Clinical Case Report

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Prenatal diagnosis for a Chinese family with a de novo *DMD* gene mutation

A case report

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Abstract

Background: Patients with Duchenne muscular dystrophy (DMD) usually have severe and fatal symptoms. At present, there is no effective treatment for DMD, thus it is very important to avoid the birth of children with DMD by effective prenatal diagnosis. We identified a de novo *DMD* gene mutation in a Chinese family, and make a prenatal diagnosis.

Methods: First, multiplex ligation-dependent probe amplification (MLPA) was applied to analyze *DMD* gene exon deletion/duplication in all family members. The coding sequences of 79 exons in *DMD* gene were analyzed by Sanger sequencing in the patient; and then according to *DMD* gene exon mutation in the patient, *DMD* gene sequencing was performed in the family members. On the basis of results above, the pathogenic mutation in *DMD* gene was identified.

Results: MLPA showed no *DMD* gene exon deletion/duplication in all family members. Sanger sequencing revealed c.2767_2767delT [p.Ser923LeufsX26] mutation in *DMD* gene of the patient. Heterozygous deletion mutation (T/-) at this locus was observed in the pregnant woman and her mother and younger sister. The analyses of amniotic fluid samples indicated negative Y chromosome sex-determining gene, no *DMD* gene exon deletion/duplication, no mutations at c.2767 locus, and the inherited maternal X chromosome different from that of the patient.

Conclusion: The pathogenic mutation in *DMD* gene, c.2767_2767delT [p.Ser923LeufsX26], identified in this family is a de novo mutation. On the basis of specific conditions, it is necessary to select suitable methods to make prenatal diagnosis more effective, accurate, and economic.

Abbreviations: ABD = actin-binding domain, CK = creatine kinase, MLPA = multiplex ligation-dependent probe amplification, NGS = next-generation sequencing, SRY = sex-determining region on Y, STR = short tandem repeat.

Keywords: de novo mutation, *DMD* gene, prenatal diagnosis, pseudohypertrophic muscular dystrophy, Sanger sequencing

1. Introduction

Pseudohypertrophic muscular dystrophy, a severe X-linked recessive hereditary disorder mainly affecting the male, includes Duchenne muscular dystrophy (DMD, OMIM #310200) and Becker muscular dystrophy (BMD, OMIM #300376) with the incidences of 1 in 3600 and 1 in 185,181, respectively, in live baby boys.[1] BMD is featured by mild symptoms and long survival time that is close to the normal human lifespan in most patients. However, DMD patients usually have severe and fatal symptoms mainly including progressive muscular atrophy and myasthenia complicated with gastrocnemius muscle pseudohypertrophy. DMD onset usually occurs between 3 and 5 years old, followed by loss of standing and walking ability before the age of 12 years and death of heart failure or respiratory failure before the age of 20 years.[2] DMD severely affects young men's health and brings heavy mental and economic burdens to both the family and society. At present, there is no effective treatment for DMD, thus it is very important to avoid the birth of children with DMD by effective prenatal diagnosis.

The pathogenic gene of *DMD*, the dystrophin gene located in X p21.2, is one of the largest human genes with a total length of 2.4 MB containing 79 exons. In clinical practice, the short tandem repeat (*STR*) gene linkage analysis is commonly used to

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determine the chromosome X with disease risk, multiplex ligation-dependent probe amplification (MLPA) is applied for *DMD* gene exon deletion/duplication detection, and Sanger sequencing and next-generation sequencing (NGS) are used to detect *DMD* gene exon point mutations, but each method has its own advantages and shortcomings. In this study, we carried out gene diagnosis for the Chinese family with de novo *DMD* gene mutation and the prenatal diagnosis for the patient's mother using MLPA technology, Sanger sequencing, and *STR* gene linkage analysis.

2. Subjects and methods

All study methods were approved by the Ethics Committee of Henan Provincial Peoples Hospital. All the subjects enrolled into the study gave written informed consent to participate.

2.1. Subjects

The Chinese family with familial DMD history visited the Prenatal Diagnosis Center of Henan Province for *DMD* gene diagnosis and prenatal diagnosis. Two elder brothers of the pregnant woman died at the ages of 16 and 18 years, respectively. The 5-year-old boy (III1) was diagnosed with DMD with creatine kinase (CK) of 16,230 U/L and myogenic damage showed by electromyogram, but muscle biopsy was not performed in the boy with DMD due to family refusal. The 31-year-old woman (II4) with mid-pregnancy is the mother of the boy with DMD (III1). The genealogical tree is shown in Fig. 1.

2.2. Specimen collection and DNA extraction

Peripheral blood (3–5 mL) was collected and mixed with EDTA-K2 for anticoagulation in family members. Amniotic membrane puncture was performed to obtain fetal exfoliated cells from the pregnant woman under ultrasound guidance. Meanwhile, the peripheral blood samples (3 to 5 mL) were also collected and mixed with EDTA-K2 for anticoagulation during physical examination of new staffs of our hospital including 50 males and 50 females who had no stories of DMD and neuromuscular disease, and no blood relationship with this DMD family. These new staffs gave consent to participate in this study. 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, Madison) followed by storage at −20°C.

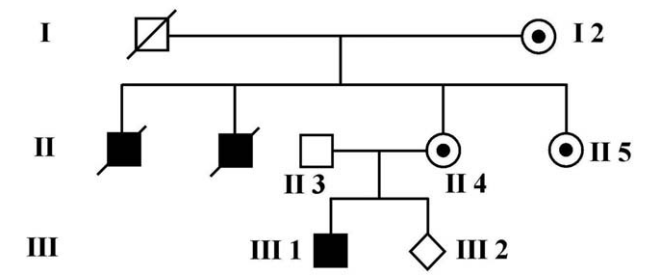

Figure 1. Genealogical tree of the DMD family. DMD = Duchenne muscular dystrophy; : Dead normal male; : Female DMD carrier; : Dead male DMD patient; : Normal male; : Male DMD patient and : Fetus.

2.3. Maternal cell contamination detection in amniotic fluid

To make sure the accuracy of results, DNA samples from the pregnant woman and its amniotic fluid were amplified using PowerPlex 21 HS genotyping system (Promega, Madison) according to the manufacturer's instructions to analyze if there was the maternal cell contamination in amniotic fluid. Amplification was performed in a volume of 25 μL including 5 μL of PowerPlex 21 5X Master Mix, 5 μL of PowerPlex 21 5X Primer Pair Mix, 5 ng of DNA and H_2O. The cycling conditions were as follows: 96°C for 1 minute, 94°C for 10 seconds, 59°C for 1 minute, 72°C for 30 seconds, 30 cycles; 60°C for 10 minutes.

2.4. 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 (*SRY*) gene was performed using 5′-GAATATTCCCGCTCTCCGGA-3′ and 5′-GCTGGTGCTC-CATTCTTG AG-3′ primers in triplicate.[3]

2.5. MLPA

MLPA kits, SALSA P034-B1 and P035-B1 (MRC-Holland, Netherlands), were used to detect the deletion/duplication mutations in 79 exons of *DMD* gene in the patient (III1), the pregnant woman (II4) and her mother (I2) and younger sister (II5), and the amniotic fluid sample (III2), respectively, according to the instructions of MLPA kits.

2.6. Sanger sequencing

Primers for 79 DMD exons were designed according to the *DMD* gene sequence, and detailed information about the sequences and primers could be found from www.dmd.nl and previous publications.[4] PCR amplification products were sequenced by Songon Biotech Co. Ltd (Shanghai, China) to analyze the patient's (III1) *DMD* gene exon mutations. *DMD* genes of family members and the amniotic fluid were sequenced according to the patient's *DMD* gene exon mutation. Also, to exclude the possibility of gene polymorphism, the sequences at the mutation locus in *DMD* genes from 50 normal males and 50 normal females without blood relationship with the patient were also detected.

2.7. STR gene linkage analysis

The 5′-STR, 3′-STR, 45- STR, 49-STR, and 50- STR primers of *DMD* gene were designed.[3] Linkage analysis was performed in the family members, including the patient's father (II3), the pregnant woman (II4), the patient (III1), and the amniotic fluid sample (III2). Prenatal diagnosis was based on the results of linkage analysis and Sanger sequencing.

3. Results

3.1. Results detected by PowerPlex 21 HS system

PowerPlex 21 HS system indicated that in the amniotic fluid, no maternal DNA typing was found, sex chromosomes were X,X, demonstrating that the amniotic fluid had no maternal contamination and might be used in subsequent texts (Fig. 2).

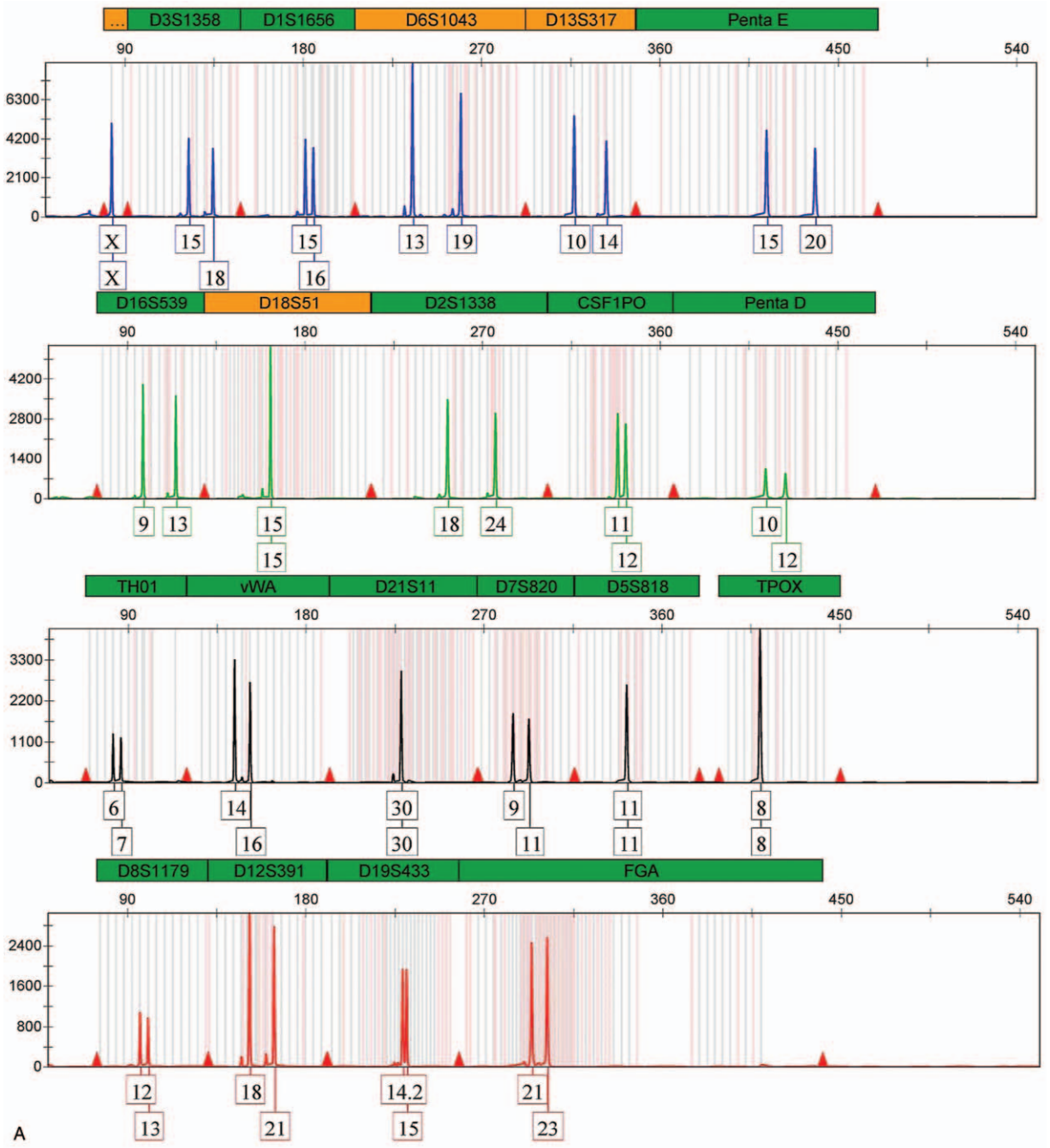

Figure 2. Detection of maternal components in the amniotic fluid using PowerPlex 21 HS system. (A) Pregnant woman's DNA typing; (B) Fetal DNA typing. In the amniotic fluid, no maternal DNA typing is found, demonstrating that the amniotic fluid has no maternal contamination.

3.2. SRY gene

Agarose electrophoresis indicated that SRY band failed to be amplified, suggesting that *SRY* gene was negative in the amniotic fluid sample.

3.3. MLPA results

MLPA (P034-B1, P035-B1) showed that the ratios of 79 exons of *DMD* gene all were about 1 in the patient (III1), suggesting that there were no heterozygous deletion or/and duplication mutations in the 79 exons of *DMD* gene in the patient (III1). As the same, MLPA (P034-B1, P035-B1) also showed no heterozygous deletion or/and duplication mutations in the 79 exons of *DMD* gene in the family members including pregnant woman (II4), and her mother (I2) and younger sister (II5), and the amniotic fluid sample (III2) (Fig. 3). This suggests that *DMD* gene mutation was not deletion or/and duplication mutations in this family.

3.4. Sanger sequencing analysis results

One mutation c.2767_2767delT [p.Ser923LeufsX26] was detected in *DMD* gene of the patient (III1). This mutation induced *DMD* gene frame shift mutation, which led to early termination of *DMD* gene translation because there was a

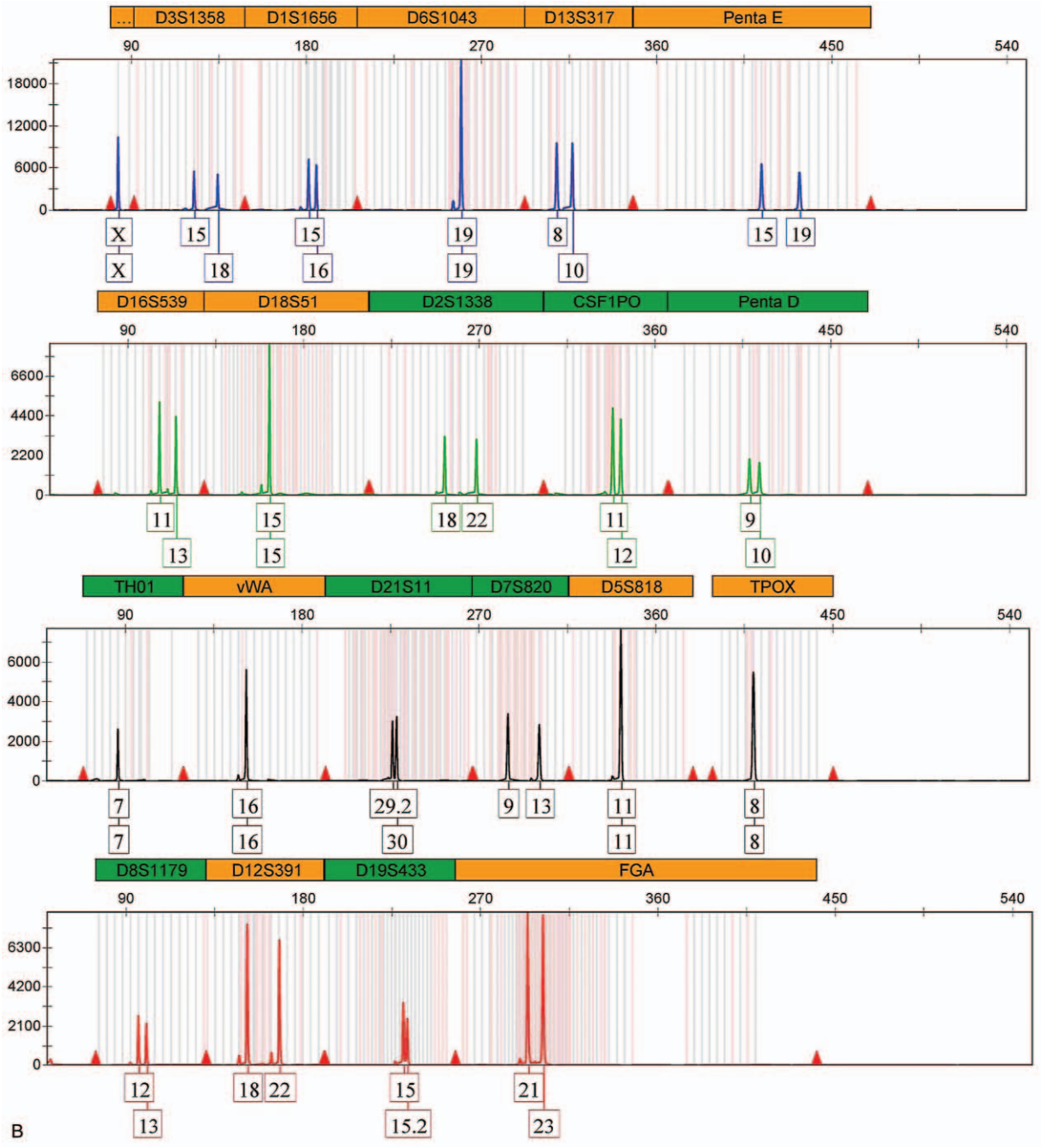

Figure 2. Continued

termination codon TGA at the 26th codon after the mutational site. Heterozygous deletion mutation (T/−) at this locus was observed in the pregnant woman and her mother and younger sister; but in the amniotic fluid sample and pregnant woman's husband (II3), no mutations (T/T) at this locus were found (Fig. 4). The mutations at the c.2767 locus in *DMD* gene from 50 normal males and 50 normal females without blood relationship with the patient were also not detected (T/T) (Fig. 5).

3.5. STR gene linkage analysis

Linkage analysis was performed in the family, including the patient's father (II3), the pregnant woman (II4), the patient (III1), and the amniotic fluid sample (III2), and the loci, 49-STR, and 50-STR, showed that the inherited maternal X chromosome in the amniotic fluid (III2) was different from that of the patient with DMD (III1) (Fig. 6), which was consistent with sequencing results. On the basis of the results above, we concluded that the fetus was female and had no DMD.

4. Discussion

DMD is the most common X-linked recessive life-threatening hereditary disease, and its pathogenic gene is *DMD* gene. The *DMD* gene was first cloned in 1987[5] and encodes cytoskeletal proteins that play a role in maintaining the stability of

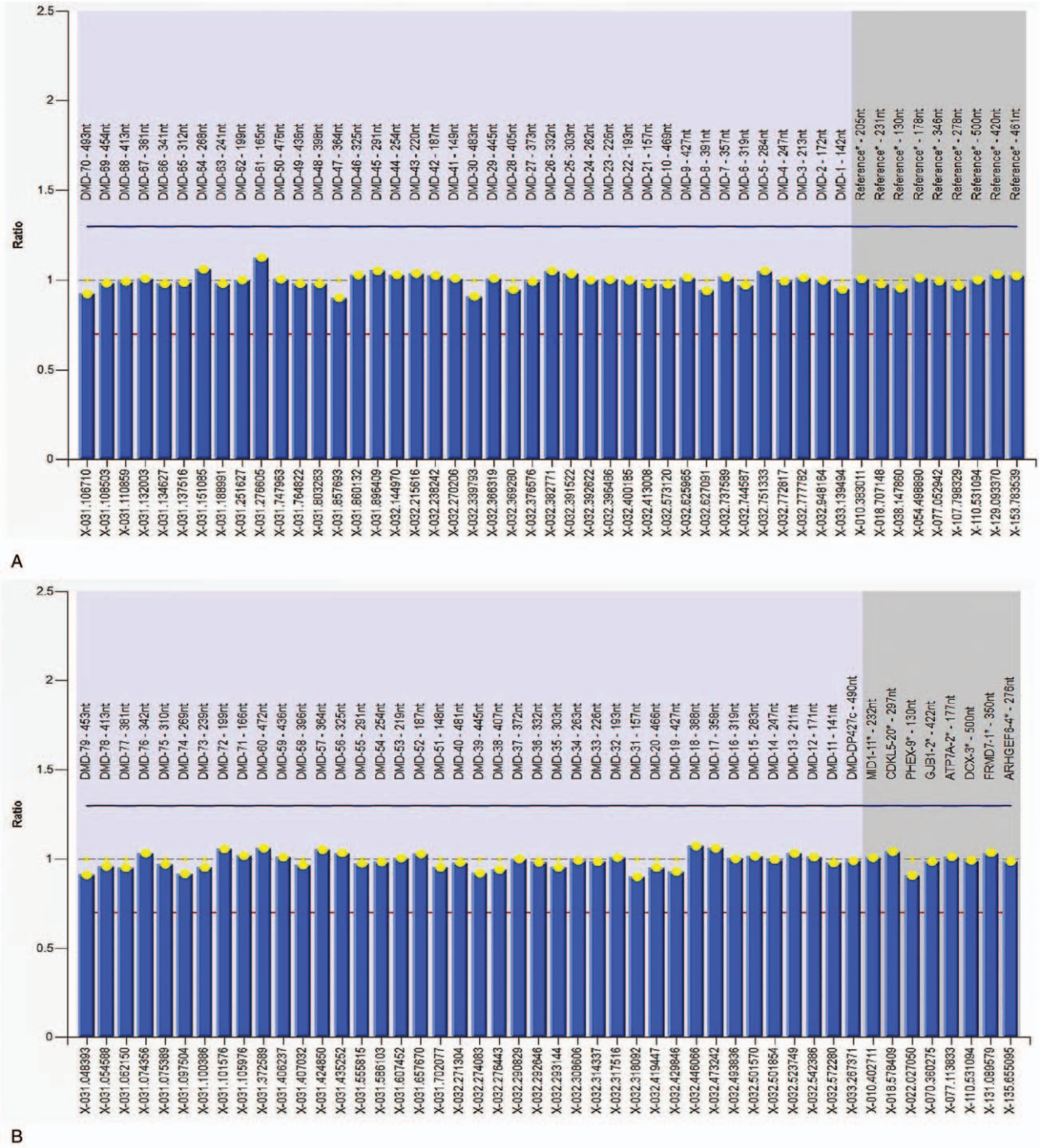

Figure 3. Detection of deletion/duplication mutations in *DMD* gene exons by MLPA. (A) Patient (MLPA SALSA P-034); (B) Patient (MLPA SALSA P-035); (C) Amniotic fluid sample (MLPA SALSA P-034); (D) Amniotic fluid sample (MLPA SALSA P-035). Note: DMD = Duchenne muscular dystrophy; MLPA = multiplex ligation-dependent probe amplification. MLPA shows that the ratios of 79 exons of *DMD* gene all are about 1 in the patient (III1) and the fetus (III2), suggesting that there were no heterozygous deletion or/and duplication mutations in the 79 exons of *DMD* gene in the patient (III1) and the fetus (III2).

cytoskeleton and muscle fiber. Current researches revealed that 60% to 65% DMD cases were caused by large fragment deletion in *DMD* gene, and other pathogenic mutations include duplication (5–10%) and point mutation (25–30%).[6,7]

The most common methods for clinical *DMD* gene analysis include multiplex PCR method and MLPA method, which can effectively detect deletion/duplication mutations in *DMD* gene exons, but cannot found point mutations in all exon regions. Thus, multiplex PCR and MLPA methods are not applicable for gene testing in the DMD family without deletion/duplication mutation in *DMD* gene exon regions and cannot provide necessary clinical genetic counseling. *STR*-gene linkage analysis can determine the source of X chromosomes with disease risk, but it is likely to produce wrong conclusion while not knowing that the pregnant woman is DMD carrier or not in sporadic families or families with genital mosaicism.[8] Sanger sequencing can be used for gene testing in the family with *DMD* gene point mutations and provide reliable diagnostic basis for clinical

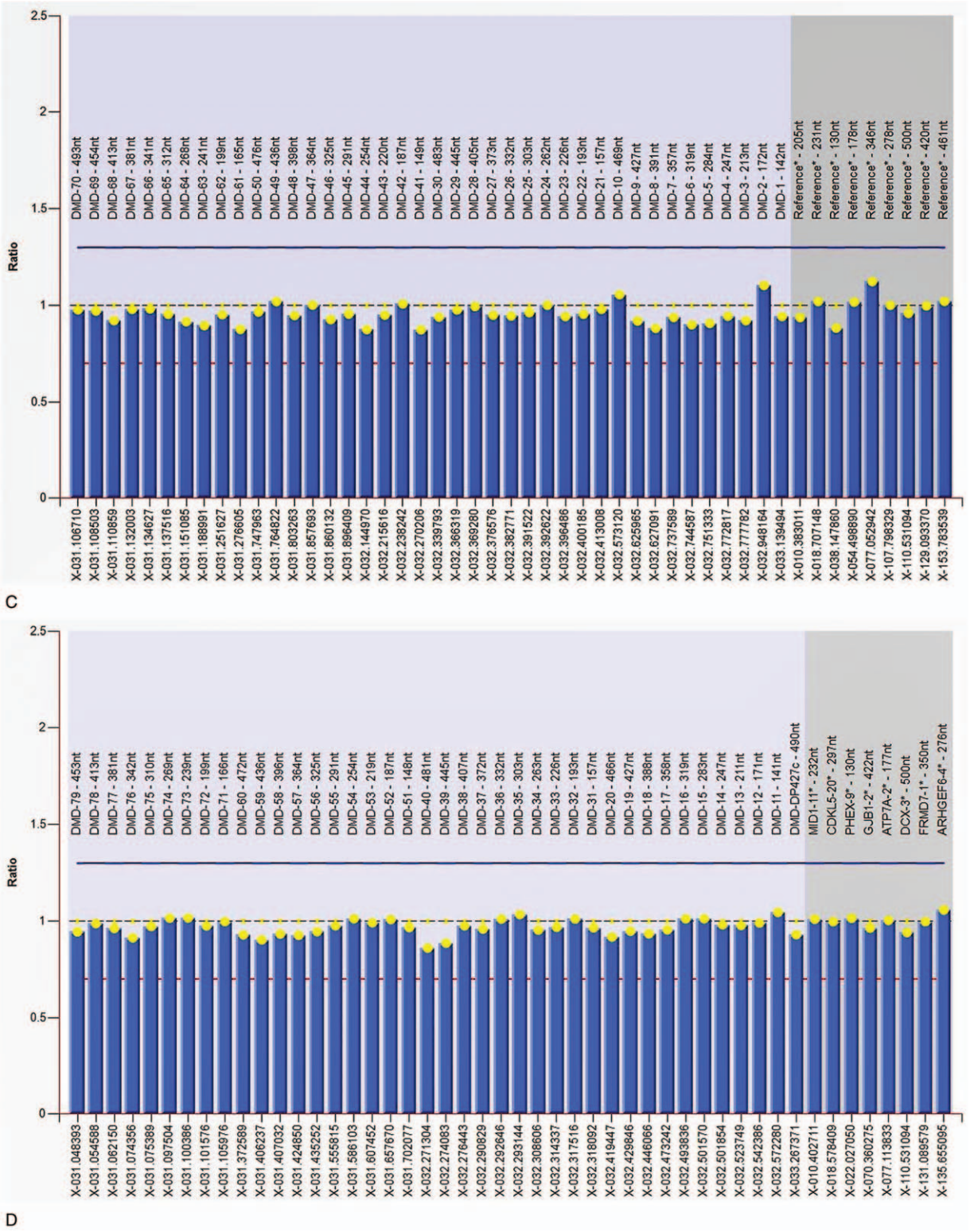

Figure 3. Continued

genetic counseling. With the development and progress of NGS technology, some scholars have applied NGS technology in *DMD* gene diagnosis.[9] For hereditary disorders caused by multiple pathogenic genes or/and having multiple inheritance modes, NGS technique has great advantages in detecting multiple genes simultaneously in 1 test, such as familial hereditary cataract[10] and retinitis pigmentosa.[11] However, the application of NGS has been limited by its high requirement for the conditions of clinical laboratory and extremely complex data processing, so it is difficult to use NGS widely in clinical practice

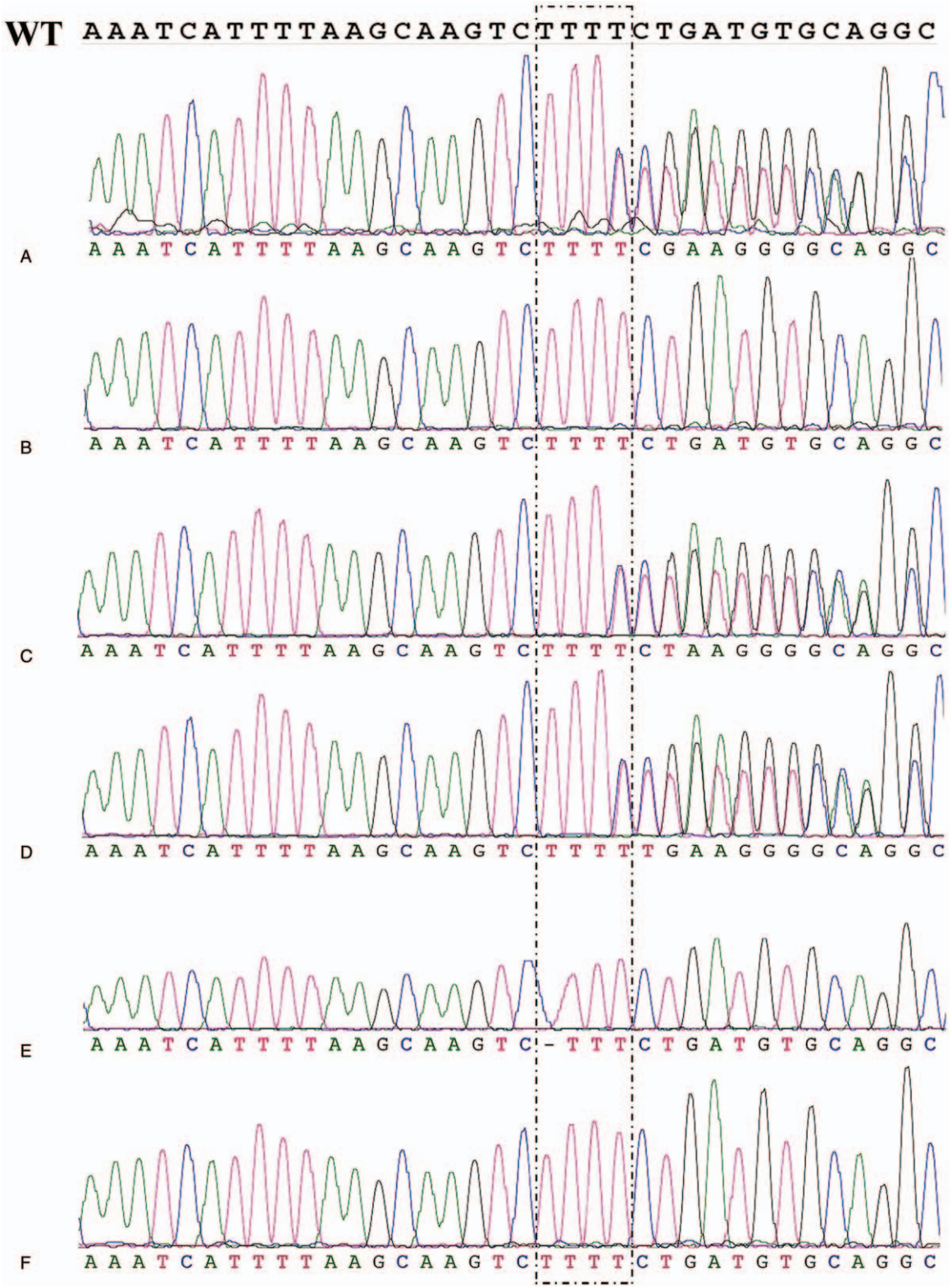

Figure 4. Results of Sanger sequencing. (A) Pregnant woman's mother (I2) with c.2767_2767delT heterozygous mutation in *DMD* gene; (B) Pregnant woman's husband (II3) without mutations at c.2767 locus in *DMD* gene; (C) Pregnant woman (II4) with c.2767_2767delT heterozygous mutation in *DMD* gene; (D) Pregnant woman's younger sister (II5) with c.2767_2767delT heterozygous mutation in *DMD* gene (II5); (E) Patient (III1) with c.2767_2767delT mutation in *DMD* gene; and (F) Fetus (III2) without mutations at c.2767 locus in *DMD* gene. Note: DMD = Duchenne muscular dystrophy; WT = wild type. The mutations are in the black dashed frame.

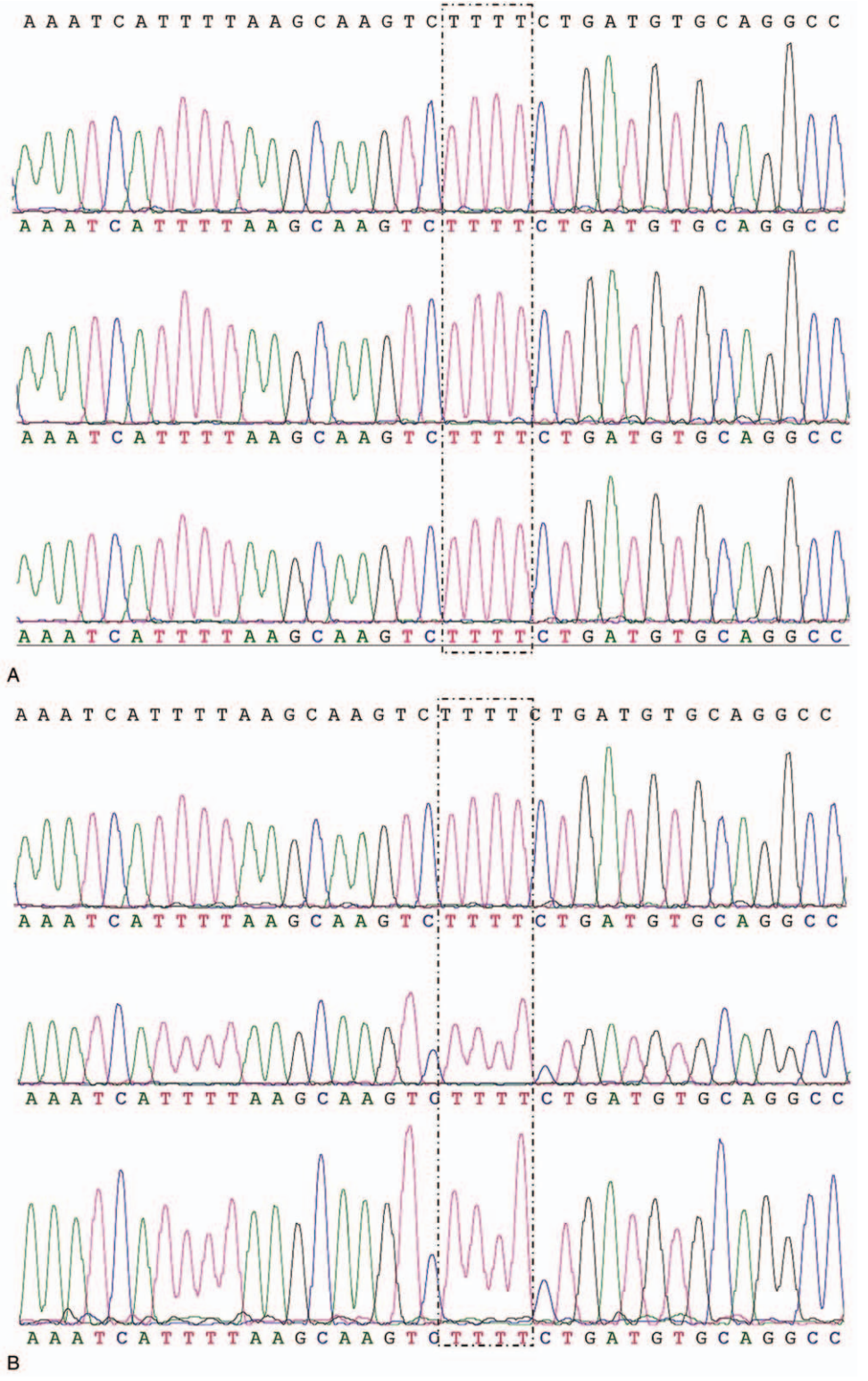

Figure 5. Results of Sanger sequencing. (A) No mutations at c.2767 locus in *DMD* gene in 3 from 50 normal males; (B) No mutations at c.2767 locus in *DMD* gene in 3 from 50 normal females. DMD = Duchenne muscular dystrophy. The mutation area in *DMD* gene in the family is marked with dashed frame.

in developing countries. Furthermore, to guarantee the accuracy of NGS results, Sanger sequencing is still needed to confirm NGS results. *DMD* gene diagnosis may be made using Sanger sequencing method with lower requirement for the laboratory hardware and software conditions, because it does not require simultaneous detection of multiple genes due to definite clinical diagnosis of DMD with clear inheritance mode and known pathogenic gene. Sanger sequencing is featured by relatively low requirements for the laboratory conditions and the ability of sequencing all exon regions in *DMD* gene, which could detect

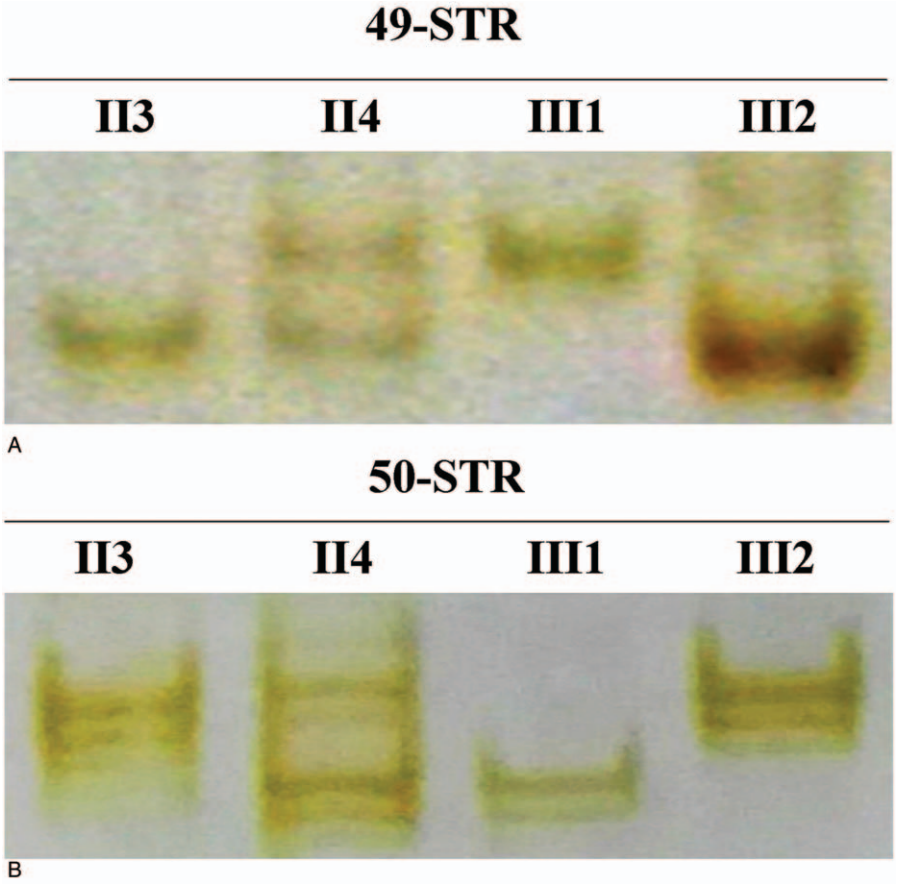

Figure 6. *STR* gene linkage analysis of the DMD family. The 2 sites, 49-STR (6A) and 50-STR (6B), show that the bands are not at the same level between amniotic fluid (III2) and the patient with DMD (III1), demonstrating that the inherited maternal X chromosome in the amniotic fluid (III2) is different from that of the patient with DMD (III1). DMD = Duchenne muscular dystrophy; STR = short tandem repeat.

mutation sites in *DMD* genes and amino acid residue alteration. Therefore, Sanger sequencing combined with *DMD* gene point mutation databases such as www.umd.be/DMD/, www.genome.utah.edu/DMD/mutation_tables.cgi, https://www.ncbi.nlm.nih.gov/clinvar, and http://www.hgmd.cf.ac.uk/ac/index.php can provide reliable reference for clinical genetic counseling in most clinical laboratories. Good results have been obtained in noninvasive DMD prenatal diagnosis,[1] but *DMD* gene detection and prenatal diagnosis are rarely accepted by patients in clinical practices currently. 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 only the cause of advanced technology. Thus, we suggest that suitable methods be selected to make prenatal diagnosis more effective, accurate, and economic.

In this study, we analyzed maternal components in amniotic fluid using PowerPlex 21 HS system to avoid the interference of maternal contamination to the subsequent detection[12] and guarantee the accuracy of detection results. Beyond that, the fetus gender can be determined according to *SRY* gene test, but the mother–child relationship should also be confirmed using samples from the pregnant woman and fetus. These methods should be suggested in prenatal diagnosis of hereditary disorders. We analyzed the *DMD* gene exon mutations in the family members using MLPA technology, and found no deletion/duplication mutations in DMD exons. Thus, combined with clinical symptoms, hematology analysis, electromyography results, and inheritance mode, we think that this is a DMD family caused by *DMD* gene point mutation. Therefore, we sequenced all *DMD* gene exon regions of this family by Sanger sequencing technology and discovered a c.2767_2767delT mutation in *DMD* gene of the patient as well as heterozygous mutation at the same locus in the pregnant woman and her mother and younger sister. Furthermore, no mutation at this locus was detected in the 50 healthy males and 50 healthy females without blood relationship with this patient. Also, this mutation has not been previously reported according to databases such as http://www.genome.utah.edu/DMD/mutation_tables, www.umd.be/DMD, https://www.ncbi.nlm.nih.gov/clinvar, and http://www.hgmd.cf.ac.uk/ac/index.php, and literature retrieval.[4,13–21] One previous study has shown that the Dystrophin protein was an 427-kDa Rod protein composed of 3685 amino acid residues, which contain 4 structural domains[22]:① N-terminal actin-binding domain (amino acid 14–240); ② triple-helix spectrin-like domain (amino acid 253–3040); ③ cysteine-rich region domain (amino acid 3080–3360), and ④ C-terminal domain (amino acid 3361–3685). The c.2767_2767delT [p.Ser923LeufsX26] mutation found in the *DMD* gene of this family is a frame shift mutation, which produces termination codon TGA at the 26th codon after the mutation site. Combined with references,[17,18] we think that this

mutation induces early termination of *DMD* gene translation, namely, that only 947 amino acid residues were translated from the mutant *DMD* gene, and residues 923 to 947 were ill-matched amino acids, which results in truncated dystrophin protein. Therefore, the truncated dystrophin protein only contains the N-terminal actin-binding domain (ABD) and partial triple-helix spectrin-like domain, but loses the cysteine-rich region domain and C-terminal domain, which have significant functions,[23,24] leading to DMD. The CK value of the patient in this family was 16,230 U/L, a relatively high CK value in DMD patients that we have analyzed in this laboratory. Until the date of manuscript submission, the patient was at the age of 7 years and 1 month, with significantly limited action ability and more severe symptoms compared with other DMD children. We speculate that relatively severe symptoms in the patient might be attributed to the loss of long critical domains in Dystrophin proteins. Moreover, this patient also suffered from congenital heart disease, and whether it is associated with the loss of long domains in *DMD* gene remains to be further investigated.[25] Subsequently, gene testing was performed in the amniotic fluid sample (III2). SRY was negative, MLPA indicated no deletion/duplication mutation in DMD exons, and no mutation at c.2767 locus was found by Sanger sequencing. Also, *STR* gene linkage analysis found that the inherited maternal X chromosome in the amniotic fluid sample was different from that of the patient (III1), which is consistent with sequencing results. In summary, we think that the c.2767_2767delT mutation in *DMD* gene is the cause of DMD in the patient, the pregnant woman and her mother and younger sister are *DMD* carriers, the fetus was not a *DMD* carrier, which was confirmed by postpartum follow-up.

In the present study, we combined MLPA, Sanger sequencing, and *STR* gene linkage analysis together for the gene diagnosis of DMD family without deletion/duplication mutations. Such combination brings the advantages of gene testing into full play, because it can identify probands and carriers through mutual confirmation of multiple methods, which is suitable for gene diagnosis and prenatal diagnosis of DMD families and could provide reliable references for genetic counseling. Meanwhile, we reported here a de novo pathogenic *DMD* gene mutation, providing useful information for *DMD* gene mutation database.

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