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

Suchada Mongkolchaipak1,2, Sirivipa Piyamongkol3,4, Chutithep Teekaput3, Rungrithwa Sirapat1, Wanwisa Suriya4, Tawiwant Pantasri2, Theera Tongsong4, Wirawit Piyamongkol4,5

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
5 Suchada IVF Center, Sriracha, 20130 Chon Buri, Thailand

*Correspondence: wirawit.p@cmu.ac.th (Wirawit Piyamongkol)
† These authors contributed equally.

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 Ca2+ disturbs the Ca2+-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 determination 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 give the couple a difficult decision as whether to terminate 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. Preliminary 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 consuming.

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 obtaining 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 termination 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 contamination. 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 DBL (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 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 (Bio-Active Co. Ltd., Bangkok, Thailand) according to the manufacturer’s instructions [34]. SNP genotyping information was analyzed using BlueFuse Multi software version 4.5 (Bio-Active 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 contam ination detection. Amelogenin X/Y [39] for gender determination was added. Multiple microsatellites within the dystrophin gene, including 5’-Sn4 (Intron 4) [40], DXS206 (Intron 7) [41], DXS1236 (Intron 49) [20] and DXS1214 (Intron 63) [42]) were employed. The PCR mixture consisted of 200 nM 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 HotStar-Taq 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’-Sn4, DXS206, DXS1236, DXS1214 fragments were labeled with 6FAM (blue), VIC® (green), 6FAM (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™-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™ 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™ 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 determination were exhibited.

| Primers     | Location on DMD gene | Sequences Forward | Sequences Reverse | Fragment length (bp) | References |
|-------------|----------------------|-------------------|-------------------|----------------------|------------|
| DMD c.895G>T | Exon 11 forward      | 5′-GGCCGGGTTGGTAATATTCT-3′ | 5′-CCTGAGGCATTCCCATCTT-3′ | 126 | OMIM: NM_004010 |
|             | reverse              |                   |                   |                      |            |
| DMD c.895G>T | Exon 11 mini-sequencing | 5′-TCAGAAGATGAGAAATCTGGTAATTTTCTCTCTCTCTCTGTCGT-3′ | 5′-GAATCAAGCTTCTCTCTGTCGTAATTTTCTCTCTCTCTCTGTCGT-3′ | 134–186 | [40] |
| 5′-5n4      | Intron 4 forward      | 5′-GAAGGGAAAATGATGAATAAACT-3′ | 5′-GTCAGAAGTATTTTCTCTCTCTCTGTCGT-3′ | 134–186 | [40] |
|             | reverse              |                   |                   |                      |            |
| DXS206      | Intron 7 forward      | 5′-TTCTGGTTTTTCTGTCGT-3′ | 5′-GAATCAAGCTTCTCTCTGTCGTAATTTTCTCTCTCTCTGTCGT-3′ | 218–236 | [41] |
|             | reverse              |                   |                   |                      |            |
| DXS1236     | Intron 49 forward     | 5′-GCCAGGTTCTCTCTCTGTCGT-3′ | 5′-CGATTCTCTCTCTGTCGTAATTTTCTCTCTCTCTGTCGT-3′ | 226–260 | [20] |
|             | reverse              |                   |                   |                      |            |
| DXS1214     | Intron 63 forward     | 5′-GCCAGGTTCTCTCTCTGTCGT-3′ | 5′-CGATTCTCTCTCTGTCGTAATTTTCTCTCTCTCTGTCGT-3′ | 148–162 | [42] |
|             | reverse              |                   |                   |                      |            |
| Amelogenin X/Y | forward              | 5′-GCTGAGGGCACCACACTCAG-3′ | 5′-CGTGGGCCTCTGTAAGAGATAG-3′ | X 119 | [39] |
|             | reverse              |                   |                   |                      |            |

Fig. 1. Family DA's haploblock chart of karyomapping analysis. Haploblock chart of DMD c.895G>T (E299X) from karyomapping (BlueFuse Multi software) using SNP array information (Illumina HumanKaryomap-12 DNA Analysis Kit) and mutation analysis using multiplex fluorescent PCR (F-PCR) and mini-sequencing for DMD c.895G>T for the couples at risk of having DMD c.895G>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\textsuperscript{TM}-120LIZ\textsuperscript{®} (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\textsubscript{7}® (5 s injection time, 15,000 V, 60 °C, 24 min). Data was analyzed by GeneMapper\textsuperscript{®} software version 4.0. Color of individual peaks was interpreted as A (Green, dR6G dye), C (Yellow/Black, dTAMRA\textsuperscript{TM} dye), G (Blue, dR110 dye) and T (Red, dROX\textsuperscript{TM} dye) \cite{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.
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 unimutational 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 Ilu 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 unimutational 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 eliminating 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. Procedures 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. Advantages 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.
| Embryo No. | Mini-Sequencing
| DMD \( c^{895G>T} \) 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 | Alleles |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Father | G | - | Pat | Mat | Pat | Mat | Pat | Mat | 119X | 125Y | Male | Normal | P1 | Normal |
| Mother | G | T | 134 | 148 | 228 | 228 | 254 | 232 | 162 | 152 | Female | Carrier | M1/M2 | Carrier |
| Mother's mum | G | T | 150 | 148 | 234 | 228 | 252 | 232 | 160 | 152 | Female | Carrier | M1/M3 | Carrier |
| DA1 | G | T | 156 | 148 | 232 | 228 | 232 | 156 | 152 | 119 | Female | Carrier | M1 | 46,XX | Carrier |
| DA2 | G | - | 156 | 134 | 232 | 228 | 232 | 156 | 152 | 119 | Female | Normal | M1/M2 | 46,XX | Carrier or Normal |
| | | | | | | | | | | | | | | | Ambiguous* | Intragenic recombination |
| DA3 | - | T | - | 148 | - | 228 | - | 232 | - | 152 | 119 | 125 | Male | Affected | M1 | 46,XY | Affected |
| DA4 | G | - | - | 134 | - | 228 | - | 254 | - | 162 | 119 | 125 | Male | Normal | M2 | 46,XY | Normal |
| DA5 | - | T | - | 148 | - | 228 | - | 232 | - | 152 | 119 | 125 | Male | Affected | M1 | 46,XY | Affected |
| DA6 | G | - | 156 | 134 | 232 | 228 | 254 | 232 | 162 | 119 | Female | Normal | M2 | 46,XY | Normal |
| DA7 | G | - | - | 134 | - | 228 | - | 254 | - | 162 | 119 | 125 | Male | Normal | M2 | 46,XY | Normal |
| DA8 | G | T | 156 | 148 | 232 | 228 | 232 | 156 | 152 | 119 | Female | Carrier | M1/M2 | 46,XX | Carrier or Normal |
| | | | | | | | | | | | | | | | Ambiguous* | Intragenic recombination |
| DA9 | G | T | 156 | 148 | 232 | 228 | 232 | 156 | 152 | 119 | Female | Carrier | M1 | 46,XX | Carrier |
| DA10 | - | T | - | 148 | - | 228 | - | 232 | - | 152 | 119 | 125 | Male | Affected | | Affected |
| DA11 | G | T | 156 | 148 | 232 | 228 | - | 232 | - | 152 | 119 | 125 | Female | Carrier | | Carrier |
| DA12 | G | T | 156 | 148 | 232 | 228 | 232 | 156 | 152 | 119 | Female | Carrier | | | Carrier |
| DA13 | G | T | 156 | 148 | 232 | 228 | 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 | 232 | 156 | 152 | 119 | Female | Carrier | | | Carrier |
| DA16 | G | - | 156 | 134 | 232 | 228 | 254 | 232 | 162 | 119 | Female | Normal | | | Normal |
| DA17 | G | T | 156 | 148 | 232 | 228 | 232 | 156 | 152 | 119 | Female | Carrier | | | Carrier |
| DA18 | G | - | 156 | 134 | 232 | 228 | 254 | 232 | 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 | 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 | 232 | 156 | 152 | 119 | Female | Carrier | | | Carrier |
| DA24 | G | - | 156 | 134 | 232 | 228 | 254 | 232 | 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. Trophoectoderm 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 |
|------------|----------------|----------------|----------------|----------------|-------|--------|-------------|-----------------------|-------------------|---------------------|-------------------|-------|
| Father     | 156 232        | 254 156        | 119 125        | Male           | Normal | -      | Normal     | Normal                | Normal            | Normal              | Carrier           |       |
| Mother     | 148 152        | 228 226        | 234 238        | 156 158        | Female  | Carrier | M1/M2      | Affected              | Affected          | Affected            | Carrier           |       |
| Son        | - 152          | - 226          | - 238          | - 158          | Male    | Affected | M1         | 46,XY                 | Affected          | Affected            | Affected          |       |
| DB1        | - 152          | - 226          | - 238          | - 158          | Female  | Carrier | M1         | 46,XY                 | Affected          | Affected            | Affected          |       |
| DB2        | - 152          | - 226          | - 238          | - 158          | Male    | Affected | M1         | 46,XY                 | Affected          | Affected            | Affected          |       |
| DB3        | 156 148        | 232 228        | 254 234        | 156 156        | Female  | Normal  | M2         | +2p,-22               | Normal            | Normal*             | * chromosomal un-balanced |       |
| DB4        | 156 152        | 232 226        | 254 238        | 156 158        | Female  | Carrier | M1         | 46,XX                 | Carrier           | Carrier             |       |
| DB5        | 156 148        | 232 226        | 254 238        | 156 158        | Female  | Normal  | M2         | 46,XX                 | Normal            | Normal              |       |
| DB6        | 156 152        | 232 226        | 254 238        | 156 158        | Female  | Carrier | M2/M1      | 46,XX                 | Carrier           | Ambiguous           | Intragenic recombination |       |
| DB7        | 156 152        | 232 226        | 254 238        | 156 158        | Female  | Carrier | M1         | 46,XX                 | Carrier           | Carrier             |       |
| DB8        | 156 148        | 232 228        | 254 234        | 156 156        | Female  | Normal  | M2         | 46,XX                 | Normal            | Normal              |       |
| DA9        | 156 148        | 232 228        | 254 234        | 156 156        | 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 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).

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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.

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