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Original Article

Rapid method for targeted prenatal diagnosis of Duchenne muscular dystrophy in Vietnam

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Abstract

Objective: Since there is no effective curative treatment for Duchenne muscular dystrophy (DMD), prevention mostly depends on genetic counseling and prenatal diagnosis. About two-thirds of the affected patients have large deletions or duplications, which can be detected by multiplex ligation-dependent amplification (MLPA). The remaining cases include small mutations, which cannot be easily identified by routine techniques. In such cases, linkage analysis may be a useful tool for prenatal diagnosis. Here we compared results obtained from linkage using short tandem repeats (STRs) with those by MLPA and sequencing analysis.

Materials and methods: Eight Vietnamese pregnant women at risk of having a baby with DMD and requesting prenatal diagnosis were recruited in this study. MLPA and direct sequencing were applied to screen large rearrangements and point mutations in the dystrophin gene in the DMD probands and the fetal samples. STR linkage was also performed to analyze fetal mutation status.

Results: By MLPA and sequencing analysis, five DMD patients showed deletions of the dystrophin gene, and no deletions of exons were detected in seven amniotic fluid cell samples; one patient harbored the out-of-frame small deletion of exon 43, which was also found in the fetal sample of this family. STR analysis revealed the transmission of a mutant allele inside each family.

Conclusion: Our results suggest that the combination of STR and MLPA could be a rapid, reliable, and affordable detection protocol for determination of the carrier's status and prenatal diagnosis of DMD in a developing country such as Vietnam.

Keywords: Duchenne muscular dystrophy; MLPA; prenatal diagnosis; STR analysis

Introduction

The dystrophinopathies encompass a spectrum of muscle diseases caused by mutations in the *DMD* gene, which encodes the protein dystrophin (OMIM 300377, Online Mendelian Inheritance in Man) [1]. The severe end of the spectrum includes progressive muscle diseases that are classified as Duchenne (DMD, OMIM 310200) or the allelic milder Becker muscular dystrophy (BMD, OMIM 300376) when skeletal muscle is primarily affected and as Duchenne muscular dystrophy (DMD) associated dilated cardiomyopathy when the heart is primarily affected. The inheritance is X-linked recessive; while both sexes can carry the mutation, females rarely exhibit signs of the disease.

DMD is the most frequent inherited muscle disease in children, affecting around one in 3500 boys [2,3]. DMD is characterized by progressive muscle weakness, tissue inflammation, replacement of healthy muscle with fibro-fatty tissue, and muscle wasting with contractures [4]. Typically, all DMD patients are wheelchair bound in their early teens and few survive beyond the third decade, with respiratory complications and cardiomyopathy being common causes of death.

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Since the discovery of the dystrophin gene in the late 1980s, the molecular mechanism of the disease has been extensively investigated and many attempts have focused on treatment at the molecular level, including gene transfer, cell therapies, exon skipping, and stop codon mutation suppression [5,6]. Nevertheless, a curative treatment for DMD patients is not likely to be achieved in the near future. Therefore, genetic counseling and targeted prenatal diagnosis will still be a cornerstone of the DMD family management in the foreseeable future.

Identifying the dystrophin mutation in all DMD cases would permit a molecular diagnosis as the basis for proper genetic counseling. However, the enormous size of the dystrophin gene and a large variety of mutations are two major challenges for the clinical molecular diagnostic laboratory [7]. The dystrophin gene, located at locus Xp21.1, is the largest human gene identified, spanning more than 24 Mb of genomic DNA. Dystrophin consists of 79 exons, forming a 14-kb mRNA transcript, and has lengthy introns (up to 250 kb) [8]. The majority of identified mutations are deletions, accounting for approximately 60–65% of DMD cases, and duplications have been observed in 8–15% [9,10]. The remaining cases include small mutations, such as microdeletions, microinsertions, point mutations, or splicing mutations [9].

The standard molecular genetic protocol for detection of deletion and duplication in the dystrophin gene is, nowadays, by multiplex ligation-dependent probe amplification (MLPA). It is considered to be the most powerful single technology to identify gross rearrangements in the large dystrophin gene [9,11,12]. Point mutation detection has been improved by the availability of techniques, such as denaturing gradient gel electrophoresis, protein truncation test, Sanger sequencing, and next generation sequencing [13–15]. However, these methods are too labor intensive, time consuming, and expensive for a developing country to be applied in prenatal diagnosis of DMD within a few days. Linkage analysis, based on short tandem repeats (STRs) at polymorphic loci in the dystrophin gene, is a rapid method and can be widely used for carrier detection and in the prenatal diagnosis of DMD families in which the causative mutations cannot be or were not determined in the probands [16–18].

In this study, we compared STR analysis results with those of MLPA and sequencing in eight Vietnamese pregnant women at risk of having a child with DMD, requesting prenatal diagnosis to establish a reliable and convenient prenatal diagnosis protocol for Vietnamese DMD families.

Materials and methods

Patients

Genetic analysis was performed in six unrelated Vietnamese DMD families including eight pregnant women at risk of having an affected baby. The family histories were kindly provided by the Vietnam National Hospital of Pediatrics. Amniocentesis was performed at 16–22 weeks of gestation under ultrasound guidance. In this study, all participants signed a written informed consent as required by the Vietnam Medical Ethics Council.

In each case, 10 mL of amniotic fluid was taken during amniocentesis from a pregnant woman. Three to four milliliter of peripheral blood was collected from all of the DMD pedigree's relevant members. Genomic DNA was extracted from the peripheral blood of DMD patients, as well as their family members, using the QIAamp DNA blood Mini Kit (Qiagen Inc., Hilden, Germany). The QIAamp DNA Mini kit was used to extract DNA of the fetal amniotic fluid cells following the manufacturer's recommendation. The genomic DNA was visualized by electrophoresis on a 0.8% agarose gel and quantified using the Nanodrop system (1000 spectrophotometer, Thermo Scientific, Wilmington, DE, USA).

MLPA analysis

The MLPA reaction was performed to screen the dystrophin gene using the SALSA MLPA probe sets P034 and P035 (MRC-Holland, Amsterdam, The Netherlands) according to the manufacturer's instructions. Briefly, 50–100 ng of genomic DNA in 5 μL deionized water was denatured and hybridized overnight with the probe mix P034 or P035. Ligation was performed with Ligase 65 enzyme and finally, polymerase chain reaction (PCR) amplification was performed with the specific SALSA FAM PCR primers. Amplification products and Size Standard 600 were mixed completely and run by capillary electrophoresis on the Beckman CEQ-8000 genetic analytic system (GenomeLab GeXP, Genetic Analysis System, Beckman Coulter, Brea, CA, USA). The peaks obtained after capillary electrophoresis were analyzed by the GeneMapper v3.2 software (GeneMarker, Softgenetics, LLC State College, PA, USA).

Sequencing analysis

PCR amplification of all 79 exons and eight promoters of the dystrophin gene were performed using Takara *Ex Taq* DNA Polymerase (Takara Inc, Otsu, Shiga, Japan). The sequence of the primers and the respective annealing temperatures for PCR were obtained from the Leiden Muscular Dystrophy website (www.dmd.nl, Center for Human and Clinical Genetics, Leiden University Medical Center). In total, 87 segments of size ranging from 170 to 600 base pairs were amplified.

Purified PCR products were subjected to cycle sequencing with the ABI Bigdye Terminator V1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). The purified products were then sequenced on an ABI PRISM 3100 Genetic Analyzer (Model 373A, Applied Biosystems, Foster City, CA, USA).

STR analysis

Four dystrophin intragenic STRs (DSTR44, DnSTR44, DSTR49, DSTR50) were amplified using the Mastermix and the previously reported primer oligonucleotides (Leiden Muscular Dystrophy website). The forward primers were

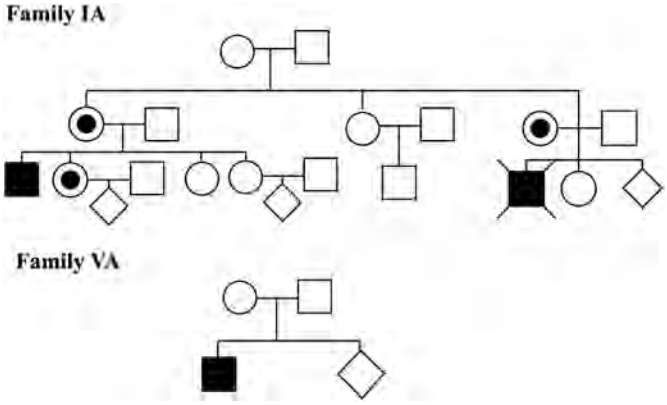

Fig. 1. Pedigree of family IA and VA.

labeled with 5-carboxyfluorescein FAM. Amplification reactions were performed with the denaturation at 95°C for 4 minutes, 35 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds, extension at 72°C for 30 seconds, and final extension 72°C for 7 minutes. PCR products (0.5 μL) were mixed with 0.3 μL SS600 and 32 μL SLS. The mixture was separated by capillary gel electrophoresis (15 kV at 60°C for 30 minutes) on Beckman CEQ-8000 genetic analytic system. The data was then analyzed with GeneMapper v3.2 software.

Results

MLPA analysis results

MLPA was performed in six DMD patients for the deletion and duplication diagnosis. Five patients showed deletions of the dystrophin gene (case IA, IIA, IIIA, IVA, VIA, Fig. 1, Table 1). In all mothers of the affected patients, MLPA analysis determined their carrier status as the signal of the mutated exons was about half that of the control value.

The fetal gender was rapidly determined by SRY gene detection in the amniotic fluid samples of eight pregnant women. All cases were found to be male and required further prenatal diagnosis for DMD.

For seven pregnant women belonging to families in which the proband had a deletion, MLPA was applied to detect potential deletion. The analysis results showed that no deletion of exons was detected in seven fetal amniotic fluid cell samples: these fetuses were diagnosed as non-affected within 1 week of sampling.

Sequencing analysis results

One proband who did not show any deletion or duplication was analyzed by direct sequencing. Sequencing of exon 43 showed a novel 2 bp deletion (6213_6214 delCC) (Fig. 3), causing a frameshift mutation. His mother was a carrier as the sequencing analysis was found to harbor a 2 bp heterozygous deletion. Exon 43 of the dystrophin gene was also sequenced in the fetal DNA (case VA - at the 21st week of pregnancy), and the 2 bp deletion was again found: the fetus was thus diagnosed as affected after 3 weeks from sampling.

STR analysis results

The STR analysis results clearly showed that the genomic DNA from amniotic fluid cells used in all experiments was not significantly contaminated by DNA from their mothers.

In all cases, it could be reasonably determined which alleles of the mother harbored the mutant dystrophin gene. In five cases (IA, IIA, IIIA, IVA, VIA), the fetuses inherited their mother's normal allele and were thus diagnosed as non-affected, whereas in case VA, the fetus inherited the mother's mutant allele and was diagnosed as affected. The STR analysis required 1 week to run and interpret and showed results consistent to those of MLPA or sequencing. Two analysis results of typical DMD families in this series are illustrated in Figs. 2 and 3.

Discussion

DMD is an X-linked, inherited neuromuscular disease characterized by a relatively high incidence and lethality rate. Currently, there is no effective treatment for DMD patients; therefore, the mainstay of prevention is detection of female carriers and diagnosis of affected fetuses. Performing prenatal diagnosis can provide sufficient and valuable genetic information that allows DMD families to make appropriate reproductive choices. The best strategy for a comprehensive DMD prenatal diagnosis depends on the realistic circumstances of each country. A balance must be found between diagnostic accuracy, costs of initial mutation detection, and the time

Table 1
Mutation characteristics of the dystrophin gene detected in Duchenne muscular dystrophy (DMD) patients.

DMD patients/family	Mutation detected	Pregnant woman at risk no.	Prenatal diagnosis results	
			By MLPA and sequencing	By STR analysis
I/IA	Del. Ex11–39	23	Non affected	Inherited normal allele
II/IIA	Del. Ex44	30 and 32	Non affected	Inherited normal allele
III/IIA	Del. Ex46–50	39	Non affected	Inherited normal allele
IV/IVA	Del. Ex3–8	20	Non affected	Inherited normal allele
V/VA	6213_6214 del. CC	12	Affected	Inherited mutant allele
VI/VIA	Del. Ex8–12	9 and 10	Non affected	Inherited normal allele

Del. = deletion; Ex = exon; MLPA = multiplex ligation-dependent probe amplification; STR = short tandem repeat.

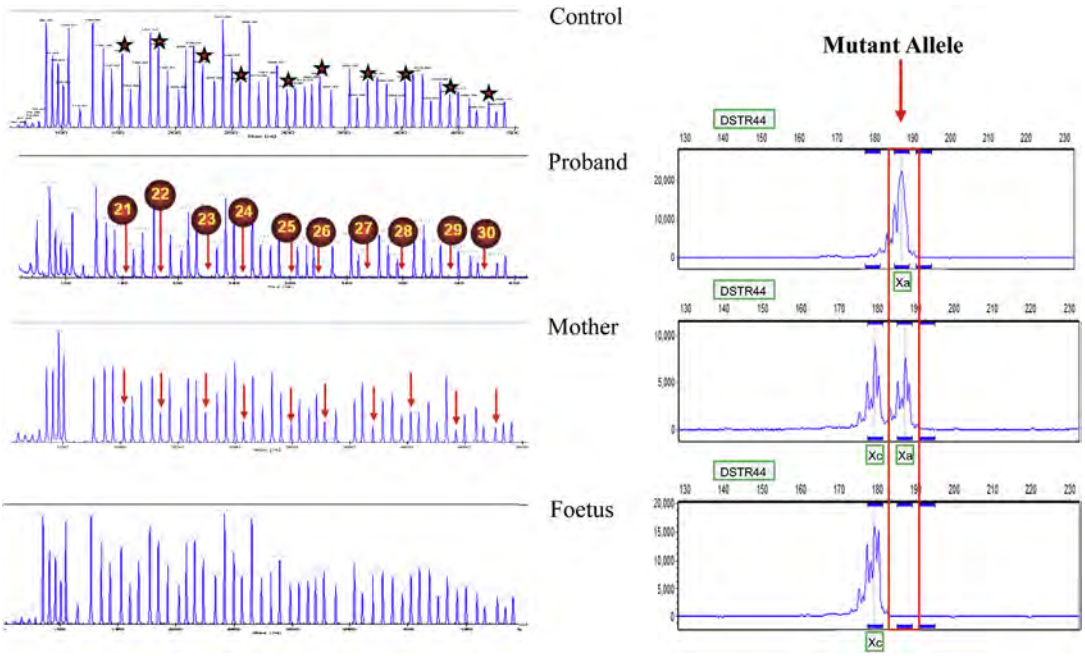

Fig. 2. Multiplex ligation-dependent probe amplification (MLPA) and short tandem repeat (STR) analysis results of family IA. These MLPA-SALSA probe mix P034 showed deletion of exon 21–30 of the dystrophin gene in the proband and his mother. MLPA showed no deletion of these exons in the fetal amniotic fluid cell samples. STR analysis indicated that the fetus inherited the mother's normal allele. The fetus was diagnosed as non-affected.

taken from sample collection to obtaining the results (turn-around time).

Mutation diagnosis for the proband is always the first step of a standard prenatal diagnosis procedure. In this study, the MLPA technique was applied to screen large rearrangements of the dystrophin gene in DMD probands. No duplication, but five deletions were identified, including one case (patient IIA) harboring a single deletion of exon 44 (Table 1). Recently, Lee

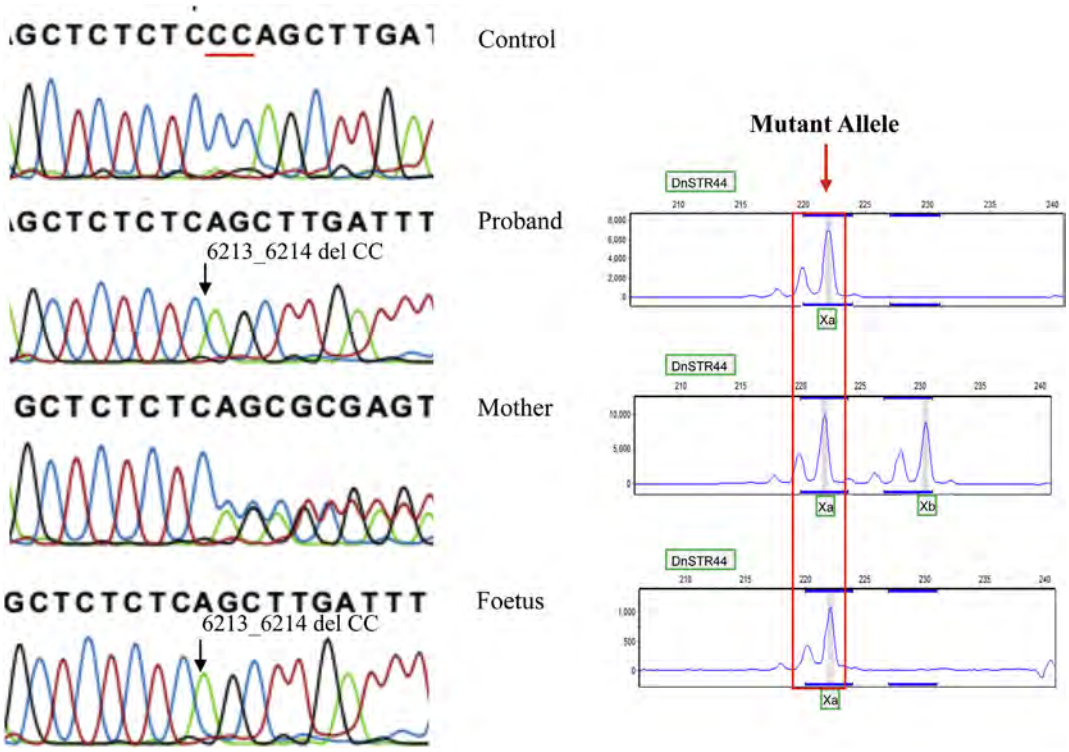

Fig. 3. Sequencing and short tandem repeat (STR) analysis results of family VA. Direct sequencing of exon 43 showed a novel 2 bp deletion (6213_6214 delCC) in the proband and the fetus. His mother was a carrier as the sequencing analysis was found to harbor a 2 bp heterozygous deletion. STR analysis indicated that the fetus inherited the mother's mutant allele. The fetus was diagnosed as affected.

et al [19] reported that single exon deletion could be a false positive MLPA result due to the presence of the nucleotide change obstructing probe annealing. A confirmatory experiment by monoplex PCR was performed to eliminate this possibility in our case, as the PCR result reconfirmed the single deletion of exon 44 of patient IIA (data not shown). Deletion can happen almost anywhere in the dystrophin gene. However, one location towards the central part of the gene (exons 44−55) and the other site towards the 5′ end (exons 2−20) have been previously reported as the two hot-spot regions [20,21]. Our data are in agreement with those studies. We also apply the MLPA technique for the carrier detection in DMD families; MLPA analysis determined the carrier status of seven pregnant women as the signals of the mutated exons were about half the value of controls. Only 2−3 days were required to perform MLPA analyses and interpret the results. For the patient with no deletion or duplication on MLPA (patient VA), full sequence analysis showed a novel 2-bp deletion (6213_6214 delCC) causing a frameshift mutation of the dystrophin gene. His mother was a 2-bp heterozygous deletion carrier as determined by sequencing analysis. The mutation was also found in the fetal DNA; the sequencing analysis required 3 weeks to identify the mutation and interpret prenatal diagnosis results. This amount of time illustrated the fact that full exon sequencing was time consuming and too costly.

It is important to note that one of the most common causes of diagnostic errors is due to the contamination of amniotic fluid by maternal blood cells at amniocentesis, which could lead to the amplification of the maternal gene and result in a false positive. This problem can be controlled by carrying on the STR analysis. Besides that, the linkage analysis could identify the transmission of the mutant allele inside each DMD families in which the (CA)n STR in the 3′ end loci of intron 44 and 49 was amplified using fluorescent PCR. In five of our DMD families (IA, IIA, IIIA, IVA, VIA), it can be reasonably inferred that the maternally inherited X chromosome of the fetuses comes from the mother's normal X chromosome, while the fetus in family VA received his mother's mutant X chromosome. STR analysis produced results in agreement with those obtained by MLPA or sequencing, but required much less time to conduct. From our experience, STR analysis is fast (PCR was amplified in ∼2.5 hours, and the capillary gel electrophoretic run took ∼30 minutes), accurate, inexpensive, and easy to perform. However, the linkage analysis should not be applied as the only technique in every case of prenatal diagnosis, due to the high recombination rate of the dystrophin gene. Some previous studies reported that intragenic recombination in the dystrophin gene has been shown to occur with a frequency as high as 10−12% [22−24], which illustrates the limitation of linkage analysis for prenatal diagnosis of DMD.

In summary, our study showed that a combined strategy that includes MLPA and STR analysis could minimize diagnostic errors in linkage analysis and offer advantages over the approach of MLPA or sequencing alone. This combination may become a method of choice in Vietnam for prenatal diagnosis in familial cases of DMD.

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