Clinical and Experimental Obstetrics & Gynecology

IMR Press

Original Research

Successful strategy of comprehensive pre-implantation genetic testing for Duchenne muscular dystrophy and chromosome balance using karyomapping and fluorescent PCR

Suchada Mongkolchaipak[1,†], Sirivipa Piyamongkol[2,†], Chutithep Teekaput[3], Rungthiwa Sirapat[1], Wanwisa Suriya[4], Tawiwan Pantasri[4], Theera Tongsong[4], Wirawit Piyamongkol[4,*]

[1] *Suchada IVF Center, Sriracha, 20130 Chon Buri, Thailand*

[2] *Department of Pharmaceutical Sciences, Faculty of Pharmacy, Chiang Mai University, 50200 Chiang Mai, Thailand*

[3] *Department of Internal Medicine, Faculty of Medicine, Chiang Mai University, 50200 Chiang Mai, Thailand*

[4] *Department of Obstetrics and Gynaecology, Faculty of Medicine, Chiang Mai University, 50200 Chiang Mai, Thailand*

*Correspondence: wirawit.p@cmu.ac.th (Wirawit Piyamongkol)

† These authors contributed equally.

DOI:10.31083/j.ceog4805187

Submitted: 12 April 2021 Revised: 25 April 2021 Accepted: 18 May 2021 Published: 15 October 2021

Background: Duchenne muscular dystrophy (DMD) is major childhood muscular dystrophy. Pre-implantation genetic testing (PGT) is an alternative to prenatal diagnosis. This study performed SNP microarray with karyomapping PGT of DMD in comparison to PCR-based techniques for validation. *Methods*: Two families at risk of having DMD offspring decided to have karyomapping PGT. PCR protocol using mini-sequencing and intragenic microsatellites-based linkage analysis was developed and applied. *Results*: Karyotyping results of family DA (DMD c.895G>T) exhibited three normal, two carriers, two affected and two with intragenic recombination. Karyomapping results of family DB (DMD exon 8 and 9 duplication) showed four normal, two carriers, two affected and one with intragenic recombination. One embryo was chromosome unbalanced and one was uniparental disomy. *Conclusion*: Successful karyomapping PGT for DMD was successfully performed. Limited number of embryos were tested due to its expensive consumables. Intragenic recombination precluded haplotyping. Karyomapping provides advantages of CNV and parental origin information.

Keywords

Duchenne muscular dystrophy (DMD); Embryo selection; Haplotyping; Karyomapping; Pre-implantation genetic testing for monogenic disease (PGT-M)

1. Introduction

Duchenne muscular dystrophy (DMD, MIM #310200, Xp21.2-p21.1), an X-linked recessive disorder, is one of the most common muscular dystrophies in childhood with the prevalence of 2.9 per 10,000 live male births [1]. DMD gene possesses 79 exons covering over 2.3 Mb. DMD is associated with loss or abnormal function of the key muscular protein called dystrophin, one of spectrin protein superfamily with 427 kilodalton (kDa), and it is expressed in skeletal muscles, cardiac muscles, cerebral cortical neurons and Purkinje cerebellar neurons [2, 3]. Mutations within DMD gene cause deficiency of dystrophin and are associated with muscular membrane instability [4], leading to apoptosis and necrosis of muscle cells. DMD patients usually express gross motor delay, proximal muscle weakness, calf hypertrophy (at 3 to 5 years old), and loss of ambulation (by the age of 12 to 13) [5]. The patients suffer progressive proximal muscle weakness and are at increased risk of death from respiratory failure and cardiomyopathy occurring in their 30's [6, 7]. Gross deletion (60–65%) and duplication (8–15%) which lead to frameshift are major mutations of the DMD gene while the remainder are caused from point mutations [8–10] with approximately one-third of DMD mutations being de novo [3].

Mutations of DMD gene cause dystrophin protein deficiency and absence of dystrophin-associated glycoprotein complex (DGC). Following the dysregulated signaling pathways and abnormal membrane structure, the reactive oxygen species (ROS) develop and lead mitochondrial dysfunction. The increased intracellular Ca^{2+} disturbs the Ca^{2+}-dependent pathways and results in apoptosis and necrosis of muscle cells. The final result is a fibrotic process and adipose tissue replacement in skeletal muscles causing the loss of skeletal muscle function [11].

Similar to other chronic and progressive conditions, the patients and parents encounter psychologic and social difficulties. Affected children suffer from diseases associated with discomfort and pain, restricted physical activities, school absences and learning delay. The parents worry about disease advancement and disability. The parent may need to leave their job in order to take care of affected child leading to a decrease in quality of life and income loss. Additional expenses such as nursing, food, school support, and transport are greater than for a healthy child [1]. The patients suffer progressive proximal muscle weakness and die from respiratory failure and cardiomyopathy in their fourth decade of life [6, 7].

Since there is no specific and curative treatment available, genetic counselling and prenatal diagnosis are recommended. Female carrier identification can be done using a commercial DMD mutation screening test by multiplex ligation-dependent probe amplification (MLPA) [12]. A primary test is carried out to detect large deletions or duplication within the DMD gene. If the primary test is negative, sequence analysis of DMD encoding region is performed to look for minor frameshift or nonsense mutations using denaturing gradient gel electrophoresis (DGGE), protein truncation test and Sanger sequencing [13, 14]. For those without an answer, next generation sequencing (NGS) can be used to check for all possible mutations [15].

Early prenatal diagnosis (PND) of DMD employed fetal blood sampling and the measurement of plasma creatine phosphokinase activity [16]. However, this technique is not specific [17]. First trimester fetal sex deter mination was employed in pregnancies at risk for DMD [18]. In the early molecular era, prenatal diagnosis and carrier detection of DMD utilized closely linked restriction fragment length polymorphism (RFLPs) markers [19]. Linkage analysis based using dinucleotide repeat polymorphisms was also applied for PND of DMD [20]. In utero fetal muscle biopsy for PND of DMD was performed in cases of problems with molecular genetic analysis [21, 22].

First trimester chorionic villous sampling (CVS) [23], second trimester amniocentesis [24] and fetal blood sampling (FBS or cordocentesis) [25] are choices of invasive PND procedures for prenatal diagnosis. Use of non-invasive cell free fetal DNA (cffDNA) for monogenic disorders requires further study. Prenatal diagnosis is able to provide fetal samples for genetic analysis. Normal results reassure parents that their baby will be unaffected. However, abnormal results gives the couple a difficult decision as whether to ter minate or continue the pregnancy and prepare for postnatal affected infant [26]. In addition, irrespective of the results, some pregnancies may miscarry following the procedures [23–25].

Pre-implantation genetic testing (PGT) [27] is an alternative to traditional PND allowing the parents a chance to initiate a pregnancy with the confidence that the baby will be unaffected. However, the extremely large size of the DMD gene and the diversity of mutations make molecular genetic testing difficult and labor intensive. Preli minary PGT protocols using single cell multiplex PCR amplifying five dystrophin gene exons in combination with sex identification has been introduced [28]. A similar strategy has been applied in clinical PGT for DMD [29, 30]. PGT for DMD using interphase fluorescence in situ hybridization (FISH) to detect deletions of specific exons within the dystrophin gene has been previously reported [31]. The application of multiple displacement amplification (MDA) prior to PCR has also been applied in PGT of DMD [32]. PGT protocols incorporating the analysis of five or seven exons, four polymorphic markers distributed along the dystrophin gene situated in the two deletion hotspots, and the analysis of amelogenin fragments for sex identification has been described [33]. For PGT-M of DMD, developing PCR protocols for each family are expensive, labor intensive, and time consu ming.

Karyomapping is an advanced molecular method using single nucleotide polymorphism array (aSNP) for simultaneously haplotyping and copy number variation (CNV) analysis [34]. The techniques were first demonstrated using samples from previous PCR based PGT-M of cystic fibrosis in 2009. Karyomapping was also tested on embryo samples of previous PGT cases, including Huntington disease, Peutz-Jeghers syndrome, and Crigler-Najjar syndrome [35]. These procedures gave successful results in 97.7% of samples. However, the studies did not carry out genuine clinical PGT. The first live birth after PGT-M using karyomapping from polar body biopsy was for Smith-Lemli-Opitz syndrome in 2014 [36]. Since the study employed polar body biopsy techniques, postzygotic CNV cannot be detected. Specifically, the transferred unaffected embryo was not tested by karyomapping. Therefore, the claim that this was the first karyomapping PGT-M live birth was inaccurate. Successful PGT-M using karyomapping for Marfan syndrome was also reported in 2015 [37]. The study performed a SNP on single blastomeres from day 3 embryo biopsy with a SNP demonstrating results in 78–82% and PCR giving results in 87.5% with a healthy male infant as a result. Although there have been several publications regarding PGT-M using karyomapping, most were retrospectively performed from samples of previous PCR based PGT. Only a few had prospective clinical PGT cycles with unimpressive results. Therefore, more details need to be explored regarding the clinical applications of karyomapping.

This study aims to apply a SNP and karyomapping for PGT-M of DMD and PGT-A in 2 clinical PGT cycles in comparison to PCR techniques.

2. Materials and methods

2.1 Patient details

Two families at risk of having an affected DMD offspring joined the project following extensive counselling and the obtaining of informed consent. The project was approved by the Research Ethics Committee of Faculty of Medicine, Chiang Mai University (OBG-2562-06117).

The patient of family DA was 31 years old and her husband was 32 years old. She and her mother carried DMD c.895G>T (E299X within Exon 11) mutation. She experienced one pregnancy ter mination following positive PND. Her husband did not have any DMD mutations. Her mother's DNA was used as a reference in karyomapping analysis. The patient of family DB was 32 years old and her husband was 34 years old. She carried DMD Exon 8 and 9 duplication mutation. Her husband did not have any DMD mutations. Her son who was 11 years old, was affected with DMD Exon 8 and 9 duplication mutation. Her 4 year old daughter was a carrier of DMD Exon 8 and 9 duplication mutation. Her son's DNA was used as a reference for karyomapping analysis.

2.2 In vitro fertilization (IVF) and embryo biopsy

Both patients underwent IVF procedures with routine ovarian stimulation. Intracytoplasmic sperm injection (ICSI) was performed to avoid sperm DNA conta mination. Blastocysts of good quality were chosen for biopsy with a laser on day 5 post-fertilization. Five trophectoderms were taken for whole genome amplification (WGA) and subsequent molecular testing including e. karyomapping and mutation analysis. After biopsy, all embryos were cryopreserved.

2.3 Cell lysis and whole genome amplification (WGA)

Biopsied trophectoderms were washed thoroughly in phosphate buffered saline (PBS, Cell Signaling Technology, Theera Trading Co. Ltd. Bangkok, Thailand) with 0.1% polyvinyl alcohol (PVA, Sigma-Aldrich, Chiangmai VM Co., Ltd., Chiang Mai, Thailand) before transferring to microcentrifuge tubes with a total volume of 4 μL. DNA extraction was performed using alkaline lysis buffer protocol [38]. Three μL of denaturation buffer (0.25 μL of 1M DTT and 2.75 μL of buffer DLB (REPLI-g® Single Cell Kit, Chiangmai VM Co. Ltd., Chiang Mai, Thailand)) was added and mixtures were incubated at 65 °C for 10 min. This was followed by addition of a en neutralization buffer (3 μL of stop solution). WGA by multiple displacement amplification (MDA, REPLI-g® Single Cell Kit) was done according to the manufacturer's instruction. A mixture of 9 μL of water, 29 μL of reaction buffer and 2 μL of DNA polymerase (REPLI-g® Single Cell Kit) was added to the extracted DNA, resulting in a total volume of 50 μL. Mixtures were incubated at 30 °C for 8 h and at 65 °C for 3 min to inactivate the reaction. SNP array with karyomapping analysis was performed for haplotyping and CNV [34].

2.4 SNP array and karyomapping analysis

Amplified MDA samples were tested with SNP array using Illu mina HumanKaryomap-12 DNA Analysis Kit (BioActive Co. Ltd., Bangkok, Thailand) according to the manufacturers instructions [34]. SNP genotyping information was analyzed using BlueFuse Multi software version 4.5 (BioActive Co. Ltd., San Diego, CA, USA) for karyomapping analysis and molecular cytogenetics. Haplotyping analysis from SNP genotyping information of the couples together with an offspring or an informative relative serving as a reference reveals inheritance of unaffected or affected genes in the embryos allowing the diagnosis of a monogenic disorder of the embryos. Additionally, SNP genotyping provides CNV details of every chromosome. These results were compared with those of PCR.

2.5 Multiplex fluorescent PCR and mini-sequencing

Mutation analysis was performed to confirm diagnosis results. Aliquots of amplified WGA products were used for multiplex fluorescent PCR and mini-sequencing analysis. For family DA, 0.5 μL of amplified WGA products were amplified using primers covering DMD c.895G>T and polymorphic linked markers for linkage analysis and conta mination detection. Amelogenin X/Y [39] for gender deter mination was added. Multiple microsatellites within the dystrophin gene, including 5'-5n4 (Intron 4) [40], DXS206 (Intron 7) [41], DXS1236 (Intron 49) [20] and DXS1214 (Intron 63) [42]) were employed. The PCR mixture consisted of 200 mM of each primer, 5 μL of 2x QIAGEN® Multiplex PCR Master Mix (QIAGEN® Multiplex PCR Kit, Chiangmai VM Co. Ltd., Chiang Mai, Thailand) and was made up to a total volume of 10 μL with distilled deionized water. The amplifications were performed with the following conditions: 94 °C for 30 s, annealing at 60 °C for 1 min 30 s and extension at 72 °C for 1 min 30 s for 37 cycles. These were preceded by primary denaturation at 95 °C for 15 min to activate HotStarTaq DNA Polymerase (QIAGEN® Multiplex PCR Kit) followed by final extension at 72 °C for 10 min. The multiplex amplified products were each tagged with two different fluorochromes using labeled primers [43]. This allowed analysis to be performed on an automated laser fluorescent sequencer ABI Prism® 3130 (GenePlus Co., Ltd., Bangkok, Thailand). 5'-5n4, DXS206, DXS1236, DXS1214 fragments were labeled with 6'FAM (blue), VIC® (green), 6'FAM (blue) and NED® (yellow/black) fluorescent dyes. Mini-sequencing [44] was performed for mutation analysis of DMD c.895G>T mutation. The same multiplex fluorescent PCR was employed for family DB, except for the DMD c.895G>T primers. The results were analyzed and compared with karyomapping results. Details of the primers are summarized in Table 1 (Ref. [20, 39–42]).

2.6 Fragment analysis on ABI Prism® 3130

A mixture of 1 μL multiplex fluorescent PCR product, 10 μL deionized formamide (GenePlus Co., Ltd.) and 0.1 μL Genescan^{TM}-500LIZ® size standard (GenePlus Co., Ltd.) was prepared and denatured at 95 °C for 5 min. The denatured sample was subjected to capillary electrophoresis using Performance Optimized Polymer 7 (POP-7®, GenePlus Co., Ltd.; 5 s injection time, 15,000 V, 60 °C, 20 min) on an automated laser fluorescent sequencer ABI Prism® 3130. The data was analyzed by GeneMapper® software version 4.0 (GenePlus Co., Ltd.).

2.7 Mini-sequencing

Mini-sequencing techniques were employed for mutation analysis of DMD c.895G>T. Amplified PCR products were treated with Exonuclease I/Alkaline Phosphatase using ExoProStar^{TM} 1-Step (Bang Trading 1992 Co., Ltd., Bangkok, Thailand) to remove unincorporated primers and dNTPs from PCR reactions prior to DNA sequencing. 2.14 μL of PCR products were added into 0.2-mL microcentrifuge tubes containing 0.86 μL of ExoProStar^{TM} 1-Step and incubated at 37 °C for 30 min, followed by 80 °C for 15 min. The mini-sequencing reaction mixture was set up on ice and comprised of 5.0 μL of SNaPshot® Multiplex Kit (GenePlus Co., Ltd.), 0.5 μL of mini-sequencing primer (0.2 μM stock) (Table 1), 3.0 μL of the purified template and distilled deionized water in a total volume of 10 μL. The thermal cycles were performed with the conditions of 96 °C for 10 s, 50 °C for 5 s and 60 °C for 30 s for 25 cycles.

Table 1. Primers details for multiplex fluorescent PCR and mini-sequencing in the PGT-M protocol for DMD. Primers covering DMD c.895G>T mutation, Polymorphic linked markers to DMD gene for linkage analysis assay and Amelogenin X/Y for gender deter mination were exhibited.

Primers	Location on DMD gene		Sequences	Fragment length (bp)	References
DMD c.895G>T	Exon 11	forward	5′-GGCCGGGTTGGTAATATTCT-3′	126	OMIM: NM_004010
		reverse	5′-CCTGAGGCATTCCCATCTT-3′		
DMD c.895G>T	Exon 11	mini-sequencing	5′-TCAGAAGATGAAGAAACTGAAGTACAA-3′		
5′-5n4	Intron 4	forward	5′-GAAGGGAAAATGATGAATAAAACT-3′	134–186	[40]
		reverse	5′-GTCAGAACTTTGTCACCTGTC-3′		
DXS206	Intron 7	forward	5′-TTCTGGTTTTCTGGTCTG-3′	218–236	[41]
		reverse	5′-GAATCAATCTCTCTGTCAAG-3′		
DXS1236	Intron 49	forward	5′-GGCAAGTTTCTCTTCGTCGT-3′	226–260	[20]
		reverse	5′-CGATTCGTGTTTTGCATTGT-3′		
DXS1214	Intron 63	forward	5′-GCCAGCGTATACCCATTTTG-3′	148–162	[42]
		reverse	5′- CAGGTAGAAAGATAGCAGGCAAC-3′		
Amelogenin X/Y		forward	5′-GCTTGAGGCCAACCATCAG-3′	X 119	[39]
		reverse	5′-CCTGGGCTCTGTAAAGAATAG-3′	Y 125	

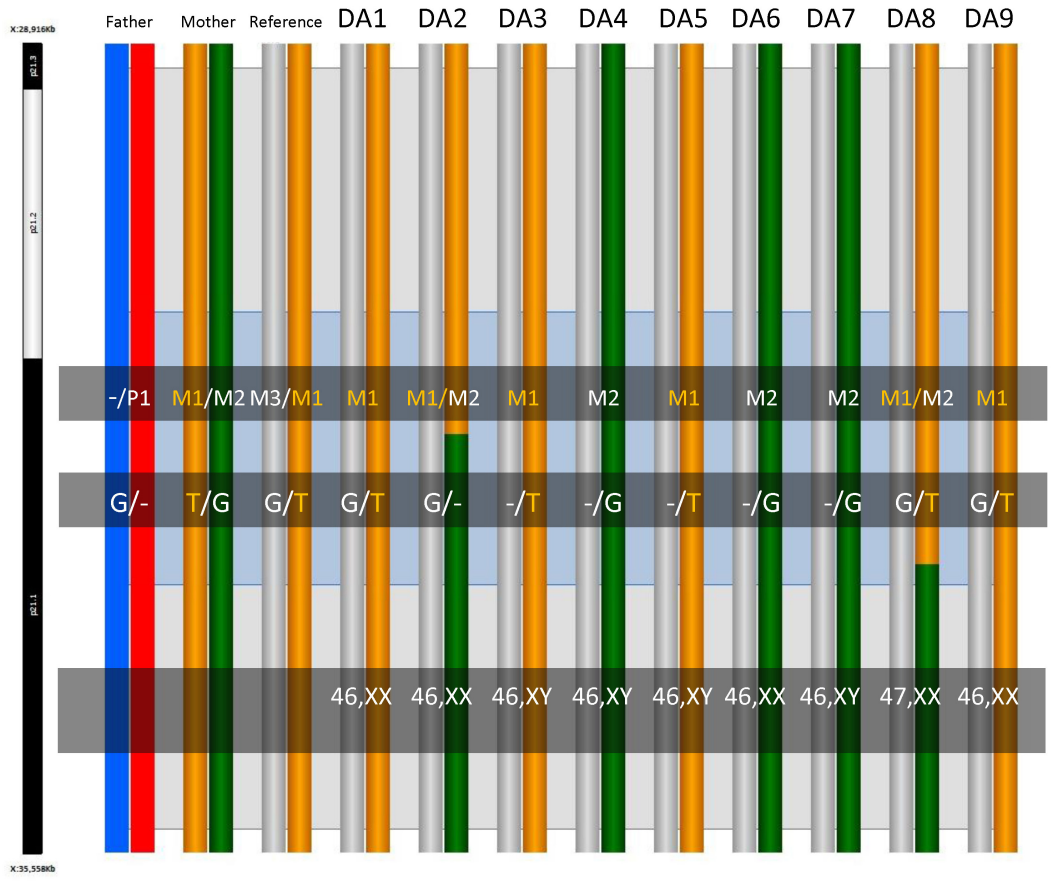

Fig. 1. Family DA's haploblock chart of karyomapping analysis. Happloblock chart of DMD c895G>T (E299X) from karyomapping (BlueFuse Multi software) using SNP array information (Illu mina HumanKaryomap-12 DNA Analysis Kit) and mutation analysis using multiplex fluorescent PCR (F-PCR) and mini-sequencing for DMD c895G>T for the couples at risk of having DMD c895G>T (E299X) offspring (family DA). DNA of the mother of the patient who is a carrier was employed as the reference. Haplotyping of DMD gene was demonstrated together with PCR results and chromosome analysis results.

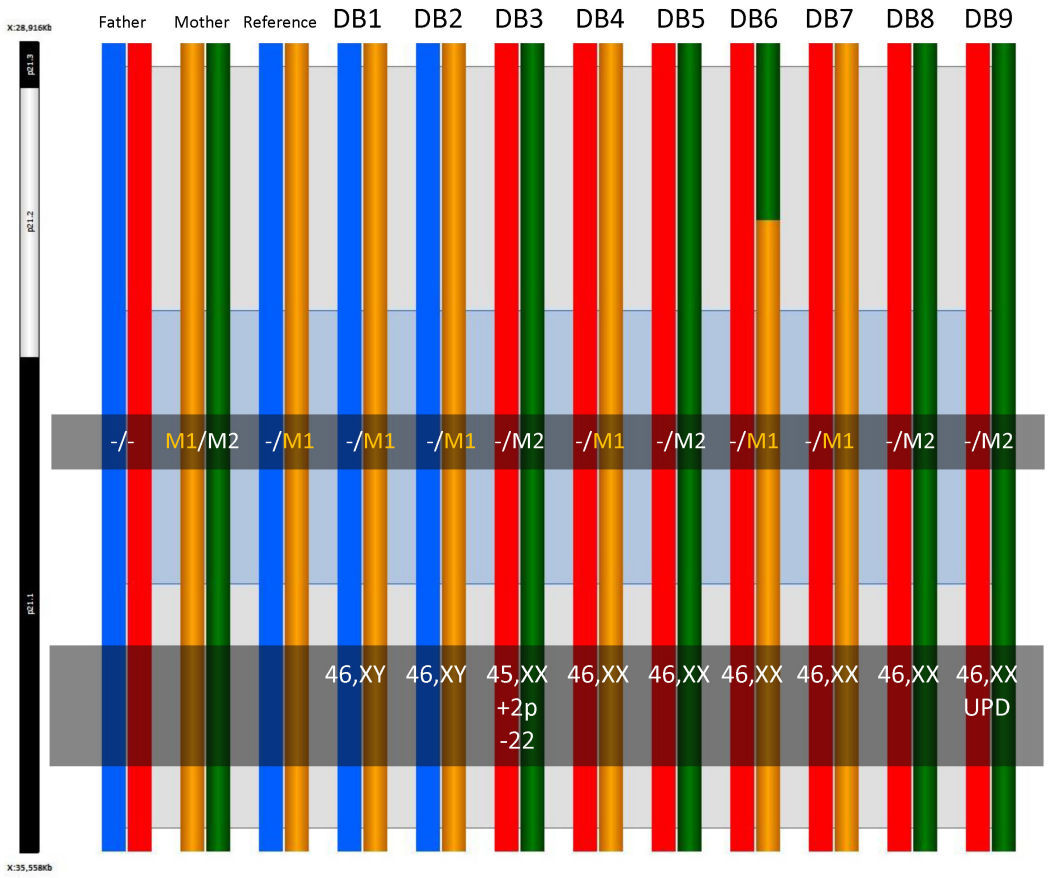

Fig. 2. Family DB's haploblock chart of karyomapping analysis. Haploblock chart of DMD exon 8–9 duplication from karyomapping (BlueFuse Multi software) using SNP array information (Illumina HumanKaryomap-12 DNA Analysis Kit) and multiplex fluorescent PCR (F-PCR) for the couples at risk of having DMD exon 8–9 duplication offspring (family DB). DNA of the affected son was employed as the reference. Haplotyping of DMD gene was demonstrated together with linkage analysis of short tandemly repeats (STR) and chromosome analysis results.

A mixture of 1 μL of purified mini-sequencing product, 1 μL of GeneScan^{TM}-120LIZ® (GenePlus Co., Ltd.) size standard and 10 μL of deionised formamide (GenePlus Co., Ltd.) was prepared and heated to 95 °C for 5 min. Denatured samples were subjected to capillary electrophoresis using POP-7® (5 s injection time, 15,000 V, 60 °C, 24 min). Data was analyzed by GeneMapper® software version 4.0. Color of individual peaks was interpreted as A (Green, dR6G dye), C (Yellow/Black, dTAMRA^{TM} dye), G (Blue, dR110 dye) and T (Red, dROX^{TM} dye) [44].

3. Results

Two clinical PGT-M cycles for DMD were performed. Nine embryos with good morphology from each patient were chosen for PGT-M using a SNP with karyomapping analysis. DNA of the mother of the patient who is a carrier was employed as the reference. Karyomapping results of family DA (DMD c.895G>T (E299X)) revealed three normal (embryos No. DA4, DA6 and DA7), two carriers (embryos No. DA1 and DA9), two affected (embryos No. DA3 and DA5) and two with intragenic recombination (embryos No. DA2 and DA8) (Fig. 1 and Table 2). Mutation analysis using multiplex fluorescent PCR incorporating with mini-sequencing and microsatellites-based linkage analysis confirmed haplotyping results in all 9 embryos (Tables 2 and Supplementary Table 1). In addition, PCR provided genotyping results in the further 15 embryos (5 normal, embryos No. DA14, DA16, DA18, DA22 and DA24; 7 carriers, embryos No. DA11, DA12, DA13, DA15, DA17, DA21 and DA23; and 3 affected, embryos No. DA10, DA19 and DA20). Polymorphic marker analysis revealed the absence of extraneous DNA contamination.

According to CNV information from karyomapping, three normal (two male, embryos No. DA4 and DA7 and one female, embryo No. DA6) and two carrier (both female, embryos No. DA1 and DA9) embryos with chromosomal balance were fulfilled for transfer (Table 2). During the first embryo transfer, one normal female embryo (embryo No.

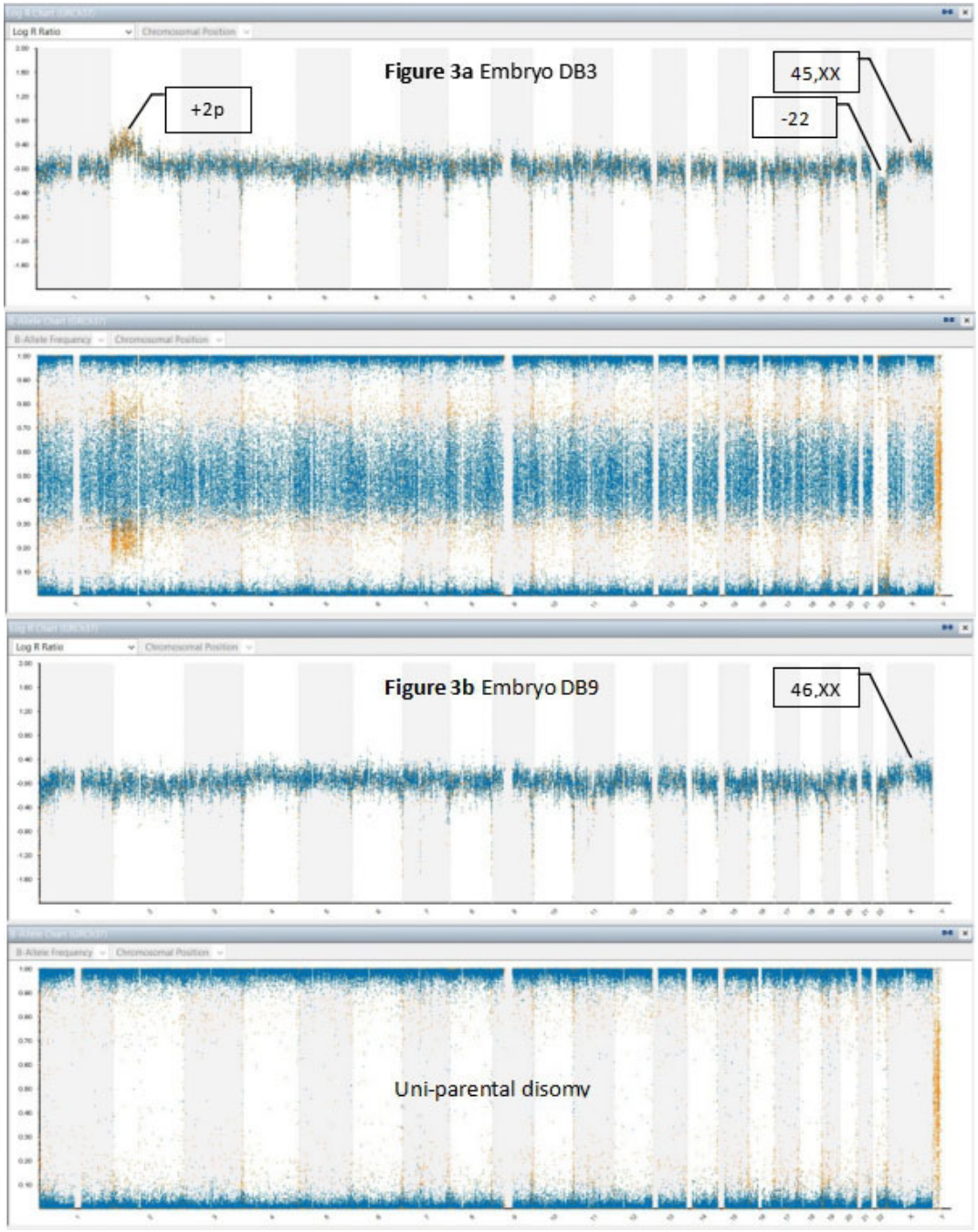

Fig. 3. Family DB's chromosome balance analysis from aSNP results. (a) shows copy number variation (CNV) of embryo DB3, 45,XX, +2p, –22. (b) shows CNV of embryo DB9, 46,XX, uniparental disomy of every chromosome.

DA6) was chosen with no resulting pregnancy. In the second transfer, one normal male embryo (embryo No. DA4) was transferred with a resulting normal male infant. Considering that PND procedures result in increased miscarriage rate, the patient refused PND. Postnatal DNA analysis confirmed the PGT results.

Nine embryos of family DB were chosen for PGT-M using a SNP with karyomapping analysis. DNA of the affected son was employed as the reference. Karyomapping results of family DB (DMD exon 8–9 duplication) revealed four normal (embryos No. DB3, DB5, DB8 and DB9), two carriers (embryos No. DB4 and DB7), two affected (embryos No. DB1 and DB2) and one with intragenic recombination (embryo No. 6) (Fig. 2). Microsatellites-based linkage analysis confirmed haplotyping results in all embryos. Additionally, cytogenetic analysis from SNP information demonstrated one normal embryo chromosomally unbalanced, i.e., 45,XX, +2P, –22 (embryo No. DB3) (Fig. 3a) and one normal embryo with uniparental disomy of every chromosome (UPD, embryo No. DB9) (Fig. 3b). Both sets of the chromosomes were maternal,

ie unimaternal disomy. Therefore, two normal (both female, embryos No. DB5 and DB8) and two carrier (both female, embryos No. DB4 and DB7) embryos that were chromosomally balanced were fulfilled for transfer (Table 3). All are being cryopreserved for later embryo transfer. Polymorphic marker analysis revealed the absence of extraneous DNA contamination.

4. Discussion

In this study, high resolution SNP array provided haplotyping-based diagnosis of DMD in 2 clinical PGT-M cycles. Karyomapping results were verified by PCR-based analysis. Novel multiplex PCR incorporating with mini-sequencing and fluorescent PCR was developed for DMD c895G>T (E299X) mutation detection and STR-based linkage analysis (family DA). STR-based linkage analysis was employed for DMD exon 8 and 9 duplication mutation (family DB). PCR-based analysis confirmed haplotyping in all embryos. Interestingly, three ambiguous embryos with intragenic recombination from karyotyping analysis were revealed to be normal (embryo No. DA2) and carrier (embryos No. DA8 and DB6) embryos from PCR. This demonstrated that PCR-based techniques provided more definitive results in some embryos. Based on karyomapping alone, these three embryos will not be transferred.

PCR can analyze as many embryos as needed with lower extra expense, while high additional expense is major concern for microarray and prevents some embryos from analysis as in family DA's. In family DA, from all 24 embryos with good quality, only nine were analyzed by karyomapping, while all 24 were analyzed by PCR. One kit of Illu mina HumanKaryomap-12 DNA Analysis Kit can analyzed 12 samples at a time, sparing three samples for the parents and one close relative as references, it is possible to analyze up to 9 embryos in one kit. Analyzing more embryos will double or triple the cost of diagnosis. Therefore, karyomapping results were available for only 9 embryos, while PCR results were presented for all embryos of family DA in this study (Table 2).

The advantages of karyomapping over the conventional PCR-based diagnosis include the diagnosis of duplication or large insertion (i.e., family DB's) and deletion mutations with unknown breakpoints, rescuing PCR results with ADO and the additional CNV information. Due to the advantage of SNP, origins of chromosomal gain and loss and UPD can be revealed. In this study, embryos No. DB3 and DB9 were found to be 45,XX, +2p, –22 and uni-maternal disomy, respectively. All gained and lost chromosomes belonged to the mother. It was demonstrated that karyomapping can analyze both point mutation (family DA) and large duplication (family DB) without the need to have protocol modifications. Therefore, karyomapping is a widely applicable PGT-M protocol.

DMD is an X-linked recessive disorder, therefore, male offspring can be either normal or affected, while female offspring can be either normal, carrier or affected. In this study, the mothers of both families were carriers of mutant genes, but the fathers did not carry any mutant genes. Therefore, PGT-M for DMD focused on maternal DMD alleles as there was no need to analyze paternal allele.

Interestingly, when the pathogenic variant is known, karyomapping can be used independently for PGT because aCGH can identify any recombination events eli minating any misdiagnosis from recombination. In families with a history of muscular dystrophy with no known pathogenic variants, a third are new mutations where the mothers' pathogenic variant is not known. Pprocedures of PGT for these families are very challenging. Types of particular muscular dystrophy need to be confirmed by clinical geneticists using clinical criteria, biochemical assays and histology. When the particular type of muscular dystrophy was confirmed, whole exome sequencing (WES) is performed in the members of the family including probands. Candidate genes for the particular type of muscular dystrophy are focused. Bioinformatics can be helpful in accelerating mutation identification process with PGT then being performed for the families.

The strength of this study is the ability of performed karyomapping along with PCR analysis for PGT of DMD in two families. Karyomapping provided haplotyping based diagnosis and chromosome balance information of the embryos. PCR revealed direct mutation analysis (family DA) and microsatellites based linkage analysis (both families) results. DMD families in this study possessed single nucleotide mutation (c895G>T (E299X)) and large duplication (exon 8–9 duplication) mutation. Therefore, karyomapping was validated for both types of mutations. The limitation of this study is that a CGH with karyomapping is expensive. Therefore, limited number of embryos were analyzed due to fiscal restraints.

5. Conclusions

Two clinical PGT-M cycles using karyomapping were performed for both families at risk of having DMD (c895G>T (E299X) and exon 8–9 duplication mutations) offspring. One healthy baby was resulted from the evaluation. This study exhibits that, in addition to monogenic haplotyping, a SNP provides the benefit of extra information of chromosome balance and parental origins, i.e., uniparental disomy in one of the embryos. Karyomapping can omit the risk of transfer of chromosomally unbalanced embryos. This is particularly useful in patients with advanced age. Disadvantages of karyomapping are that some embryos may be reported as ambiguous due to intra-genic recombination and the expensive consumables utilized restrict the number of analyzed embryos. The newly developed multiplex fluorescent PCR and mini-sequencing protocol confirmed karyomapping results. The practical application of karyomapping is providing a haplotyping based PGT-M with additional CNV to families at risk of having offspring affected with DMD.

Table 2. Family DA's PGT-M results for DMD c895G>T (E299X(from karyomapping analysis (BlueFuse Multi software) using SNP-Array information (Illu mina HumanKaryomap-12 BeadChip) and mutation analysis using multiplex fluorescent PCR (F-PCR) and mini-sequencing for DMD c895G>T and short tandemly repeat (STR) linked markers i.e., 5'-5n4, DXS206, DXS1236 and DXS1214 and Amelogenin X/Y. Trophectoderm cells were biopsied from 24 day-5 embryos of the couples at risk of having DMD c895G>T offspring and went through whole genome amplification using multiple displacement amplification (MDA). Samples from 9 embryos were analyzed using karyomapping, all were analyzed by mini-sequencing and multiplex F-PCR.

Embryo No.	Mini-Sequencing DMDc895G>T analysis		STR1		STR2		STR3		STR4		AMXY		Gender	PCR results	Karyomapping analysis		Chromosome analysis	Karyomapping results	Conclusion results	Notes
			Intron4		Intron7		Intron49		Intron63											
			5'-5n4		DXS206		DXS1236		DXS1214											
	Alleles		Alleles		Alleles		Alleles		Alleles		Alleles									
	Normal	Mutant	Pat*	Mat*	Pat	Mat	Pat	Mat	Pat	Mat	119X	125Y			Pat	Mat				
Father	G	-	156		232		238		156		119	125	Male	Normal	P1				Normal	
Mother	G	T	134	148	228	228	254	232	162	152	119	-	Female	Carrier		M1/M2			Carrier	
Mother's mum	G	T	150	148	234	228	252	232	160	152	119	-	Female	Carrier		M1/M3			Carrier	
DA1	G	T	156	148	232	228	238	232	156	152	119	-	Female	Carrier		M1	46,XX	Carrier	Carrier	
DA2	G	-	156	134	232	228	238	232	156	152	119	-	Female	Normal		M1/M2	46,XX	Carrier or Normal	Ambiguous*	*Intragenic re-combination
DA3	-	T	-	148	-	228	-	232	-	152	119	125	Male	Affected		M1	46,XY	Affected	Affected	
DA4	G	-	-	134	-	228	-	254	-	162	119	125	Male	Normal		M2	46,XY	Normal	Normal	
DA5	-	T	-	148	-	228	-	232	-	152	119	125	Male	Affected		M1	46,XY	Affected	Affected	
DA6	G	-	156	134	232	228	238	254	156	162	119	-	Female	Normal		M2	46,XX	Normal	Normal	
DA7	G	-	-	134	-	228	-	254	-	162	119	125	Male	Normal		M2	46,XY	Normal	Normal	
DA8	G	T	156	148	232	228	238	232	156	152	119	-	Female	Carrier		M1/M2	46,XX	Carrier or Normal	Ambiguous*	*Intragenic re-combination
DA9	G	T	156	148	232	228	238	232	156	152	119	-	Female	Carrier		M1	46,XX	Carrier	Carrier	
DA10	-	T	-	148	-	228	-	232	-	152	119	125	Male	Affected					Affected	
DA11	G	T	156	148	232	228	-	232	-	152	119	-	Female	Carrier					Carrier	
DA12	G	T	156	148	232	228	238	232	156	152	119	-	Female	Carrier					Carrier	
DA13	G	T	156	148	232	228	238	232	156	152	119	-	Female	Carrier					Carrier	
DA14	G	-	-	134	-	228	-	254	-	162	119	125	Male	Normal					Normal	
DA15	G	T	156	148	232	228	238	232	156	152	119	-	Female	Carrier					Carrier	
DA16	G	-	156	134	232	228	238	254	156	162	119	-	Female	Normal					Normal	
DA17	G	T	156	148	232	228	238	232	156	152	119	-	Female	Carrier					Carrier	
DA18	G	-	156	134	232	228	238	254	156	162	119	-	Female	Normal					Normal	
DA19	-	T	-	148	-	228	-	232	-	152	119	125	Male	Affected					Affected	
DA20	-	T	-	148	-	228	-	232	-	152	119	125	Male	Affected					Affected	
DA21	G	T	156	148	232	228	238	232	156	152	119	-	Female	Carrier					Carrier	
DA22	G	-	-	134	-	228	-	254	-	162	119	125	Male	Normal					Normal	
DA23	G	T	156	148	232	228	238	232	156	152	119	-	Female	Carrier					Carrier	
DA24	G	-	156	134	232	228	238	254	156	162	119	-	Female	Normal					Normal	

*Pat, Paternal; *Mat, Maternal.

Table 3. Family DB's PGT-M results of DMD exon 8–9 duplication from karyomapping analysis (BlueFuse Multi software) using SNP Array information (Illumina HumanKaryomap-12 BeadChip) and multiplex fluorescent PCR (F-PCR) for short tandemly repeat (STR) linked markers for contamination detection and linkage analysis. Trophectoderm cells were biopsied from 9 day-5 embryos of the couples at risk of having DMD exon 8–9 duplication offspring and went through whole genome amplification using multiple displacement amplification (MDA). Samples from 9 embryos were analyzed using karyomapping and multiplex F-PCR.

Embryo No.	STR1 Intron 4		STR2 Intron 7		STR3 Intron 49		STR4 Intron 63		AMXY		Gender	PCR results	Karyomapping analysis		Chromosome analysis	Karyomapping results	Conclusion results	Notes
	Alleles		Alleles		Alleles		Alleles		Alleles									
	Pat*	Mat*	Pat	Mat	Pat	Mat	Pat	Mat	119	125			Pat	Mat				
Father	156		232		254		156		119	125	Male	Normal	-				Normal	
Mother	148	152	228	226	234	238	156	158	119	-	Female	Carrier		M1/M2			Carrier	
Son	-	152	-	226	-	238	-	158	119	125	Male	Affected	-	M1			Affected	
DB1	-	152	-	226	-	238	-	158	119	125	Male	Affected		M1	46,XY	Affected	Affected	
DB2	-	152	-	226	-	238	-	158	119	125	Male	Affected		M1	46,XY	Affected	Affected	
DB3	156	148	232	228	254	234	156	156	119	-	Female	Normal		M2	45,XX +2p,-22	Normal	Normal*	*chromosomal un-balanced
DB4	156	152	232	226	254	238	156	158	119	-	Female	Carrier		M1	46,XX	Carrier	Carrier	
DB5	156	148	232	228	254	234	156	156	119	-	Female	Normal		M2	46,XX	Normal	Normal	
DB6	156	152	232	226	254	238	156	158	119	-	Female	Carrier		M2/M1	46,XX	Carrier	Ambiguous	Intragenic recombination
DB7	156	152	232	226	254	238	156	158	119	-	Female	Carrier		M1	46,XX	Carrier	Carrier	
DB8	156	148	232	228	254	234	156	156	119	-	Female	Normal		M2	46,XX	Normal	Normal	
DA9	156	148	232	228	254	234	156	156	119	-	Female	Normal		M2	46,XX UPD	Normal	Normal**	**uni-maternal disomy of every chromosome

*Pat, Paternal; *Mat, Maternal.

This study demonstrated that karyomapping provides an accurate, quick, time saving procedure for protocol development and universal PGT-M method for monogenic disorders of various types of mutations (i.e., point mutation and duplication). Moreover, karyomapping offers the advantage of CNV and parental origin information which is a common abnormality in pre-implantation embryos.

Abbreviations

ADO, allele drop out; aSNP, single nucleotide polymorphism microarray; CNV, copy number variation; DMD, Duchenne muscular dystrophy; ICSI, intracytoplasmic sperm injection; IVF, *in vitro* fertilization; MDA, multiple displacement amplification; PCR, polymerase chain reaction; PGT-A, pre-implantation genetic testing for aneuploidy; PGT-M, pre-implantation genetic testing for monogenic disorders; PND, prenatal diagnosis; WGA, whole genome amplification.

Author contributions

Study conception and design were performed by SM, SP and WP. Ovarian stimulation, oocytes collection and embryology laboratory were performed by TP and SM. aSNP and karyomapping analysis were performed by RS, SM, SP and WP. PCR analysis and mini-sequencing were performed by WS, SP and WP. Data collection and analysis were performed by SP, SM and WP. CT took care of clinical diagnosis and assessment. Prenatal and postnatal diagnosis were performed by TT and WP. SM and SP contributed equally to this work. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Informed consent of all participants were obtained. The project was approved by the Research Ethics Committee of Faculty of Medicine, Chiang Mai University (OBG-2562-06117).

Acknowledgment

Thanks to all the peer reviewers for their opinions and suggestions.

Funding

This study was supported by the National Research Council of Thailand and Chiang Mai University Research Fund (No. CMU-2563).

Conflict of interest

The authors declare no conflict of interest.

Supplementary material

Supplementary material associated with this article can be found, in the online version, at https://ceog.imrpress.com/EN/10.31083/j.ceog4805187.

References

[1] Ryder S, Leadley RM, Armstrong N, Westwood M, de Kock S, Butt T, *et al.* The burden, epidemiology, costs and treatment for Duchenne muscular dystrophy: an evidence review. Orphanet Journal of Rare Diseases. 2017; 12: 79.

[2] Hoffman EP, Brown RH Jr, Kunkel LM. Dystrophin: the protein product of the Duchenne muscular dystrophy locus. Cell. 1987; 51: 919–928.

[3] Koenig M, Hoffman EP, Bertelson CJ, Monaco AP, Feener C, Kunkel LM. Complete cloning of the duchenne muscular dystrophy (DMD) cDNA and preli minary genomic organization of the DMD gene in normal and affected individuals. Cell. 1987; 50: 509–517.

[4] Thangarajh M. The Dystrophinopathies. Continuum. 2019; 25: 1619–1639.

[5] Perloff JK. Cardiac rhythm and conduction in Duchenne's muscular dystrophy: a prospective study of 20 patients. Journal of the American College of Cardiology. 1984; 3: 1263–1268.

[6] Harper PS. Isolating the gene for Duchenne muscular dystrophy. British Medical Journal. 1986; 293: 773–774.

[7] Duchenne. The Pathology of Paralysis with Muscular Degeneration (Paralysie Myosclerotique), or Paralysis with Apparent Hypertrophy. British Medical Journal. 1867; 2: 541–542.

[8] [8] England SB, Nicholson LV, Johnson MA, Forrest SM, Love DR, Zubrzycka-Gaarn EE, *et al.* Very mild muscular dystrophy associated with the deletion of 46% of dystrophin. Nature. 1990; 343: 180–182.

[9] [9] Takeshima Y, Yagi M, Okizuka Y, Awano H, Zhang Z, Yamauchi Y, *et al.* Mutation spectrum of the dystrophin gene in 442 Duchenne/Becker muscular dystrophy cases from one Japanese referral center. Journal of Human Genetics. 2010; 55: 379–388.

[10] Wang H, Xu Y, Liu X, Wang L, Jiang W, Xiao B, *et al.* Prenatal diagnosis of Duchenne muscular dystrophy in 131 Chinese families with dystrophinopathy. Prenatal Diagnosis. 2017; 37: 356–364.

[11] Verhaart IEC, Aartsma-Rus A. Therapeutic developments for Duchenne muscular dystrophy. Nature Reviews Neurology. 2019; 15: 373–386.

[12] Gatta V, Scarciolla O, Gaspari AR, Palka C, De Angelis MV, Di Muzio A, *et al.* Identification of deletions and duplications of the DMD gene in affected males and carrier females by multiple ligation probe amplification (MLPA). Human Genetics. 2005; 117: 92–98.

[13] Cremonesi L, Firpo S, Ferrari M, Righetti PG, Gelfi C. Double-Gradient DGGE for Optimized Detection of DNA Point Mutations. BioTechniques. 1997; 22: 326–330.

[14] Gardner RJ, Bobrow M, Roberts RG. The identification of point mutations in Duchenne muscular dystrophy patients by using reverse-transcription PCR and the protein truncation test. American Journal of Human Genetics. 1995; 57: 311–320.

[15] Xie S, Lan Z, Qu N, Wei X, Yu P, Zhu Q, *et al.* Detection of truncated dystrophin lacking the C-ter minal domain in a Chinese pedigree by next-generation sequencing. Gene. 2012; 499: 139–142.

[16] Mahoney MJ, Haseltine FP, Hobbins JC, Banker BQ, Caskey CT, Golbus MS. Prenatal diagnosis of Duchenne's muscular dystrophy. New England Journal of Medicine. 1977; 297: 968–973.

[17] Emery AE, Burt D, Dubowitz V, Rocker I, Donnai D, Harris R, *et al.* Antenatal diagnosis of Duchenne muscular dystrophy. Lancet. 1979; 1: 847–849.

[18] Williams H, Brown CS, Thomas NS, Harper PS, Roberts A, Ppadhyaya M, *et al.* First trimester fetal sexing in pregnancy at risk for Duchenne muscular dystrophy. Lancet. 1983; 2: 568–569.

[19] Bakker E, Hofker MH, Goor N, Mandel JL, Wrogemann K, Davies KE, *et al.* Prenatal diagnosis and carrier detection of Duchenne muscular dystrophy with closely linked RFLPs. Lancet. 1985; 1: 655–658.

[20] Clemens PR, Fenwick RG, Chamberlain JS, Gibbs RA, de Andrade M, Chakraborty R, *et al.* Carrier detection and prenatal diagnosis

in Duchenne and Becker muscular dystrophy families, using dinucleotide repeat polymorphisms. American Journal of Human Genetics. 1991; 49: 951–960.
[21] Evans MI, Greb A, Kunkel LM, Sacks AJ, Johnson MP, Boehm C, *et al.* In utero fetal muscle biopsy for the diagnosis of Duchenne muscular dystrophy. American Journal of Obstetrics and Gynecology. 1991; 165: 728–732.
[22] Kuller JA, Hoffman EP, Fries MH, Golbus MS. Prenatal diagnosis of Duchenne muscular dystrophy by fetal muscle biopsy. Human Genetics. 1992; 90: 34–40.
[23] Srisupundit K, Tongsong T, Piyamongkol W, Sirichotiyakul S, Tongprasert F, Leuwan S, *et al.* Chorionic villous sampling-related complications: a cohort study. Journal of Maternal-Fetal & Neonatal Medicine. 2020; 33: 1901–1905.
[24] Tongsong T, Wanapirak C, Sirivatanapa P, Piyamongkol W, Sirichotiyakul S, Yampochai A. Amniocentesis-related fetal loss: a cohort study. Obstetrics and Gynecology. 1998; 92: 64–67.
[25] Piyamongkol W, Wanapirak C, Sirichotiyakul S, Srisupundit K, Tongsong T. A comparison of cordocentesis outcomes between early and conventional procedures. Journal of Maternal-Fetal & Neonatal Medicine. 2012; 25: 2298–2301.
[26] Beksac MS, Tanacan A, Aydin Hakli D, Orgul G, Soyak B, Balci Hayta B, *et al.* Gestational Outcomes of Pregnant Women who have had Invasive Prenatal Testing for the Prenatal Diagnosis of Duchenne Muscular Dystrophy. Journal of Pregnancy. 2018; 2018: 9718316.
[27] Handyside AH, Lesko JG, Tarín JJ, Winston RM, Hughes MR. Birth of a normal girl after in vitro fertilization and preimplantation diagnostic testing for cystic fibrosis. New England Journal of Medicine. 1992; 327: 905–909.
[28] Ray PF, Vekemans M, Munnich A. Single cell multiplex PCR amplification of five dystrophin gene exons combined with gender deter mination. Molecular Human Reproduction. 2001; 7: 489–494.
[29] Girardet A, Hamamah S, Déchaud H, Anahory T, Coubes C, Hédon B, *et al.* Specific detection of deleted and non-deleted dystrophin exons together with gender assignment in preimplantation genetic diagnosis of Duchenne muscular dystrophy. Molecular Human Reproduction. 2003; 9: 421–427.
[30] Malcov M, Ben-Yosef D, Schwartz T, Mey-Raz N, Azem F, Lessing JB, *et al.* Preimplantation genetic diagnosis (PGD) for Duchenne muscular dystrophy (DMD) by triplex-nested PCR. Prenatal Diagnosis. 2005; 25: 1200–1205.
[31] Malmgren H, White I, Johansson S, Levkov L, Iwarsson E, Fridström M, *et al.* PGD for dystrophin gene deletions using fluorescence in situ hybridization. Molecular Human Reproduction. 2006; 12: 353–356.
[32] Ren Z, Zeng HT, Xu YW, Zhuang GL, Deng J, Zhang C, *et al.* Preimplantation genetic diagnosis for Duchenne muscular dystrophy by multiple displacement amplification. Fertility and Sterility. 2009; 91: 359–364.
[33] Girardet A, Fernandez C, Claustres M. Rapid and powerful decaplex and dodecaplex PGD protocols for Duchenne muscular dystrophy. Reproductive Biomedicine Online. 2009; 19: 830–837.
[34] Handyside AH, Harton GL, Mariani B, Thornhill AR, Affara N, Shaw MA, *et al.* Karyomapping: a universal method for genome wide analysis of genetic disease based on mapping crossovers between parental haplotypes. Journal of Medical Genetics. 2010; 47: 651–658.
[35] Natesan SA, Bladon AJ, Coskun S, Qubbaj W, Prates R, Munne S, *et al.* Genome-wide karyomapping accurately identifies the inheritance of single-gene defects in human preimplantation embryos *in vitro*. Genetics in Medicine. 2014; 16: 838–845.
[36] Natesan SA, Handyside AH, Thornhill AR, Ottolini CS, Sage K, Summers MC, *et al.* Live birth after PGD with confirmation by a comprehensive approach (karyomapping) for simultaneous detection of monogenic and chromosomal disorders. Reproductive Biomedicine Online. 2014; 29: 600–605.
[37] Thornhill AR, Handyside AH, Ottolini C, Natesan SA, Taylor J, Sage K, *et al.* Karyomapping-a comprehensive means of simultaneous monogenic and cytogenetic PGD: comparison with standard approaches in real time for Marfan syndrome. Journal of Assisted Reproduction and Genetics. 2015; 32: 347–356.
[38] Sermon K, Lissens W, Nagy ZP, Van Steirteghem A, Liebaers I. Simultaneous amplification of the two most frequent mutations of infantile Tay-Sachs disease in single blastomeres. Human Reproduction. 1995; 10: 2214–2217.
[39] Handyside AH, Pattinson JK, Penketh RJ, Delhanty JD, Winston RM, Tuddenham EG. Biopsy of human preimplantation embryos and sexing by DNA amplification. Lancet. 1989; 1: 347–349.
[40] King SC, Roche AL, Passos-Bueno MR, Takata R, Zatz M, Cockburn DJ, *et al.* Molecular characterization of further dystrophin gene microsatellites. Molecular and Cellular Probes. 1995; 9: 361–370.
[41] McNaughton JC, Hughes G, Jones WA, Stockwell PA, Klamut HJ, Petersen GB. The evolution of an intron: analysis of a long, deletion–prone intron in the human dystrophin gene. Genomics. 1997; 40: 294–304.
[42] Dib C, Fauré S, Fizames C, Samson D, Drouot N, Vignal A, *et al.* A comprehensive genetic map of the human genome based on 5,264 microsatellites. Nature. 1996; 380: 152–154.
[43] Piyamongkol W, Vutyavanich T, Piyamongkol S, Wells D, Kunaviktikul C, Tongsong T, *et al.* A successful strategy for Preimplantation Genetic Diagnosis of beta-thalassemia and simultaneous detection of Down's syndrome using multiplex fluorescent PCR. Journal of the Medical Association of Thailand. 2006; 89: 918–927.
[44] Bermudez MG, Piyamongkol W, Tomaz S, Dudman E, Sherlock JK, Wells D. Single-cell sequencing and mini-sequencing for preimplantation genetic diagnosis. Prenatal Diagnosis. 2003; 23: 669–677.