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RESEARCH ARTICLE

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A retrospective analysis of 237 Chinese families with Duchenne muscular dystrophy history and strategies of prenatal diagnosis

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Background: To offer 4-year clinical prenatal diagnosis experience of Duchenne muscular dystrophy (DMD).

Methods: Denaturing high-performance liquid chromatography (DHPLC) and Sanger sequencing were used for molecular diagnosis of 237 DMD families.

Results: In the study, deletions, duplications, complex rearrangement and small mutations accounted for 47.3%, 8.4%, 1.7% and 42.6% of 237 families, respectively. Sixty-six different deletion patterns were identified in 112 families. Fourteen different duplication patterns were identified in 20 families and 4 complex rearrangements were identified. About 87.1% different small mutation patterns were identified, including 37.6% different nonsense mutation patterns, 24.8% different frameshift mutation patterns, 7.9% different missense mutation patterns, and 16.8% different splice site mutation patterns. There was no significant difference in the age of onset and mutation patterns ($P > .05$). The follow-up examinations revealed that the pregnancies of 14 cases were interrupted. Two cases were preterm births, 151 cases were delivered at term, 63 cases continued to pregnancy, and 7 cases were lost to follow-up.

Conclusion: DHPLC and Sanger sequencing technique are efficient, sensitive, and specific in screening for *DMD* gene mutations. And pre-pregnancy *DMD* gene examination is an important step to assess mutation type of family with suspected DMD and guides exactly prenatal diagnosis in high-risk families.

KEYWORDS
denaturing high-performance liquid chromatography, dystrophin gene, prenatal diagnosis, Sanger sequencing

1 | INTRODUCTION

Duchenne muscular dystrophy (DMD) is a severe type of muscular dystrophy and presents in early childhood with proximal muscle weakness. Most patients cannot walk by the age of 12 and die because of cardiorespiratory complications at their late teens. DMD is inherited in an X-linked recessive fashion, which affects approximately 1/5000 boys. Most of females are asymptomatic due to one single copy of the defective gene, however, 20% also show mild symptoms.[1,2] DMD is related to the mutation of *DMD* gene mutation, including deletions, duplications, small mutations, or other smaller gene rearrangements.[3,4] DMD severely affects the survival and life quality of the patients. Currently, comprehensive management including, corticosteroid therapy, surveillance of the respiratory, cardiac, orthopedic, nutritional, and general medical issues, greatly improve the life expectancy and quality of life.[2] Gene therapy and

stem cell therapy also bring great promises for the disease, however, there is still a long way.[5-7] So the disease prevention is still the key, especially screening of female carrier and prenatal diagnosis.

The diagnosis of DMD relies on postnatal genetic testing using MLPA analysis,[8] DHPLC[9,10] or next-generation sequencing technology[11,12] or direct sequencing methods.[13] Performing prenatal diagnosis can offer sufficient and valuable genetic information for DMD families, to make appropriate reproductive choices. Prenatal molecular diagnosis for DMD was firstly introduced by the Netherlands in 1985.[14] At present, multiplex PCR, MLPA, and other techniques were tested to make prenatal diagnosis.[15-17] However, there are also barriers associated with prenatal diagnosis of DMD, which include probands and carries screening, pre and post-test counseling, and early completion of fetal diagnosis. In this study, we retrospectively analyzed clinical data on 237 DMD cases and the clinical application of DHPLC and direct Sanger sequencing in DMD families and assessed the validity of the strategy.

2 | MATERIALS AND METHODS

2.1 | Subjects

The study was approved by the Chinese People Liberation Army General Hospital medical ethics committee (Approval no. S2016-120-02). This retrospective cohort study included 237 DMD families, who underwent prenatal diagnostic testing in the prenatal diagnostic center of Shaanxi Xi Jing Hospital from November. 2011 to May 2016. Each family signed informed consent. Inclusion criteria were as follows: (i) a history of Duchenne muscular dystrophy or (ii) diagnosed as DMD by physical and biochemical examination finding or muscle biopsy (iii) from Nov. 2011 to May 2016. The exclusion criteria were as follows: (i) indefinite diagnosis (ii) wheelchair-bound after age 13 years.

2.2 | Karyotype analysis

Amniocentesis was performed under ultrasonographic guidance. Amniotic fluid samples (20 mL) were collected and centrifuged at 395 *g* for 8 minutes. After discarding the supernatant, the cells were suspended in 6 mL culture medium (GIBCO AmnioMAX-II complete, Grand Island, NY, USA) and cultured for 9 days at 37°C and 5% CO2.[18] Karyotype analysis was performed to exclude fetal abnormal karyotype.

2.3 | Gene mutation examination

Genomic DNA was extracted from peripheral blood specimens or fetal samples using the NP986-S nucleic acid extraction system (Tian Long, Xi'an, China). The major rearrangement of *DMD* gene was examined by denaturing high-performance liquid chromatography (DHPLC) and multiplex PCR (mPCR). The copy number variation of samples was performed according to Zou et al.[10] If a large deletion/duplication was not detected by DHPLC and multiplex PCR, PCR amplification, and Sanger sequencing using primer sets were used to confirm the diagnosis. The mutations were described according to the Leiden Muscular Dystrophy database (http://www.dmd.nl/). The nomenclature system was in 2000 on human mutation (reference sequence GenBank fileNM_004006.1).[19]

2.4 | Gene mutation analysis

For prenatal diagnosis of monogenic disorders, thirteen STR markers within the dystrophin gene were applied to rule out maternal contamination (markers AMEL, D3S1358, D5S818, D8S1179, D13S317, D16S539, D18S51, D21S11, FGA, CSF1PO, TPOX, VWA, and TH01).

These sequences were analyzed by preliminary comparison with sequences in the GenBank database and software Vector NTI. The nucleotide position was assessed using the *DMD* reference sequence (RefSeq NM_004006.2) and mutation nomenclature followed the guidelines of the Human Genome Variation Society (HGVS). Small mutations can be determined according to LOVD database (Leiden Open Variation Database: www.dmd.nl), dbSNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/), and clinVar database (http://www.ncbi.nlm.nih.gov/clinvar/?term=DMD%5Bgene%5D)[20] as references. Unreported missense mutation or splice site mutation was evaluated using the Polyphen-2 (http://genetics.bwh.harvard.edu/pph2/), SIFT algorithms (http://sift.jcvi.org/), and Alamut 2.7.2 to analyze pathogenicity. All substitutions with a Polyphen-2 score below 1 and a SIFT score below 0.05 were considered damaging substitutions. In addition, when an amino acid substitution was detected in any of the 100 healthy controls, pathogenicity was rejected.

2.5 | Genetic counseling and follow-up

All pregnant women received counseling by genetic counselor according to a standardized protocol consistent with national guidelines.[21] The pregnancy outcomes were acquired from delivery records if the pregnant women were delivered at our hospital; otherwise, they were gained by telephone follow-up.

2.6 | Statistical analysis

Statistical analyses for clinical data were performed using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA). Between-group comparisons were calculated using a chi-square test. All values are described as mean ± SD. For all tests, *P*-value less than .05 was considered as significant difference.[22]

3 | RESULTS

3.1 | Case characteristics

During the 4 years, we identified a total of 237 DMD families (214 with alive patients and 23 not alive patients) in our center. A total

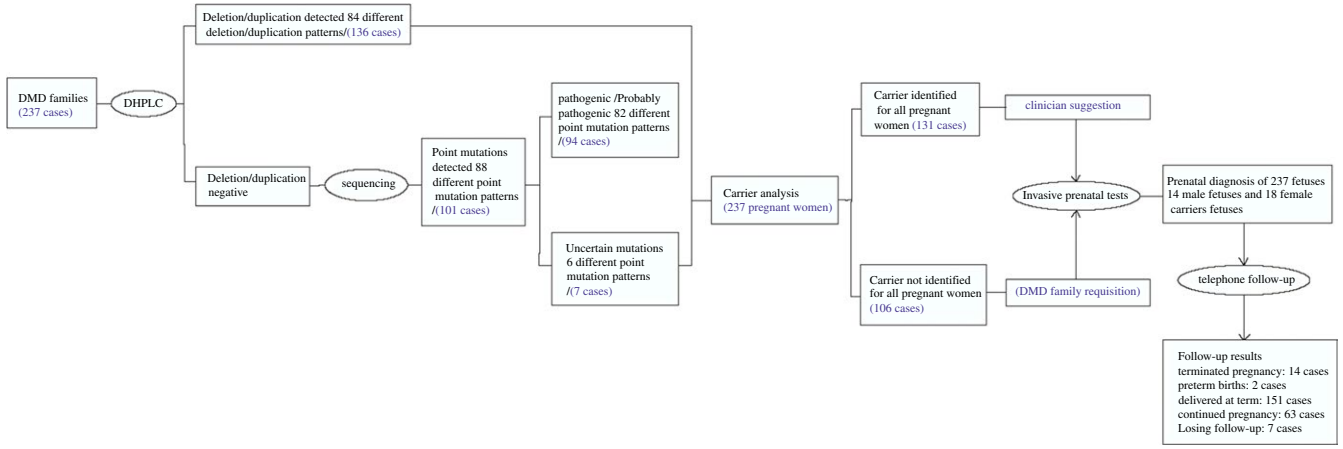

FIGURE 1 Stepwise duchenne muscular dystrophy patients mutation analysis and fetuses prenatal gene diagnosis results

of 23 cases were primarily diagnosed as DMD by their relatives' description and case history. The mean gestational age at prenatal diagnostic testing was 26 ± 2 weeks (range of 18-32 weeks). All fetuses had normal karyotype results.

3.2 | Mutation spectrum

The diagnostic process and spectrum of mutations of 237 DMD families were summarized as a flowchart in Figure 1. In the 237 DMD families detected by DHPLC, deletions and duplications accounted for 47.3% (112/237) and 8.4% (20/237) of 237 families, respectively. We detected 1.7% (4/237) complex rearrangements in DMD families. The remaining 42.6% (101/237) were small mutations. 172 DMD different mutations were found in 237 cases.

3.3 | Detection and duplication mutations

About 48.5% (66/136) different deletion patterns were detected in this study. The commonly observed deletion patterns were deletion of exons 49-50 (6.3%, n = 7), deletion of exons 45-50 (3.6%, n = 4), deletion of exons 46-55(3.6%, n = 4), and deletion of exon 51 (3.6%, n = 4). 10.3% (14/136) different duplication patterns were identified in 20 families. The most common duplication patterns were duplication of exon 2 (20.0%, n = 4), followed by duplication of exon 66 (15%, n = 3), and duplication of exons 3-4 (10%, n = 2). 2.9% (4/136) different complex rearrangements (Del 45-47 and Dup 72; Del 49-50 and Dup 66; Del 51 and Dup 48; Del 3-5 and Dup 66) were identified in this study. Both deletions and duplications showed a great heterogeneity, because 52 of the 136 identified deletions and duplications were tested only once (Figure 2).

3.4 | Small mutations

We identified 87.1% (88/101) different small mutation patterns including 37.6% (38/101) different nonsense mutation patterns, 24.8% (25/101) different frameshift mutation patterns, 7.9% (8/101) different missense mutation patterns, and 16.8% (17/101) different splice site mutation patterns in 101 families. Eighty-two different small mutations, which were reported to affect the *DMD* gene function, were identified in 101 families. However, six new small mutations (p.Asp556Asn, p.Gly882Asp, p.Gln2366Lys, p.Gln2806Arg, c.5586 + 18A>G, and c.9649 + 15T>C were firstly identified in this study. (Table 1).

3.5 | Analysis the association between the age of onset and mutation patterns

According to clinical data, the mean age at loss of ambulation among deletion, duplication, and small mutation were 4.7 ± 2.2,

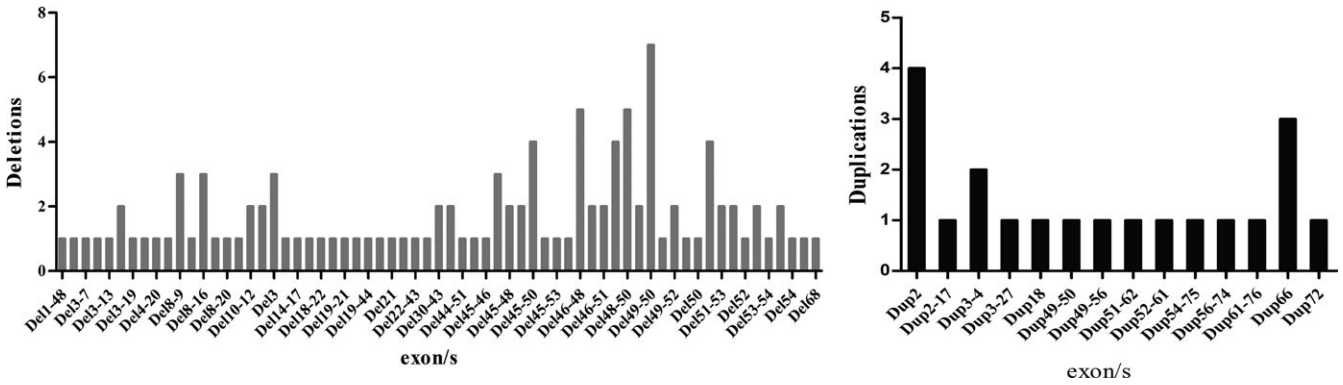

FIGURE 2 Duchenne muscular dystrophy mutations detected using DHPLC

TABLE 1 Summary of different point mutation patterns mutations in 101 DMD patients

Family No.	Disease onset	Mutation site	Protein level	Mutation type	Pathogenicity analysis
F132	4	c.4178T>A	p.Leu1393X	nonsense	(++)
F52	6	c.10033C>T	p.Arg3345X	nonsense	(+++)
F24	5	c.10108C>T	p.Arg3370X	nonsense	(+++)
F29	6	c.10141C>T	p.Arg3381X	nonsense	(+++)
F214	4	c.1033C>T	p.Gln345X	nonsense	(++)
F235	5	c.10546G>T	p.Glu3516X	nonsense	(++)
F79, F197	3,4	c.1594C>T	p.Gln532X	nonsense	(++)
F164	5	c.1886C>G	p.Ser629X	nonsense	(+++)
F152	6	c.2137C>T	p.Thr715X	nonsense	(+++)
F149	6	c.2302C>T	p.Arg768X	nonsense	(+++)
F189	3	c.2308A>T	p.Lys770X	nonsense	(+++)
F62	6	c.2665C>T	p.Arg889X	nonsense	(++)
F158	3	c.2816T>A	p.Leu939X	nonsense	(+++)
F44	10	c.2833C>T	p.Gln945X	nonsense	(++)
F147	4	c.3388G>T	p.Glu1130X	nonsense	(++)
F184	4	c.3940C>T	p.Arg1314X	nonsense	(+++)
F22, F45	2,2	c.4174C>T	p.Gln1392X	nonsense	(++)
F15	6	c.4232C>T	p.Gln1411X	nonsense	(++)
F206	5	c.4996C>T	p.Arg1666X	nonsense	(+++)
F25, F104	2	c.5287C>T	p.Arg1763X	nonsense	(+++)
F43	6	c.5633C>T	p.Gln1878X	nonsense	(++)
F30	4	c.615T>A	p.Tyr205X	nonsense	(+++)
F190	3	c.6292C>T	p.Arg2098X	nonsense	(+++)
F78, F103	6,4	c.6547G>T	p.Glu2183X	nonsense	(++)
F35	1	c.7105G>T	p.Glu2369X	nonsense	(+++)
F2, F148	2,4	c.7657C>T	p.Arg2553X	nonsense	(+++)
F4	6	c.7705C>T	p.Gln2569X	nonsense	(++)
F185	4	c.8038C>T	p.Arg2680X	nonsense	(+++)
F28	2	c.829C>T	p.Gln277X	nonsense	(++)
F100	4	c.8713C>T	p.Arg2905X	nonsense	(+++)
F156	2	c.8740G>T	p.Glu2914X	nonsense	(++)
F151	2	c.8944C>T	p.Arg2982X	nonsense	(+++)
F47	9	c.9072G>A	p.Trp3024X	nonsense	(+++)
F150	4	c.9337C>T	p.Arg3113X	nonsense	(+++)
F153	4	c.9568C>T	p.Arg3190X	nonsense	(+++)
F171	3	c.10171C>T	p.Arg3391X	nonsense	(+++)
F130	4	c.1615C>T	p.Arg539X	nonsense	(+++)
F124	3	c.3295C>T	p.Gln1099X	nonsense	(+++)
F11	5	c.10231dupT	p.Thr3411 fs	frameshift	(+++)
F229	1	c.1365_1366delGA	p.Gln455 fs	frameshift	(++)
F180	2	c.1713insT	p.Phe571 fs	frameshift	(++)
F216	4	c.1758delC	p.His586 fs	frameshift	(++)
F188	4	c.2191delC	p.Leu731 fs	frameshift	(++)

(Continues)

TABLE 1 (Continued)

Family No.	Disease onset	Mutation site	Protein level	Mutation type	Pathogenicity analysis
F159	3	c.2300delA	p.Glu767 fs	frameshift	(++)
F208	4	c.2571delC	p.Pro857 fs	frameshift	(++)
F163	2	c.264_264 + 4delTGTAA	p.Asn88 fs	frameshift	(++)
F26	5	c.3257delA	p.Lys1086 fs	frameshift	(++)
F19	9	c.4584inA	p.Gln1528 fs	frameshift	(++)
F186	6	c.4630delA	p.Arg1544 fs	frameshift	(++)
F154	5	c.4746_4747delCT	P. Val582 fs	frameshift	(++)
F155	3	c.4808_4809insGGAA	p.Met1603 fs	frameshift	(++)
F146	1	c.6033insTTAA	p.Leu2011 fs	frameshift	(++)
F187	3	c.6045delA	p.Glu2015 fs	frameshift	(++)
F82	3	c.6804_6807delACAA	p.Lys2268 fs	frameshift	(++)
F194	3	c.7327_7328insA	p.Thr2443 fs	frameshift	(++)
F40	4	c.7433inG	p.Ala2478 fs	frameshift	(++)
F32	4	c.7885delAG	p.Asp2629 fs	frameshift	(++)
F94	6	c.8104insT	p.Leu2702 fs	frameshift	(++)
F192	1	c.8147-8149insCAGAAGCTGAA ACAACTGCCAATGTCCTACA	p.Gln2716 fs	frameshift	(++)
F105	4	c.841_844delAGTC	p.Ser281 fs	frameshift	(++)
F215, F217	7,4	c.8877delC	p.Ser2959 fs	frameshift	(++)
F96	3	c.9461delT	p.Lys3154 fs	frameshift	(++)
F160	3	c.9722_9723delCT	p.Ser456 fs	frameshift	(++)
F18	3	c.1666G>A	p.Asp556Asn	missense	(+)
F57	3	c.2645G>A	p.Gly882Asp	missense	(+)
F195	8	c.3432G>T	p.Gln1144His	missense	(++)
F97	7	c.5163G>C	p.Lys1721Asn	missense	(++)
F34, F39, F107	3,7,7	c.5234G>A	p.Arg1745His	missense	(++)
F143, F173	5,3	c.5234G>A, c.7096C>A	p.Arg1745His;p.Gln2366Lys	missense	(++)
F1, F141	4,6	c.7096C>A	p.Gln2366Lys	missense	(+)
F136	6	c.8417A>G	p.Gln2806Arg	missense	(+)
F9, F51	7,3	c.10797 + 1G>A	p.spl	Splice site mutation	(++)
F12	5	c.1149 + 2T>G	p.spl	Splice site mutation	(++)
F232	6	c.1704 + 1G>C	p.spl	Splice site mutation	(+++)
F157	3	c.2169-1G>T	p.spl	Splice site mutation	(++)
F162	8	c.2803 + 1G>A	p.spl	Splice site mutation	(+++)
F167, F177	7,3	c.358-2A>G	p.spl	Splice site mutation	(+++)
F50	4	c.3602-2G>C	p.spl	Splice site mutation	(++)
F46	3	c.3603 + 2dupT	p.spl	Splice site mutation	(++)
F127, F139	2,3	c.4518 + 3A>T	p.spl	Splice site mutation	(++)
F101	6	c.5449-2A>G	p.spl	Splice site mutation	(++)
F118	1	c.5586 + 18A>G	p.spl	Splice site mutation	(+)
F102	4	c.8027 + 1G>A	p.spl	Splice site mutation	(++)
F193	2	c.8028-1G>C	p.spl	Splice site mutation	(++)
F92	2	c.8217 + 3delAAGT	p.spl	Splice site mutation	(++)
F93	9	c.8668 + 1G>A	p.spl	Splice site mutation	(++)

(Continues)

TABLE 1 (Continued)

Family No.	Disease onset	Mutation site	Protein level	Mutation type	Pathogenicity analysis
F69	3	c.9649 + 15T>C	p.spl	Splice site mutation	(+)
F161	4	c.9807 + 5G>A	p.spl	Splice site mutation	(+++)

Pathogenicity Analysis was evaluated using the LOVD (www.dmd.nl), ClinVar database, Polyphen-2 (http://genetics.bwh.harvard.edu/pph2/), SIFT algorithms (http://sift.jcvi.org/), Alamut 2.7.2. and ExAc, 1000 Genomes. (+++), pathogenic; (++), probably pathogenic and (+), uncertainly.

TABLE 2 Prenatal diagnosis and clinical risk in 14 cases

Family	Proband	Potential Pregnant Carrier	Fetus	Clinical Risk	Pregnancy Outcome
F14	Del 45	C	+	Affected	Top
F23	Del 45-49	C	+	Affected	Top
F33	Del 5-44	C	C	Low Risk	–
F37	Del 45-47	C	+	Affected	Top
F56	Del 45-46	C	C	Low Risk	–
F61	Del 46-48	C	C	Low Risk	–
F68	Del 3-9	C	C	Low Risk	–
F73	Dup 2	C	+	Affected	Top
F87	Del 21	C	C	Low Risk	–
F98	Del 46-48	C	+	Affected	Top
F117	Del 8-16	C	+	Affected	Top
F128	Del 22-43	C	C	Low Risk	–
F134	Del 51-53	C	C	Low Risk	–
F140	Del 3-19	C	C	Low Risk	–
F170	Dup 2-17	C	+	Affected	Top
F174	Del 3-13	C	+	Affected	Top
F200	Del 49-51	C	C	Low Risk	–
F204	Dup 51-62	C	+	Affected	Top
F212	Del 45-50	C	C	Low Risk	–
F226	Del 46-48	C	C	Low Risk	–
F228	Del 45-53	C	C	Low Risk	–
F24	C.10108C>T	C	C	Low Risk	–
F29	C.10141C>T	C	+	Affected	Top
F162	C.2803 + 1G>A	C	C	Low Risk	–
F171	C.10171C>T	C	+	Affected	Top
F28	C.829C>T	C	+	Affected	Top
F40	C.7433ing	C	C	Low Risk	–
F96	C.9461delt	C	+	Affected	Top
F34	C.5234G>A	C	+	Affected	Top
F57	C.2645G>A	C	C	Low Risk	–
F97	C.5163G>C	C	C	Low Risk	–
F157	C.2169-1G>T	C	C	Low Risk	–

+, positive for DMD mutation; –, negative for DMD mutation; C, carrier for DMD; Top, termination of pregnancy.

4.3 ± 1.9, and 4.2 ± 1.9 years, respectively. No relationships were found between the age of onset and mutation patterns ($P > .05$).

3.6 | Prenatal diagnosis of 237 fetuses

For 237 families, we analyzed the carrier status of all pregnant women, among whom 131 had the same mutation as their children

or patients. Fourteen pathogenic mutations and 18 female carriers were detected in fetal samples (Table 2). Two cases (F174 and F162) with deletion asked for repeat analytical tests. DHPLC analysis was performed to confirm. Fetal echocardiography showed ventricular septal defect and congenital heart disease in family F23 and F87, respectively.

3.7 | Follow-up and genetic counseling

Among the total cases, 14 pregnant women chose the termination of pregnancy. The pedigree diagnosed as negative and carriers continued the pregnancy. In total, the pregnancies of 14 cases were interrupted, 2 were preterm births (>34 gestational weeks), 151 were delivered at term, 63 continued the pregnancy and 7 were lost to follow-up. For 18 female carrier fetuses, long time monitoring is necessary.

4 | DISCUSSION

Lacking effective therapies, DMD brings great harm to many patients and their families. Families with DMD history showed anxiety and depression and strongly requested prenatal diagnosis. It has been known that 1/3 sporadic mutations of *DMD* gene occurs in male patients.[23,24] Germinal mosaicism cases were detected in some DMD families.[25-28] So, the prenatal DMD diagnosis has been recommended as the most effective way to avoid congenital malformed children. In this retrospective cohort study, genetic analysis and prenatal diagnosis results of the 237 Chinese families with DMD history were presented. Fourteen pathogenic mutations (5.9%) and 18 female carriers (7.6%) were detected in fetal samples. The size of the samples was good for a rare disorder.

Molecular genetic analysis of DMD patients is essential for establishing a definitive diagnosis, directing appropriate clinical management, and providing for further prenatal diagnosis, and genetic counseling.[29] Cho A et al reported that the rates of deletions, duplications, and sequence variations were reported as 65.4%, 13.3%, and 12.3%, respectively.[22] Chen C et al reported that they were 79%, 19.8%, and 9.2%, respectively.[9] In our study, we found the rate of deletions, duplications, and sequence variations as 47.3%, 8.4%, 1.7%, and 42.6%, respectively. We believed that these differences between the rates were due to sample size for inspection patients and regional differences.

In recent years, the prenatal diagnosis techniques acquired great improvements. The advancement in ultrasound-monitored amniocentesis enhanced the capability for diagnosis of DMD in utero. TA et al[30] reported combination of STR and MLPA could be a rapid, reliable, and affordable detection protocol for determination of the carrier's status and prenatal diagnosis of DMD.[30] Zhang et al[31] reported that real-time PCR assay was successfully used to make prenatal diagnosis of DMD families in thirty Chinese families.[31] Yoo et al reported the single targeted sequencing platform was applied to detect carriers and make non-invasive prenatal diagnosis.[17] Parks et al reported that haplotype dosage analysis for X-linked disorders was one non-invasive prenatal diagnosis for DMD.[32] Therefore, we identified *DMD* mutations in a cohort of 237 Chinese patients using denaturing high-performance liquid chromatography (DHPLC) followed by Sanger sequencing according to local conditions and platforms in our prenatal diagnosis center. Chen et al (2014)[29] also reported DHPLC analysis exhibited high efficiency and specificity for identifying any small alterations in the *DMD* gene.[9]

In this study, 82 different small mutations were previously reported or possibly affect the *DMD* gene function (Table 1). However, the pathogenicity of six different small mutations (p.Asp556Asn, p.Gly882Asp, p.Gln2366Lys, p.Gln2806Arg, c.5586 + 18A>G, and c.9649 + 15T>C) was not clear.

This study offered 4-year clinical prenatal diagnosis experience of Duchenne muscular dystrophy, which was one relatively large sample size to perform mutational analysis. The limitation of this study was that some novel mutations had not been fully proved to be related to the DMD.

In conclusion, this study offers informative data for proper prenatal genetic counseling of pregnant women and their partner. DHPLC technology has high sensitivity and specificity.

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CONFLICT OF INTERESTS

All authors declare no conflict of interests. The authors alone are responsible for the content and writing of the paper.

ETHICAL APPROVAL

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

INFORMED CONSENT

Informed consent was obtained from all individual participants included in the study.

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