gene mutations, including point mutation, insertion, deletion,

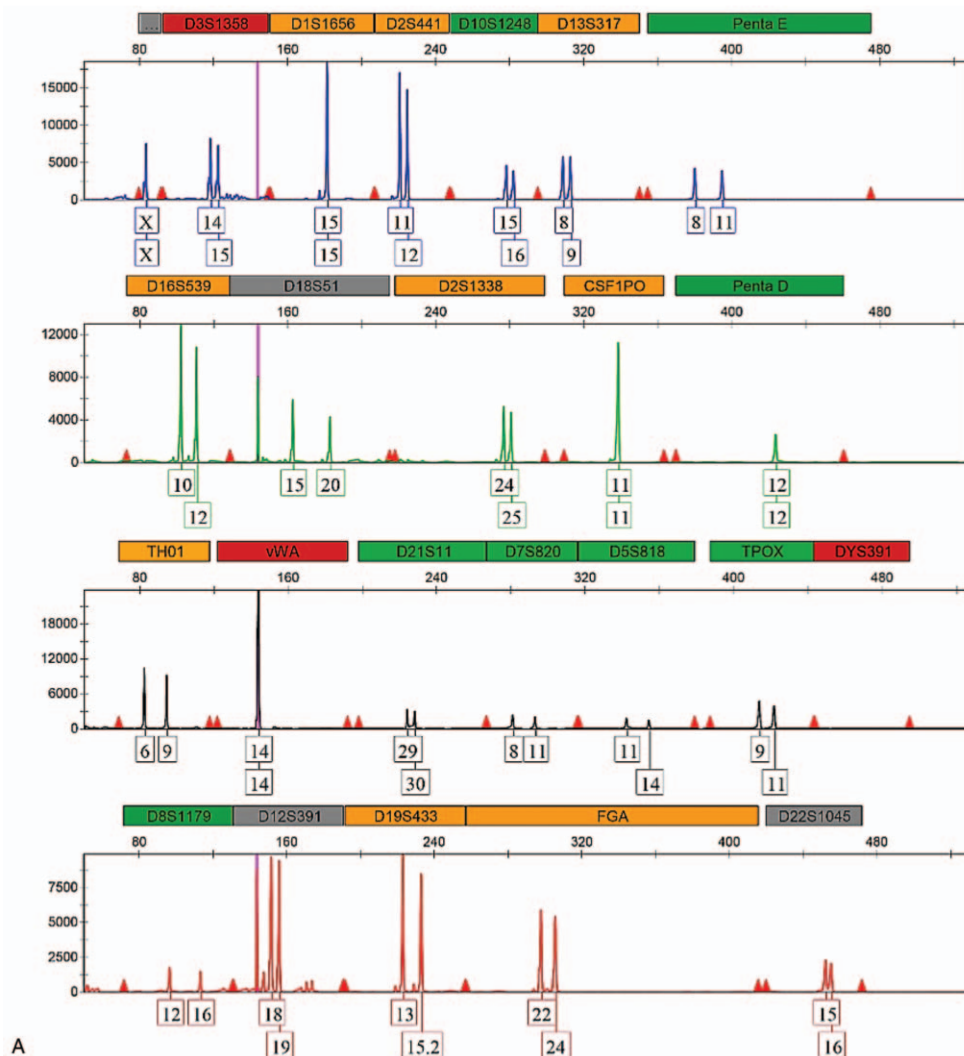

Figure 4. Alignment of genotyping between the fetus and his mother. Notes: (A) Pregnant woman's DNA typing (II3). (B) Fetal DNA typing (III2). The labels such as D3S1358, D1S1656, D2S441, etc in different colors are different gene loci and the corresponding numbers indicate the typing of different gene loci.

repetition, deletion of the whole gene,[7] and special synonymous mutation.[8] Up to November 2018, the *F9* gene mutation database (EAHD Coagulation Factor Variant Databases) has recorded a total of 1094 mutations in 3713 HB patients (http://www.factorix.org/statistics.html.php), point mutation accounts for 73.1% and the mutations occurring in serine protease domain (SPD) accounts for about 56.1%. In this study, the novel c.1232G>C mutation in *F9* gene is also a point mutation occurring in the SPD. The mRNA length of *F9* gene is 2804 bp containing 8 exons and encoding 461 amino acids. At present, Multiplex ligation dependent probe amplification (MLPA), Sanger sequencing and high throughput sequencing techniques have been used in gene test for HB[9–11] and they have their own advantages. MLPA can detect large deletion or duplication mutations in *F9* gene, but cannot find point mutations with high mutation rate. However, the second generation sequencing requires high-level hardware and software in laboratories and its subsequent data analysis is more complex, so it is not suitable for wide application in clinical practice. Sanger sequencing requires relatively simple conditions for clinical laboratory, can detect all exon sequences and flanking sequences of *F9* gene to find mutation site. Combined with *F9* gene mutation database (http://www.hgmd.cf.ac.uk/ac/index.php, https://www.ncbi.nlm.nih.gov/clinvar/, and http://www.eahad-db.org/search.html.php) and mutation pathogenicity prediction database (http://genetics.bwh.harvard.edu/pph2 and http://mutpred.mutdb.org), the analysis results of gene tests could be obtained, providing a reliable basis for clinical gene diagnosis and genetic counseling. Sanger sequencing is suitable for HB gene test in most clinical laboratories. Therefore, in clinical gene diagnosis and prenatal diagnosis, different clinical laboratories should select suitable detection methods based on their various conditions, and it is not necessary to choose the most advanced detection method just for the sake of utilizing advanced technology. Thus, we suggest that suitable methods be selected to make gene and prenatal diagnoses more effective, accurate and economic.

In this study, Sander sequencing indicated that c.1232G>C [p.Ser411Thr] mutation was detected in *F9* gene of the patient (III1), c.1232G>C heterozygous mutation was also found in the patient's mother (II3) and grandmother (I2); but no mutations

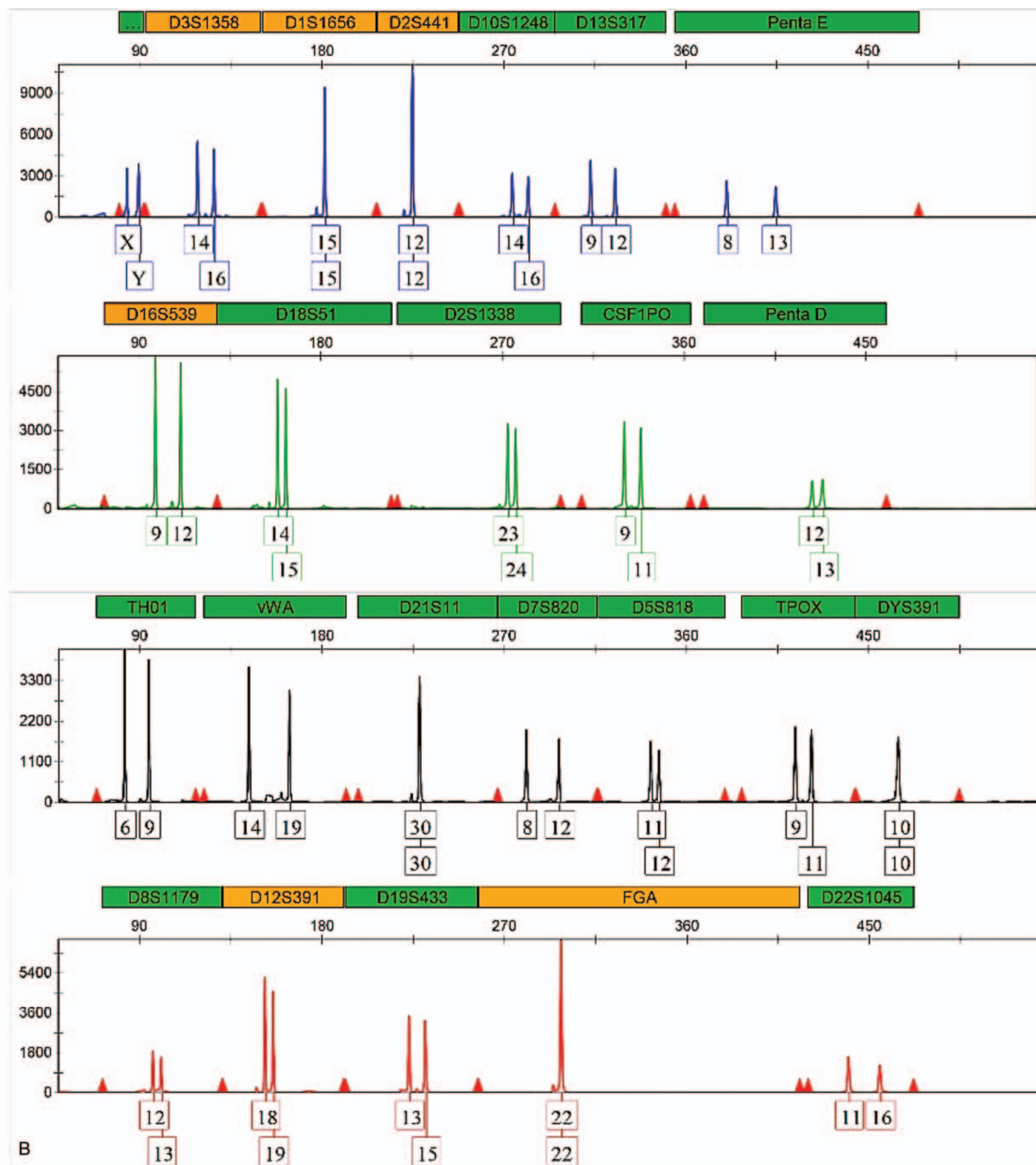

Figure 4. (Continued).

were found in the patient's uncle (II4), aunt (II5), grandfather (I1), and father (II2). Until now, the mutations occurring at 411th codon in *F9* gene have been reported, such as c.1232G>A,[12] c.1232G>T,[13] and c.1233T>A.[14] However, the c.1232G>C mutation detected in this study has not been reported.[8–23] To ascertain whether this mutation is a pathogenic mutation in this HB family, we carried out the protein phenotypic analysis of this mutation. The factor IX protein contains seven regions including signal peptide, prepeptide, Gla domain, epidermal growth factor-like domains (EGF1 and EGF2), activated peptide and SPD.[24] Mature factor IX should be cut off signal peptide and prepeptide to form a specific spatial structure which is released to blood for participating in the process of endogenous coagulation. The p. Ser411Thr mutation changes the 411th amino acid codon in *F9* gene from serine codon AGT to threonine codon ACT, which may affect the spatial structure of factor IX, retarding complex formation of factors VIII and IX. And the 411th serine of factor IX is a fundamental component of the active region of serine protease, which plays an important role in activating coagulation factor X; so this mutation directly affects the protease activity. At the same time, we used PolyPhen-2 to predict the pathogenicity of this mutation and found that this mutation is a pathogenic mutation (PROBABLY DAMAGING SCORE = 1.000) (Fig. 3A). And we did not find this mutation in *F9* gene of 100 healthy

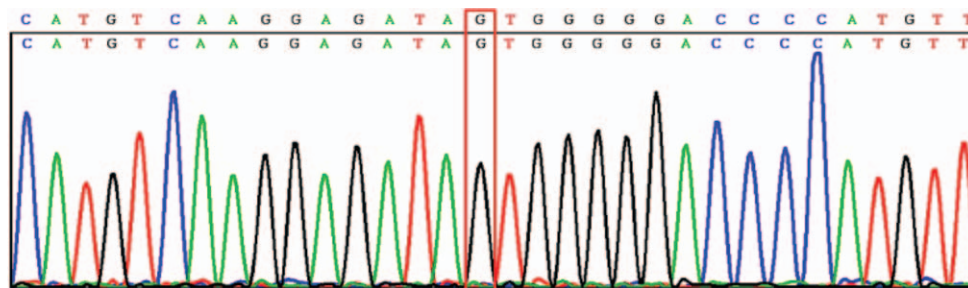

Figure 5. Sanger sequencing in the fetal sample. Note: There is no mutation at the c.1232 locus in the fetal *F9* gene (Wild type).

individuals (Fig. 2A). According to the latest standards and guidelines for the interpretation of clinical significance of ACMG sequence variation, the c. 1232G > C (p.Ser411Thr) in *F9* gene was in line with the interpretation rule of pathogenic mutation [PS1+PM1+PM5+(PP1-PP4)], namely that the new mutation causing the change in amino acids and occurring at the known pathogenic mutational and highly-conserved site, was predicted to be a pathogenic mutation by PolyPhen-2. Therefore, we think that the c.1232G > C (p.Ser411Thr) in *F9* gene is a pathogenic mutation of HB, which leads to HB in this family.

After identifying the pathogenic mutation in *F9* gene, prenatal diagnosis was performed on the pregnant women (II3) with 19-week gestation. In prenatal diagnosis, we used the PowerPlex 21 HS (Promega, Fitchburg, WI) system to detect the maternal component in the amniotic fluid sample to avoid the influence of maternal contamination on the subsequent test results,[25] and ensure the accuracy of the subsequent test results. Prenatal diagnosis showed positive *SRY* in the amniotic fluid and no mutations in *F9* gene, suggesting that the fetus was male without HB. Subsequently, we followed up the pregnant women. The pregnant women gave birth to a male baby with Apgar score of 10 at $39W^{+4}$. Until November 1, 2018, the infant had been 3 years and 10 months old and the infant was normal during follow-up, which was consistent with the results of prenatal diagnosis. We will continue to follow up the baby until his adulthood.

This study had a limitation. The c.1232G > C (p.Ser411Thr) in *F9* gene was regarded as a pathogenic mutation according to the latest standards and guidelines for the interpretation of clinical significance of ACMG sequence variation. Therefore, this conclusion remains to be further confirmed by more reports about this mutation.

In this study, we carried out gene and prenatal diagnosis for the family with a novel c.1232G>C (p.Ser411Thr) mutation in *F9* gene, and provided reliable genetic counseling for the family. At the same time, we report the c.1232G>C (p.Ser411Thr) mutation in *F9* gene, which provides useful information for *F9* gene mutation database and a theoretical basis for HB gene therapy.

Author contributions

Conceptualization: Zhi-ping Guo.
Data curation: Hao Li, Hong-yan Liu, Zhen Wang.
Writing – original draft: Xue Lv.
Writing – review & editing: Tao Li.

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