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Case Report

Noninvasive prenatal diagnosis for X-linked disease by maternal plasma sequencing in a family of Hemophilia B

Ping Hu [a, 1], Fengchang Qiao [a, 1], Yuan Yuan [b, d, e, 1], Ruihong Sun [c], Yan Wang [a], Lulu Meng [a], Ying Lin [a], Hang Li [a], Yaoshen Wang [b], Rui Han [b, d, e], Dong Liang [a], Dingyuan Ma [a], Tao Jiang [a], Hui Jiang [b, d, e], Zhengfeng Xu [a, *]

[a] State Key Laboratory of Reproductive Medicine, Department of Prenatal Diagnosis, Obstetrics and Gynecology Hospital Affiliated to Nanjing Medical University, Nanjing, China
[b] BGI-Shenzhen, Shenzhen, China
[c] Department of Laboratory Medicine, the First Affiliated Hospital of Nanjing Medical University, Nanjing, China
[d] Tianjin Translational Genomics Center, BGI-Tianjin, BGI-Shenzhen, Tianjin, China
[e] Binhai Genomics Institute, BGI-Tianjin, BGI-Shenzhen, Tianjin, China

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ABSTRACT

Objective: To apply a Hidden Markov Model to test Hemophilia B in a fetus by maternal plasma sequencing only employing proband and maternal haplotypes.
Case Report: A family at risk for Hemophilia B was recruited in this study. We performed genetic diagnosis on the proband using our targeted capture system (containing *F9* gene coding region, highly heterozygous SNPs and a 13-kb chromosome Y specific region), and revealed a causative *F9* gene mutation (c.190T>C, p.Cys64Arg). Maternal plasma cell-free DNA obtained at 8 weeks of gestation was targeted-captured and sequenced using the customized system. The fetus inherited the *F9* (c.190T>C, p.Cys64Arg) mutation according to the Hidden Markov Model. The mother continued the pregnancy.
Conclusions: This study is the first report of a haplotype-based approach in NIPD of Hemophilia B. With further evaluation, this method might be useful for NIPD of Hemophilia B and for other X-linked single-gene disorders.

Introduction

Hemophilia B is a genetic bleeding disorder with X-linked inheritance, characterized by spontaneous or provoked, often uncontrolled, bleeding into joints, muscles and other soft tissues. The disorder is caused by an anomaly of the gene responsible for the synthesis of factor IX. It affects 1:25,000 male births [1] and is characterized by varying levels of impaired blood coagulation.

Current methods of Hemophilia B treatment are expensive, challenging, and involve the regular administration of clotting factors. Prenatal diagnosis for families with Hemophilia B is an important, valuable, and effective prevention measure, because male patients will pass on the *F9* mutation to all of their daughters, and female carriers have a 50% chance to transmit the *F9* mutation to their offspring. However, conventional prenatal diagnosis of hemophilia by directly analyzing fetal DNA samples through chorionic villus sampling or amniocentesis, has a 0.5–1% risk of loss of pregnancy [1], and cannot be offered to women who decline or have a contraindication for invasive procedures.

Recently, the discovery of cell-free fetal DNA (cff-DNA) in the maternal blood circulation has opened new possibilities for the noninvasive-prenatal diagnosis of fetal genetic diseases *in utero* [2]. Notably, maternal plasma DNA sequencing using massively parallel sequencing (MPS) is feasible for the noninvasive prenatal detection of fetal common chromosome aneuploidy because it has a high detection sensitivity and specificity [3]. Noninvasive prenatal diagnosis only requires 5 ml of peripheral blood and does not require amniotic fluid puncture, which may result in fetus loss. This method can be applied to nearly all pregnant women. However, few studies have reported the use of MPS in the field of noninvasive prenatal detection of monogenic diseases to date. Thus, it is

* Corresponding author. Tianfei Street 123#, Nanjing, 210004, Jiangsu, China. Fax: +86 025 52226308.
E-mail address: zhengfeng_xu_nj@163.com (Z. Xu).
[1] Equal contribution.

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important to develop a noninvasive diagnostic method that can be used to identify a hemophilic fetus at early gestational stages.

Here, we report the noninvasive prenatal diagnosis of Hemophilia B based on the MPS of maternal plasma obtained as early as 8 gestational weeks. We used a haplotype-assisted analysis method instead of direct sequencing of the mutation site. The fetus' genotype was successfully determined by NIPD and confirmed by the Sanger sequencing of amniotic fluid cells.

Case presentation

A 29-year old woman (II2) came to our center for genetic counseling because her brother, the proband (II4) (Fig. 1), was affected by Hemophilia B.

Genetic test was performed on the proband using our targeted sequencing, and a missense mutation *F9*: NM_000133.3:exon2: c.190T>C was identified. Sanger sequencing confirmed the mutation in the proband, the grandmother, and the woman's sister. All these women were carriers with normal clotting FIX levels (Fig. 2). This mutation, which is recorded in the HGMD (accession number: CM940422), causes a Cys to Arg transition in the important glutamic acid-rich γ-carboxyglutamic acid (Gla) domain and prevents the correct folding and calcium binding of factor IX. It is a rare mutation with no frequency data in dbSNP, but is predicted to be detrimental by SIFT and PolyPhen2. Enayat MS and Ludwig M reported that patients with the *F9* gene mutation c.190T>C were affected by severe Hemophilia B [4,5].

The woman returned to our center at 8 gestational weeks. This study was designed and performed in two parts (Fig. 1). Custom-designed NimbleGenSeqCap EZ probes were used in this study. The target region was 120 kb, containing the coding region of *F9*, a 10 bp flanking region, and 1037 high heterozygous SNPs (single-nucleotide polymorphisms) distributed within the region 1 Mb upstream and downstream of the *F9* gene (Fig. 3). To determine the fetal gender, a 13 kb chromosome Y specific sequence was also captured.

A Hidden Markov Model (HMM) was established with the grandmother, proband, and maternal haplotypes and maternal plasma sequencing data was used to deduce which haplotype was inherited by the fetus (Fig. 4A). The process of fetus haplotype deduction was similar to that reported previously [2,8,10]. A Hidden Markov Model was established to deduce which haplotype was inherited by the fetus. The hidden state indicates whether the haplotype transmitted to the fetus is the same as in the proband, and is denoted as $Q = \{X1_{GM\text{-}M}, X3_{GF}\}$. The observed state is the allele ratio in each SNP in plasma data, and is denoted as $S = \{Ni\}$ $(i = 1, 2, 3,\ldots,n)$. The initial state distribution is defined as $\pi = \{1/2, 1/2\}$. The emission probability matrix is $B = \{bi,j\}$, $bi,j = P\{Xi|Nj\}$, $Xi = \{X1_{GM\text{-}M}, X3_{GF}\}$, $j = 1, 2, 3,\ldots,n.$, where the emission probabilities $P\{X1_{GM\text{-}M}|Ni\}$ and $P\{X3_{GF}|Ni\}$ follow binomial distribution and are calculated for each phased SNP as illustrated in Table 1. The transition probabilities matrix is denoted as $A = \{a\{i\}\}$, where $a\{i\} = \begin{pmatrix} 1-P & P \\ P & 1-P \end{pmatrix}$, $P = Pcomb\{i,i+1\}$, and Pcomb is the

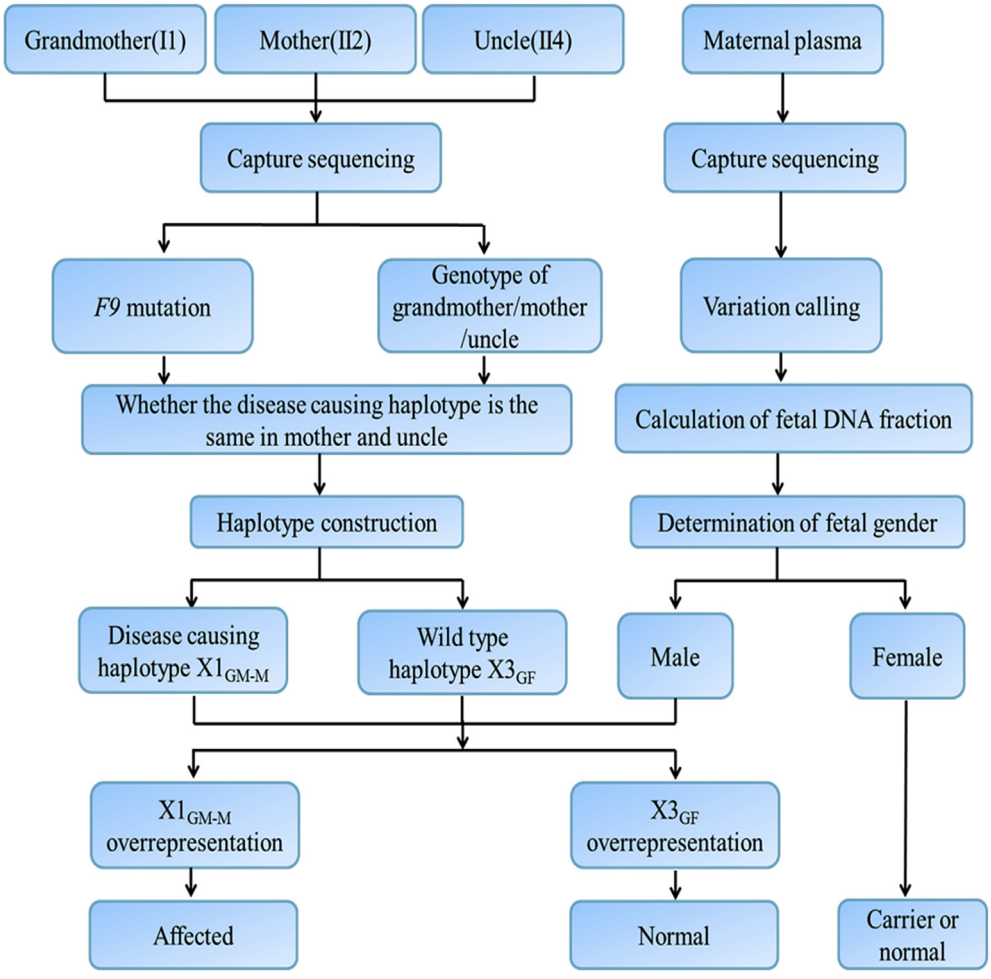

Fig. 1. Workflow of the noninvasive prenatal diagnosis of the fetal *F9* genotype.

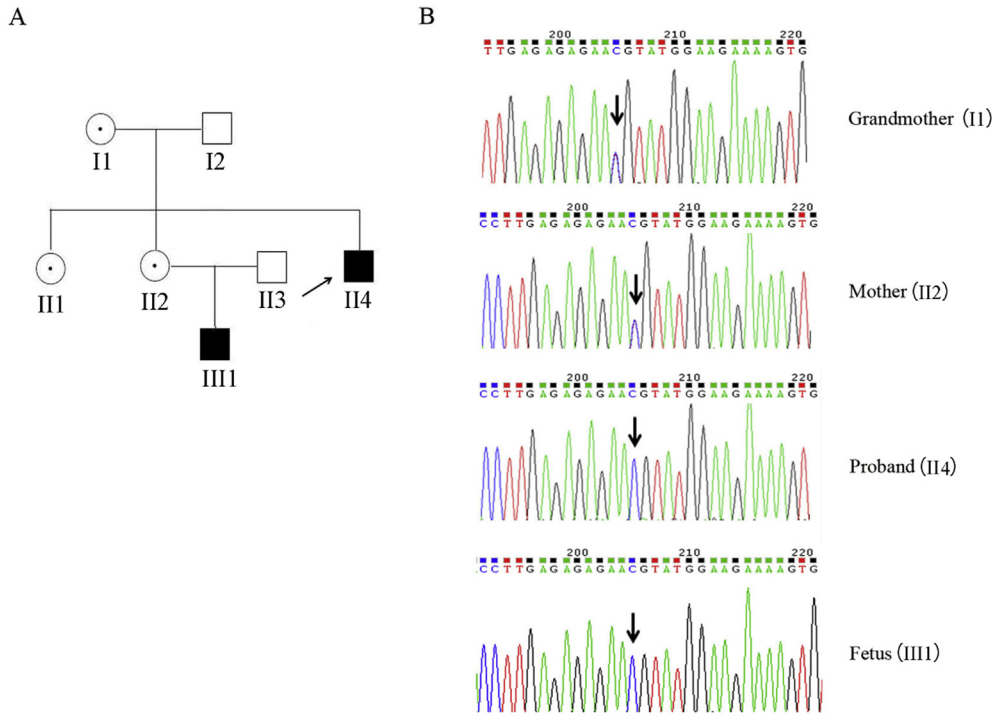

Fig. 2. Results of clinical tests for the causative mutation in this family. A. The pedigree of *F9* mutation in the family. B. Electropherogram of a segment of the *F9* gene showing the mutation c.190T>C in the family member.

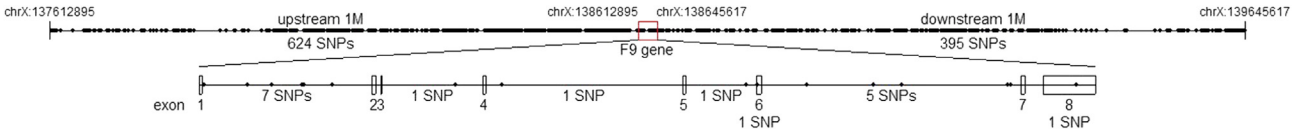

Fig. 3. Target regions surrounding the *F9* gene for enrichment and haplotyping.

probability of recombination between two neighboring SNPs calculated by the genetic distance obtained from Hapmap 3. Finally, the Viterbi algorithm was used to find the most likely path through the observed data and to deduce the inherited maternal haplotype in the fetus. The result of NIPD for the fetus is shown in Fig. 4B. The fetus was a male, who was identified as a hemizygote carrying the disease-causing mutation and was affected by Hemophilia B. Eight months after the baby was born, Sanger sequencing on peripheral blood cell DNA confirmed the newborn had inherited the missense mutation (c.190T>C) in the *F9* gene, as seen in the proband (Fig. 2). Preimplantation genetic diagnosis and prenatal diagnosis is strongly recommended for the couple's next pregnancy.

Discussion

New technologies, such as digital PCR and next-generation sequencing, have greatly accelerated the development of NIPD for clinical applications, including fetal gender estimation and RhD status determination [6,7]. However, most studies have been restricted to the identification of paternal-specific sequences. The ability to non-invasively predict whether the genetic variants have been passed from the mother to the fetus remains challenging, mainly because of the high background of maternal DNA in the plasma mixture. Few studies on the noninvasive diagnosis of single-gene disorders have been reported [8–16]. In this study, we reported a noninvasive prenatal diagnosis of Hemophilia B, a classical X-linked disease, and highlighted the feasibility and importance of haplotype-assisted NIPD in the prevention of Hemophilia B.

Non-invasive prenatal diagnosis of hemophilia by the direct mutation analysis of plasma cff-DNA from a pregnant woman was previously reported [17]. In the direct mutation analysis strategy, the detecting probe must be individually designed and pretested before use according to the specific mutation sites and types, which are infeasible for clinical practice as they delay the turnaround time. Here, we used an indirect strategy via the haplotype construction of the *F9* gene. Using our customized targeted sequencing of genomic DNA of three family members and plasma DNA from the pregnant woman, we obtained the haplotype surrounding the *F9* gene of the fetus. Of note, this indirect strategy is a universal method for the noninvasive prenatal analysis of *F9* gene defects in different mutation sites and types. In addition, our method is powerful and accurate, because it is based on information from hundreds of informative SNPs. Taken together, our method, theoretically, is suitable for all types of *F9* gene defects, expanding its scope of application.

In the previous study, genomic information from four grandparent samples and a complicated experimental procedure were required. However, in most cases, grandparent information and samples were not available, which increased difficulties in the disease diagnosis. In our study, we deduced the fetal haplotype employing the HMM model, which has high accuracy and was

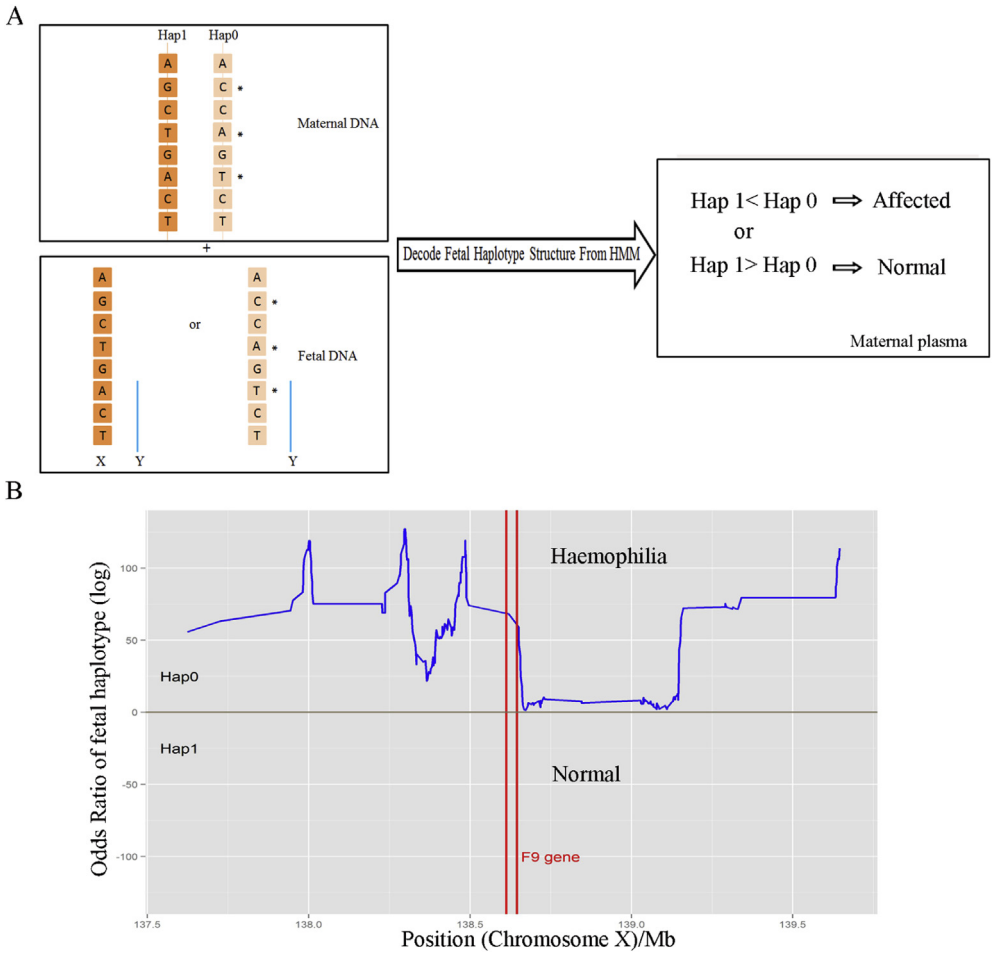

Fig. 4. Noninvasive prenatal diagnosis (NIPD) using maternal plasma DNA by massively parallel sequencing. A. a haplotype-based approach for noninvasive prenatal testing. (*, disease causing haplotype). B. The x-axis and y-axis indicate the target region surrounding the *F9* gene and the odds ratio of the fetal inherited haplotype in the maternal plasma, respectively. An odds ratio less than zero indicates that the fetus has inherited the maternal wild-type haplotype, whereas a value of greater than zero indicates inheritance of the mutant haplotype.

Table 1
Target region (*F9*) sequencing data production.

Sample	Sample ID	Reads mapped to target region (M)	Mean depth of target region in F9 ± 1M	Fraction of target covered ≥20X	ChrY depth (deduplication)	ChrY coverage (deduplication)
Mother	14D1008115	0.48	220.26	98.93%	0	0
Grandma	14D1008117	0.64	295.57	99.00%	0	0
Uncle	14D1008118	0.23	108.98	98.48%	186.04	100%
Fetus	14D1008119	0.33	153.64	98.81%	255.48	100%
Plasma	14P1008115-1	1.09	422.29	99.01%	13.34	100%

effective in CAH [8], maple syrup urine disease [9], DMD [10] and congenital deafness [11]. The strategy in this study used only genomic information from the mother, the proband and maternal plasma, which is feasible for clinical applications. Meantime, employing this strategy could reduce the cost and time for diagnosis. Thus, we believe that this method might have broader applications for all autosomal and X-linked genetic diseases with different mutant types in the future.

In this study, the plasma was obtained at 8 weeks of gestation, and the turnaround time of NIPD was 4 weeks. The NIPD result was obtained at 12 weeks, which is earlier than the gestation stage for amniocentesis. Furthermore, NIPD for Hemophilia B has several advantages for the pregnant woman and family: make earlier decisions, safer for women's health, greater privacy, less emotional distress, and etc.

In conclusion, this is the first report of the noninvasive diagnosis of Hemophilia B using targeted MPS for cell-free DNA in maternal plasma. We also demonstrated that the haplotype can be deduced by collecting genetic information from other family members, providing a one-step solution for NIPD of a single gene disorder, which might also be extended to other X-linked single gene diseases. We successfully diagnosed Hemophilia B in one family; however, large-scale prospective studies are needed to determine the sensitivity and specificity of the method. With acceptable cost and reasonable turnaround time, families may be more willing to undergo noninvasive prenatal diagnosis and

prenatal treatment for affected fetuses with appropriate genetic counseling.

Conflicts of interest

The authors have no conflicts of interest relevant to this article.

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