Prenatal diagnosis of Duchenne muscular dystrophy and cytogenetic analysis in 303 Chinese families

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Abstract

Background: Duchenne muscular dystrophy (DMD) has showed a wide spectrum of mutations in the dystrophin gene including exon deletions, duplications and small mutations. This retrospective study was to supply information of the DMD mutational spectrum in 303 Chinese families and further offer 5-year clinical experience of DMD genetic counselling and prenatal diagnosis.

Methods: In this retrospective study, 305 pregnancies in 303 pregnant women who has a birth history of DMD patients underwent prenatal diagnosis using multiplex ligation-dependent probe amplification (MLPA) followed by Sanger sequencing between 2014 and 2018. Karyotype analysis was performed to exclude fetal abnormal karyotype.

Results: The detection rate of DMD gene mutation in 303 probands was 97.7% with 7 families having a negative genetic diagnosis. The mutational spectrum comprised of large arrangements in 288/303 (95.0%) and small mutations in 8/303 (2.6%). 204 pregnant women did carrier testing among whom, 108 mothers had the same mutation as family proband. Of the 305 pregnancies underwent prenatal diagnosis, 55 of 173 male fetuses were affected. We also performed karyotype analysis and found 3 abnormal karyotypes of trisomy 21. We even found a fetus with DMD gene mutation and trisomy 21 in a same fetus by further analysis.

Conclusions: The distribution and mutation profile of 303 probands and 305 fetuses were demonstrated. Given the large samples provided in this study, the information is essential for genetic counselling and prenatal diagnosis in DMD families in China.

Background

Duchenne muscular dystrophy (DMD) is a progressive X-linked recessive, inherited myogenic disorder that affects approximate 1 in 3500 male live births[1]. Clinically, DMD is characterized by early onset with progressive proximal muscle weakness and atrophy[2]. Most of the patients will lose their ability to walk before the age of 12 years, and eventually develop respiratory insufficiency and dilated cardiomyopathy which could lead to heart failure and eventually death in the late teens or early 20s[3].

The genetic cause of DMD is due to the variants in the dystrophin gene which spans 2.4 million base
pairs on chromosome Xp21.2 and encodes dystrophin protein[4]. Dystrophin protein is a basic part of the dystrophin-glycoprotein complex (DGC), which provides a strong mechanical link between the extracellular matrix and actin cytoskeleton, maintaining the stability of muscle fibers[5]. The absence of dystrophin protein will cause progressive tissue damage, resulting in muscle weakness in DMD patients [5, 6].

A great number of studies have been described to define DMD mutation patterns in different populations[7, 8]. The majority of molecular defect in the DMD gene is the deletion of one or more exons, accounting for 65% of DMD patients and 6%-10% of DMD cases are caused by duplications[9]. Besides, the remaining patients with no detectable deletion or duplication are due to small mutations[10, 11].

Given the discovery and identified mutations of dystrophin gene, laboratory analysis of dystrophin gene has been a routine component of the clinical diagnosis of DMD[12]. With the development of technologies, there are numerous diagnostic testing methods available for genetic testing of DMD mutations[4]. MLPA or array comparative genome hybridization (aCGH) have significantly improved the detection rate of deletions and duplications in male patients and carrier females[13, 14]. Sanger sequencing of all the DMD exons or alternatively next generation sequencing (NGS) technology, in some laboratories, is used to detect point mutations. [10, 15].

Unfortunately, there is yet no effective cure for DMD to this day, which means the possible ways left to prevent DMD would be genetic counselling and prenatal diagnosis. In this study, we have described and compared DMD gene mutation pattern in 303 families, involving 305 pregnancies between 2014 and 2018 in our hospital. We shared our experience of prenatal diagnosis and genetic counselling procedure for DMD families.

Methods

**Prenatal diagnosis procedure for DMD families**

MLPA analysis covering all 79 exons of DMD gene was performed first to detect DMD deletion/duplication in 303 probands to detect the mutation type in each family. Sanger sequencing was used if the MLPA results were negative. The following procedure of carrier testing of mothers and
Prenatal diagnosis of fetuses were based on the mutation type of each proband. For the prenatal diagnosis, short tandem repeat (STR) analysis was applied in each case to rule out the mother contamination in the chorion villus and amniotic fluid and help to verify the MLPA results as a second method.

**Study population and ethics statement**

During the period between 2014 and 2018, we retrospectively enrolled 303 families who underwent genetic counseling and prenatal diagnosis for DMD at Peking Union Medical College Hospital. The inclusion criteria were as follows: (1) a history of DMD; (2) confirmed physical and biochemical examination finding or muscle biopsy; pregnancies in women related to probands with other muscular dystrophies or congenital myopathies were excluded. All the 303 families provided written informed consent to do the prenatal diagnosis and cytogenetic analysis. For the carrier testing, 204 mothers agreed and gave written informed consent to the study.

This study was approved by the ethics committee of Peking Union Medical College Hospital.

**DNA samples**

Peripheral blood samples (4 ml) in EDTA were collected from all 303 DMD probands and 204 pregnant women. Genomic DNA was then extracted from peripheral blood by using the QIAamp DNA Blood Mini Kit (Qiagen, Germany) according to the protocol of the manufacturer.

For prenatal diagnosis, chorionic villus sampling (CVS) was performed in pregnancies at 10 to 13 (+6d) weeks of gestation and amniocentesis (AC) was performed in pregnancies at 16 to 22 (+6d) weeks of gestation, under the ultrasound guidance. Chorion villus specimens were dissected under the microscope to avoid maternal contamination. All the samples were immediately transferred to the lab for further process. Fetal DNA was extracted from chorion villus and amniotic fluid using the QIAamp DNA Mini Kit (Qiagen, Germany) according to the instructions.

**MLPA analysis**

MLPA reactions were performed to detect deletions and duplications using a commercial SALSA MLPA kit from MRC Holland (Amsterdam, The Netherlands) with probe sets P034 and P035 according to the manufacturer`s instructions. The PCR products were separated by capillary electrophoresis on an ABI
3130 genetic analyzer (Applied Biosystems, USA). Data was analyzed using Coffalyser software (MRC Holland).

**Sanger sequencing**

Polymerase chain reaction (PCR) and direct sequencing was performed when no single-exon deletion or duplication was found by MLPA. PCR was carried out in a GeneAmp PCR System 9700 thermocycler (Applied Biosystems, USA). The amplified products were sequenced on ABI 3130XL DNA Analyzer (Applied Biosystems, USA) and mapped with DMD reference sequence (GenBank transcript ID: NM_004006).

**Short tandem repeat (STR) analysis**

Linkage analysis was performed to each family using STR markers scattering across the DMD gene. The forward primers were labeled with 5-carboxy fluorescein (FAM). PCR was carried out in a GeneAmp PCR System 9700 thermocycler (Applied Biosystems, USA). The PCR products were then separated by capillary electrophoresis on an ABI 3130 genetic analyzer (Applied Biosystems, USA). The GeneMapper v3.7 software (Applied Biosystems, USA) was used for data analysis.

**Karyotype analysis**

Amniotic fluid samples (20ml) were centrifuged at 1000r/min for 10 minutes. After discarding the supernatant, the cells were suspended in two culture bottles with 4ml culture medium (CHANG Amnio, USA) and cultured for 7 days at 37 °C and 5% CO2. Chorion villus was digested by 0.5% Trypsin (Life Sciences, USA) for 2 minutes and centrifuged at 1000r/min for 10minuts. The cells were then suspended in two culture bottles with 4ml culture medium (CHANG Amnio, USA) and cultured for 7 days at 37 °C and 5% CO2. Cells were then harvested and detained with Giemsa (Sigma, USA). For each sample, 5 karyograms were analyzed with 20 counts (abnormal karyotype with 50 counts). The resolution is 320 kb.

**Results**

**Mutation spectrum of probands in the DMD families**

For the 303 probands, the detection rate of DMD mutation is 97.7%. The families we included in our hospital were mainly DMD deletions and duplications. The DMD gene deletions were detected in
248/303 (81.8%) probands and DMD gene duplications were detected in 36/303 (11.9%) probands. In addition, four cases (1.3%) with both deletion and duplication were also detected. And the remaining 8/303 (2.6%) were small mutations (Figure 1).

**Deletion, duplication and small mutation patterns**

To further analyze the mutation patterns in 303 probands, each exon deletion times were accounted. We found 97 different deletion patterns. Interestingly, exon deletion hotspot was between exon 43-55 at the 3’ end of DMD gene (Figure 2A). In addition, the deletion of exon 50 was detected 102 times, which appeared to be the most detected exon deletion in the probands (Figure 2A). And the most frequent large deletion pattern was deletion of exon 48 to exon 50, which was detected 14 times (Figure 2B). Besides, the largest deletion was from exon 2 to exon 79. In 97 different deletion patterns, a single exon deletion pattern was also accounted for lots of proportion (21.6%). Furthermore, 30 duplication patterns were also detected in the probands. Likewise, two hotspots of duplication were also detected, exon 8-11 at the 5’ of the DMD gene and exon 51 at 3’ end (Figure 3A). In addition, we also found that the DMD gene exon 8-11 and exon 51-71 as the most common duplication patterns which appeared 4 times (Figure 3B). And the largest duplication extended 38 exons which is from exon 3 to exon 40. Complex rearrangements were also identified in four cases, three of which were deletion of exon 22 combined with duplication of exons 8 to 11.

However, a small part of the DMD families included in our hospital was small mutations including nonsense mutation, frameshift mutation and splice site mutation. A total of 8 (2.7%) different small mutations were identified in our population including 5 nonsense mutations, 2 frameshift mutations and 1 splice site mutation (Table 1). All in all, our findings indicate that many mutation patterns exist in the DMD gene.

**Carrier testing results for prenatal mothers**

To identify the carrier status of DMD gene mutation in pregnant mothers, 204 pregnant women who decided to do the carrier testing were screened according to the mutation types of the proband. Of the 204 cases, 108 (52.9%) cases were carriers and 96 (47.1%) showed no pathogenic mutations in the blood. A single exon deletion rate (20.5%) was much lower compared with multi exon deletions.
(79.5%) among the total deletion carriers (Table 2). And the rate of multi exon duplications showed 42.8% more inheritance than a single exon duplication among the total duplication carriers (Table 2). Even complex mutations that included deletions combined with duplications were found in two mothers. For the small mutations, the carrier rate is about 66.7% (Table 2).

**Prenatal diagnosis and cytogenetic analysis**

Among the 305 pregnancies, there were 173 (56.7%) male fetuses and 132 (43.3%) female fetuses. The mean age of mothers in pregnancies was 33.0 ±4.1 years. In order to test the heredity of the DMD gene mutation, we analyzed the mutation type of 305 pregnancies. The results showed that a total of 55 male fetuses (18%) prenatally diagnosed as disease-positive, 40 female fetuses (13.1%) carrying DMD gene mutations and the remaining fetuses (68.9%) were healthy. Among 224 collected chorion villus samples, there were 37 disease positive male fetuses (16.5%), 29 female carriers (13.0%) and 158 healthy fetuses (70.5%) (Table 3). In addition, we also collected 81 amniotic fluid samples and found 18 disease positive male fetuses (22.2%), 11 female carriers (13.6%) and 52 healthy fetuses (64.2%) (Table 3).

Surprisingly, we also found 3 fetuses with trisomy 21 (Down syndrome) by karyotype analysis and two of three fetuses were not affected by DMD. Moreover, we firstly discovered a male fetus with DMD gene mutation of exon 48 deletion and trisomy 21 (Figure 4). Thus, our analysis showed that prenatal diagnosis is crucial in DMD families to prevent the birth of DMD children. Prenatal diagnosis was suggested to mothers with a proband whether they carried the causative mutation or not.

**Discussion**

To this date, there is yet no curative treatment for DMD[16]. The medical cost of DMD has been a source of stress for affected families, compromising the life quality of the patient [17]. Given the poor prognosis for DMD patients and high cost burden for families, parental diagnosis of DMD is crucial and the key of reducing the birth of affected children in families with DMD birth history[18]. The detection of mutation pattern is essential, providing information for genetic counseling, prenatal diagnosis and gene therapy[19]. In this retrospective study, we detected DMD mutation patterns in 303 DMD probands and performed prenatal diagnosis for all the families during 5 yeas. We detected
248 exon deletions, including 97 different deletion patterns. And exon duplications were also identified in 36 probands, showing 30 different duplication distributions. Moreover, we identified 8 different small mutations including nonsense mutation, frameshift mutation and splice site mutation. And the detection rate of DMD mutation was 97.7%. The rate of deletions, duplications and small mutations were 83.8%, 12.1% and 2.7% respectively in the pedigrees. Koening et al reported the rate of deletions of one or more exons was 65% of DMD[20]. Chen et al reported the rate of deletions and small mutations were 79% and 9.2% respectively[21]. The reason for the high rate of deletion and low rate of small mutation might be that the patients enrolled in our hospital were mainly deletions and duplications. In addition, we found that deletions preferentially clustered in one hot-spot region that located between exon 43–55 of DMD gene which was in agreement with previous studies in other populations[22, 23]. Likewise, two hot spot regions were located for duplications, which were exon 8–11 and exon 51. Hotspot regions could be used to explain that DNA structure predisposes to specific breakpoint or recombination regions.

Given these, several studies have implied innovative therapeutic strategies like CRISPR/Cas 9 or oligonucleotides treatment targeting the DMD hot spot deletion regions to restore the dystrophin reading frame[24–26].

204 pregnant women decided to do the carrier testing. About 108 (52.9%) of 204 pregnant women who gave birth to a DMD patient in the past carried the same mutation as the probands in the blood. For those 96 (47.1%) did not carry the disease positive mutations in the blood, there are remained two possibilities to be considered. The mutation in probands is de novo might be one of them; germline mosaicism exists in the mother, the other. In this study, the de novo mutation rate was predicted to be 45.6% which is slightly higher than 1/3 reported in previous studies[27, 28]. This may because of the difference of enrolled population. Besides, the carrier rate of deletion mutations (47.4%) was much lower compared with the duplication rate (87.5%) among the mothers with mutations which is in accordance with previous study[29]. The identification of female carriers of DMD gene mutation is crucial to prevent the birth of DMD children. So that it is necessary to recommend carrier testing for all the mothers who have gave birth to a DMD patient during genetic counselling. If
the carrier testing result of the mother is negative, the existence of germline mosaicism should still be considered conferring the recurrence risk[30].

In this study, we summarized 305 prenatal diagnosis cases during 5 years. The sample size of our population is quite large for a rare monogenic disease. Of the 305 fetuses, 18% male fetuses were prenatally diagnosed as DMD and 13.1% female fetuses were detected carrying DMD gene mutations. Besides, karyotyping analysis was performed for all the fetuses to avoid the abnormal karyotype. Surprisingly, we found 3 fetuses with Down syndrome which is attributed to the presence of three copies of chromosome 21[31]. For the first time, we found a male fetus with DMD gene mutation and Down syndrome simultaneously.

In this study, the MLPA followed by Sanger sequencing was used for the detection of DMD mutations. MLPA is proved to be a powerful tool for the detection of deletions and duplications in patients with DMD and female carriers in affected families[34]. For small mutations, Sanger sequencing should be applied for individual exon[28]. However, with the development of new technologies, some new methods have proved to be more efficient in detecting DMD mutations. Zhang et al. has recommended the use of whole exome sequencing (WES) for the rapid and accurate molecular diagnosis of affected individuals with DMD in Chinese families[35]. Noninvasive prenatal testing has also facilitated the efficiency of genetic diagnosis for female carriers, probands and fetuses, using cell-free fetal DNA (cffDNA)[36]. Thus it is possible that next generation sequencing techniques will replace the traditional detection methods like MLPA, aCGH and Sanger sequencing in the future.

In conclusion, this study presented the spectrum of DMD mutations and 5-year clinical experience of DMD prenatal diagnosis and genetic counseling. Pregnancies in the DMD families should be prenatally tested to prevent the birth of affected children. Due to the possibility of germline mosaicism, prenatal testing is still recommended for non-carrier mothers who gave birth to a DMD patient. And MLPA approach proves to be a reliable method for the detection of DMD gene mutations.

Abbreviations

DMD
Duchenne muscular dystrophy
DGC
dystrophin-glycoprotein complex
MLPA
Multiplex ligation-dependent probe amplification
aCGH
array comparative genome hybridization
NGS
next generation sequencing
CVS
chorionic villus sampling
AC
amniocentesis

Declarations

Authors’ contributions

Mengmeng Li collected and analyzed data and prepared the manuscript; Fengxia Yao, Na Hao, Weimin Zhang, Jing Zhou and Li Tan collected the samples; Zhengqing Qiu participated in the study’s execution. Juntao Liu designed and coordinated the study. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

Datasets in this study are available from the corresponding author, upon reasonable requests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

The study was conducted according to the ethical guidelines of the Declaration of Helsinki (1975) and approved by the ethical committee of Peking Union Medical College Hospital.

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Tables

Table 1. Point mutation patterns in in 8 probands.

| Family number | Mutation       | Protein change | Mutation type | Status   |
|---------------|----------------|----------------|---------------|----------|
| F106          | c.5620G>T      | p.Glu1874X     | Non-sense     | Novel    |
| F282          | c.2566C>T      | p.Gln856X      | Non-sense     | Novel    |
| F291          | c.9337C>T      | p.Arg3113X     | Non-sense     | Reported |
| F297          | c.2257G>T      | p.Glu753X      | Non-sense     | Novel    |
| F214          | c.3196G>T      | p.Glu1066X     | Non-sense     | Novel    |
| F248          | c.4013delA     | p.Asp1337fs    | Frameshift    | Novel    |
| F195          | c.5704_5707delAGCC | p.Ala1901fs | Frameshift    | Novel    |
| F249          | c.2381-2A>G    | —              | Splice site   | Reported |
|               |                |                | mutation      |          |
Table 2. Summary of mutations in carrier mothers.

|                          | Carrier mother (n=107) | Non carrier mother (n=97) | Total (n=204) |
|--------------------------|------------------------|---------------------------|---------------|
|                          | N (%)                  | N (%)                     |               |
| **Deletion**             |                        |                           |               |
| SE                       | 17 (20.5%)             | 83 (47.4%)                | 92 (52.6%)    | 175 |
| ME                       | 66 (79.5%)             |                           |               |
| **Duplication**          |                        |                           |               |
| SE                       | 6 (28.6%)              | 21 (87.5%)                | 3 (12.5%)     | 24  |
| ME                       | 15 (71.4%)             |                           |               |
| **Deletion & duplication**|                       |                           |               |
|                          | 2 (100%)               | 0 (0)                     | 2             |
| **Small mutation**       |                        |                           |               |
|                          | 2 (66.7%)              | 1 (33.3%)                 | 3             |
Table 3. Prenatal diagnosis of fetuses in CVS and AC groups.

|                        | CVS      | AC       |
|------------------------|----------|----------|
| Average years of mother| 32.8 ± 4.1| 33.7 ± 4.1|
| Affected male fetuses  | 37 (16.5%)| 18 (22.2%)|
| Carrier female fetuses | 29 (13.0%)| 11 (13.6%)|
| Healthy fetuses        | 158 (70.5%)| 52 (64.2%)|
| Total                  | 224      | 81       |

SE: Single exon; ME: Multi exon

Figures
Figure 1

Flowchart for Duchenne muscular dystrophy (DMD) prenatal diagnosis.
Figure 2

The identified deletions in probands. (A) Deletion times of each exon in DMD gene. The X-axis shows exon position and the Y-axis shows the times of each exon deletion in probands.

(B) The 97 deletion patterns detected in probands.
The identified duplications in probands. (A) Duplication times of each exon in DMD gene. The X-axis shows exon position and the Y-axis shows the times of each exon duplication in probands. (B) The 30 duplication patterns detected in probands.
Figure 4

Karyotype (G banding) (47, XY, +21) of a fetus with DMD.