



Prenatal molecular diagnosis and carrier detection of Duchenne muscular dystrophy in Korea

Min Ji Kang^{1,2}, Moon-Woo Seong³, Sung Im Cho³, Joong Shin Park¹, Jong Kwan Jun^{1,4,*}, and Sung Sup Park³

¹Department of Obstetrics and Gynecology, Seoul National University College of Medicine, Seoul, Korea

²Department of Obstetrics and Gynecology, Gimcheon Jeil Hospital, Gimcheon, Korea

³Department of Laboratory Medicine, Seoul National University College of Medicine, Seoul, Korea

⁴Institute of Reproductive Medicine and Population, Medical Research Center, Seoul National University College of Medicine, Seoul, Korea

Purpose: Duchenne muscular dystrophy (DMD) is the most common lethal muscular dystrophy and is caused by the genetic variants of DMD gene. Because DMD is X-linked recessive and shows familial aggregates, prenatal diagnosis is an important role in the management of DMD family. We present our experience of prenatal molecular diagnosis and carrier detection based on multiplex polymerase chain reaction (PCR), multiplex ligation-dependent probe amplification (MLPA), and linkage analysis.

Materials and Methods: During study period, 34 cases of prenatal diagnosis and 21 cases of carrier detection were performed at the Seoul National University Hospital. Multiplex PCR and MLPA was used to detect the exon deletions or duplications. When the DMD pathogenic variant in the affected males is unknown and no DMD pathogenic variant is detected in at-risk females, linkage analysis was used.

Results: The prenatal molecular diagnosis was offered to 34 fetuses. Twenty-five fetuses were male and 6 fetuses (24.0%) were affected. Remaining cases had no pathogenic mutation. We had 24 (80.0%) cases of known proband results; exon deletion mutation in 19 (79.2%) cases and duplication in 5 (20.8%) cases. Linkage analysis was performed in 4 cases in which 2 cases (50.0%) were found to be affected. In the carrier testing, among 21 cases including 15 cases of mother and 6 cases of female relative, 9 (42.9%) cases showed positive results and 12 (57.1%) cases showed negative results.

Conclusion: Prenatal molecular diagnosis and carrier detection of DMD are effective and feasible. They are useful in genetic counseling for DMD families.

Key words: Prenatal diagnosis, Muscular dystrophy, Duchenne, Multiplex polymerase chain reaction, Multiplex ligation-dependent probe amplification, Linkage analysis.

Introduction

Duchenne muscular dystrophy (DMD) is one of neuromuscular diseases characterized by progressive muscular weakness

and degeneration of skeletal muscle [1]. The onset of DMD symptoms is observed ages between 2 and 3. Usually, affected boys have difficulty in motor development, approximately half of affected fail to walk until 18 months of age. It is the most

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*Corresponding author: Jong Kwan Jun, M.D., Ph.D. <https://orcid.org/0000-0002-0242-1736>

Department of Obstetrics & Gynecology, Seoul National University College of Medicine, 103 Daehak-ro, Jongno-gu, Seoul 03080, Korea.

Tel: +82-2-2072-3744, Fax: +82-2-762-3599, E-mail: jhs0927@snu.ac.kr

Conflict of interest: The authors declare that they do not have any conflicts of interest.

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common X-linked recessive fatal disease occurring in 1 in 3,500 newborns, and results from pathogenic variants in the dystrophin gene encoding the dystrophin protein [2,3]. The dystrophin gene is found in the short arm of the X chromosome (X-p21.2) [4]. Its 11.3 kb long coding sequence embraces 79 exons and 7 unique promoters and introns which comprise 99.5% of the DMD gene [5].

Prenatal diagnosis for DMD was first introduced in 1984 [6].

Prenatal tests for DMD are performed for a male fetus whereas no testing is mandate for female fetuses [7]. Traditional genetic diagnosis for DMD detects large mutations at first and smaller mutations subsequently by applying two or more analytical methods. Conservative prenatal diagnosis detects any known familial mutation in male fetal DNA by chorionic villus sampling or amniocentesis [8]. The same approach can be applied to detect the mutation of the proband and female carriers [7].

Table 1. Prenatal diagnosis of 34 fetuses in DMD high-risk families

No.	Date	Sample	Tests	Proband			Fetus	
				Results	Relation	Carrier status	Sex	Results
1	1997. 9	AF	Multiplex PCR	None			M	Normal
2	2000. 9	AF	Multiplex PCR	None	Aunt		F	
3	2002. 1	AF	Multiplex PCR	50 del	Mother		M	DMD
4	2004. 1	AF	Multiplex PCR	49-52 del	Mother	-	M	Normal
5	2004. 3	CVS	Multiplex PCR	45-52 del	Aunt	+	M	DMD
6	2004. 7	CVS	Multiplex PCR	None			F	
7	2004. 9	AF	Multiplex PCR	48 del	Mother	-	M	Normal
8	2005. 1	AF	Multiplex PCR	None	Sister		M	Normal
9	2005. 1	AF	Multiplex PCR	47-52 del	Mother	-	M	Normal
10	2005. 1	AF	Multiplex PCR	None	Aunt		M	Normal
11	2005. 3	CVS	Multiplex PCR	45-52 del	Aunt	+	M	Normal
12	2005. 5	AF	Multiplex PCR	6-8 del	Mother	-	F	
13	2005. 8	AF	Multiplex PCR	46-47 del	Mother	+	F	
14	2006. 2	AF	MLPA+Multiplex PCR	45-52dup	Aunt	-	F	
15	2006. 5	AF	MLPA+Multiplex PCR	45-54 del	Mother		M	Normal
16	2006. 9	AF	MLPA+Multiplex PCR	46-61 dup	Sister	-	M	Normal
17	2007. 1	AF	MLPA+Multiplex PCR	None	Sister		M	Normal
18	2007. 2	AF	MLPA+Multiplex PCR	2 dup	Mother		M	Normal
19	2007. 9	AF	MLPA+Multiplex PCR	54 dup	Mother	-	M	Normal
20	2007. 9	AF	MLPA+Multiplex PCR	46-48 del	Mother	-	F	
21	2007. 11	AF	MLPA+Multiplex PCR	45-50 del	Mother	+	M	DMD
22	2007. 12	AF	MLPA+Multiplex PCR	48-52 del	Mother	+	F	
23	2008. 3	AF	MLPA+Multiplex PCR	12-17 del	Mother	-	M	Normal
24	2008. 3	AF	MLPA+Multiplex PCR	8-26 del	Mother	-	F	
25	2008. 5	AF	MLPA+Multiplex PCR	41-43 del	Mother	-	F	
26	2008. 6	AF	MLPA+Multiplex PCR	46-61 dup	Sister	-	M	Normal
27	2008. 7	AF	MLPA+Multiplex PCR	44 del	Mother	+	M	Normal
28	2008. 9	AF	MLPA+Multiplex PCR	3-44 del	Mother	+	M	DMD
29	2009. 1	AF	MLPA+Multiplex PCR	3-17 del	Aunt	+	M	Normal
30	2009. 9	CVS	MLPA+Multiplex PCR	48-52 del	Mother	+	M	Normal
31	2007. 7	AF	Linkage analysis		Mother			DMD
32	2007. 9	AF	Linkage analysis		Aunt			DMD
33	2007. 11	AF	Linkage analysis		Mother			Normal
34	2008. 12	AF	Linkage analysis		Sister			Normal

DMD, Duchenne muscular dystrophy; AF, amniotic fluid; PCR, polymerase chain reaction; M, male; F, female; CVS, chorionic villi sampling; MLPA, multiplex ligation-dependent probe amplification.

If the familial mutation is not observed, haplotyping is another method for prenatal diagnosis [9].

Since the locus of the DMD gene identified in 1986 by independent experimental methods [10,11], clinicians have heavily concentrated on the molecular diagnosis of DMD. Definite treatment is not available. Therapies under investigation; pregnancy management for female carriers; genetic counseling for risk to family members and family planning; and prenatal testing and preimplantation genetic diagnosis (PGD) are all dependent on the precise molecular diagnosis of the disorders.

Many diagnostic testing procedures are employed for DMD mutation tests including multiplex polymerase chain reaction (PCR), multiplex ligation-dependent probe amplification (MLPA), array comparative genome hybridization (CGH), PCR-based Sanger sequencing, and others [12].

Some laboratories still use Multiplex PCR to identify common dystrophin deletions due to its convenience and cost-efficiency to perform [12]. Since the introduction of MLPA by Schouten in 2002, there has been a substantial increase in detection rate of dystrophin mutations as the majority of mutations developing DMD are intragenic deletions or duplications representing 65% and 5% of total cases, respectively [5].

The aim of this study is to show our clinical experience in prenatal diagnosis and carrier detection of DMD using molecular biologic techniques.

Materials and Methods

1. Study subjects

The prenatal diagnosis data of 34 at-risk pregnancies in DMD families referred to the department of obstetrics and gynecology in Seoul National University Hospital from September 1997

to January 2010 were retrospectively reviewed. Peripheral blood was collected from 24 male probands clinically diagnosed with DMD and 21 corresponding female relatives for carrier tests. This study was approved by the ethics committee of Seoul National University Hospital. Written informed consent was obtained from the patients or their guardians.

2. Specimens

For the pregnant women, chorionic villi biopsy was performed at 11 to 14 week gestation, 10–20 mL of amniotic fluid was collected via amniocentesis at 14 to 18 week gestation to obtain genomic DNA from fetus. Chorionic villi and amniocyte culture was done for prenatal genetic diagnosis. A total of 2 mL of peripheral blood was collected from patients to obtain genomic DNA for proband and carrier tests.

3. Mutation analysis

Genomic DNA was extracted from chorionic villi, amniocytes, and the patients' peripheral blood using Gentra PureGene DNA Isolation Kits (Gentra Systems Inc., Minneapolis, MN, USA). Multiplex PCR was used to detect exon deletion in 26 sites. Exon deletion or duplication of dystrophin was screened using MLPA. MLPA was performed using the SALSA P034 DMD mix 1 Kit and P035 DMD mix 2 Kit (MRC Holland, Amsterdam, The Netherlands). PCR products were analyzed on an ABI 3130 analyzer using Genemarker v1.51 (Softgenetics, State College, PA, USA). Peak heights were normalized, and a deletion or duplication was suspected when a normalized peak ratio was under 0.75 or above 1.30. Linkage analysis was done using 14 different short tandem markers spanning the DMD gene, including DXS1049, DXS1242, and etc. PCR-restriction fragment length polymorphism, and detection of ZF gene in X chromosome/ZF gene in Y chromosome were used to identify sex of fetus.

Results

The prenatal molecular diagnosis was offered to 34 fetuses

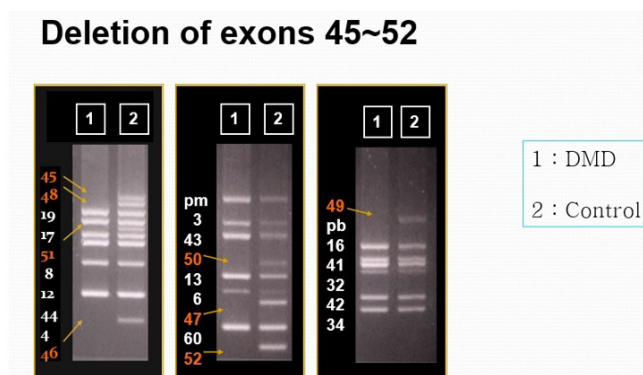


Fig. 1. Multiplex polymerase chain reaction. DMD, Duchenne muscular dystrophy.

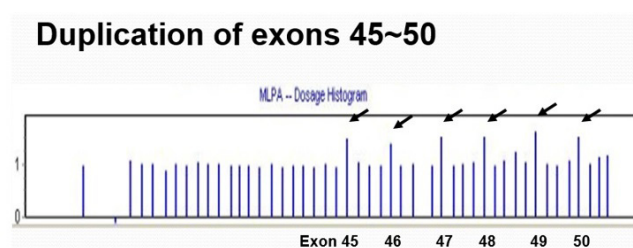


Fig. 2. Multiplex ligation-dependent probe amplification.

(Table 1). To detect the exon deletion or duplication, multiplex PCR was used (Fig. 1) and after 2005, MLPA was introduced for screening (Fig. 2). Twenty-five fetuses were male and 6 fetuses (24.0%) were affected. We had 24 (80.0%) cases of known proband results; exon deletion mutation in 19 (79.2%) cases and duplication in 5 (20.8%) cases (Fig. 3). Remaining cases had no pathogenic mutation. However, the prenatal tests could not be performed if no patients survived in their households. When the fetus was a male (Case no. 1, 8, 10, and 17), a possible test only showed that there was no known deletion/duplication of the DMD gene exon. In this context, DMD/BMD due to point muta-

tion cannot be excluded. Linkage analysis was performed in 4 cases which resulted in 2 affected cases (50.0%) (Fig. 4). In the carrier testing, among 21 cases including 15 cases of mother and 6 cases of female relative, 9 (42.9%) cases showed positive results and 12 (57.1%) cases showed negative results (Fig. 5). Of 12 cases which showed negative results in the carrier testing, 7 (58.3%) cases were male cases and all of these cases were negative in prenatal diagnosis.

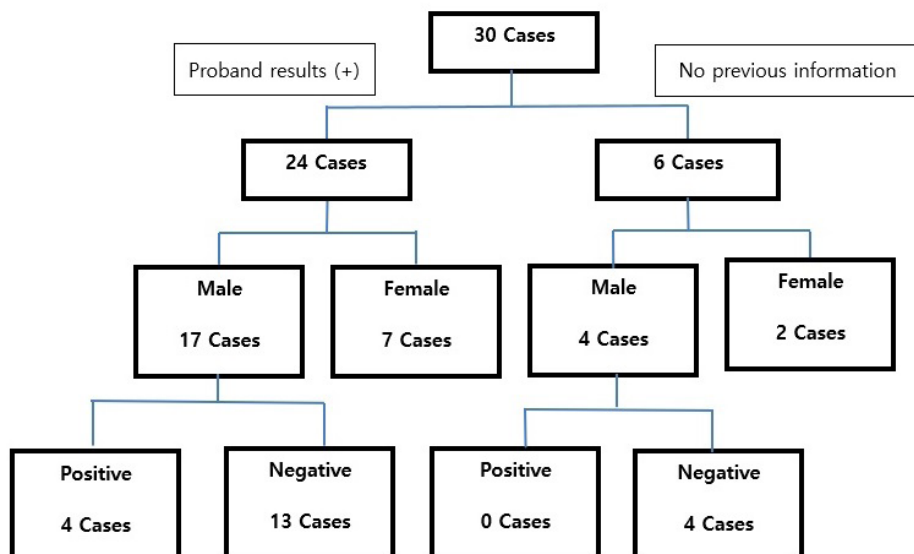


Fig. 3. Cases analyzed by multiplex polymerase chain reaction and multiplex ligation-dependent probe amplification.

Marker	Location	Mother	Fetus	Proband	Father
DXS1049		195/-	195	195	195
uniSTS98958		174/176	176	176	174
DYSII	Promotor	67/85	85	85	67
DXS1242	Promoter	206/224	224	224	206
DXS1243	Intron 1	207/-	207	207	205
DXS1238	Intron 44	185/-	185	185	195
DXS1237	Intron 45	173/177	177	177	169
DXS1236	Intron 49	231/247	247	247	251
DXS1235	Intron 50	240/244	244	244	240
DXS1036	Intron 62	145/-	145	145	147
DXS1214	Intron 63	211/215	215	215	219
DXS1234	3'UTR	132/136	132	132	132
DXS503	3'UTR	68/72	68	68	68
DXS985		128/-	128	128	128

Fig. 4. Linkage analysis.

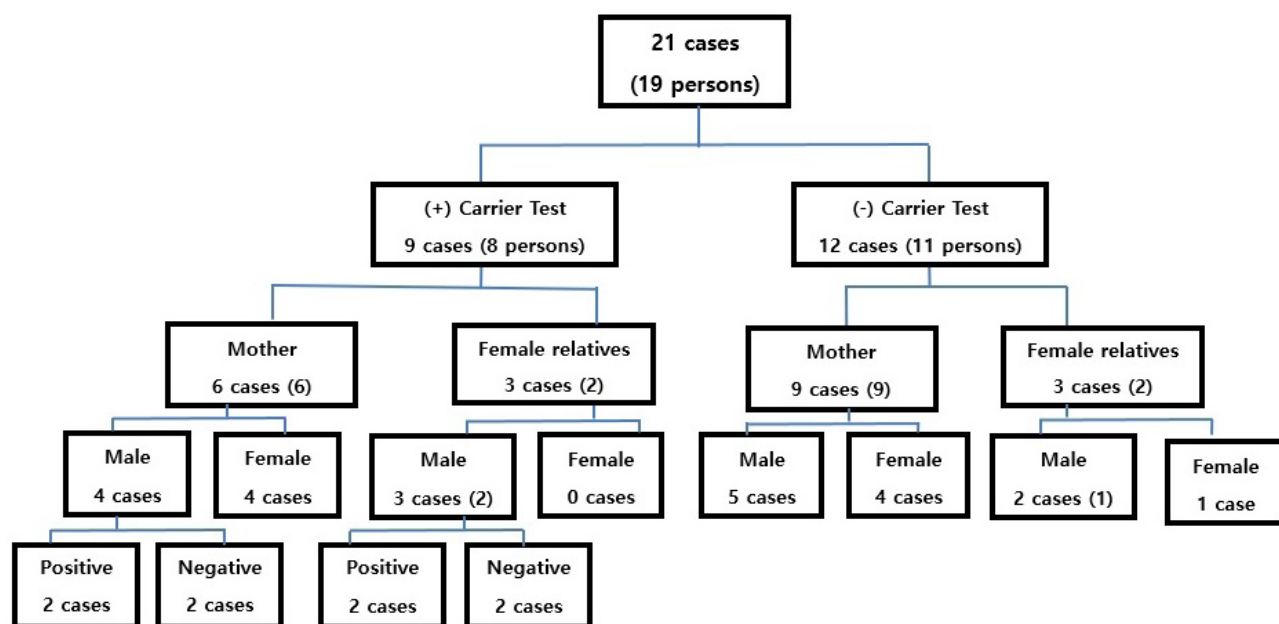


Fig. 5. Cases with or without carrier test.

Discussion

This study showed current status of prenatal molecular diagnosis and carrier detection of DMD in Korean.

As a result of the study, affected male fetus accounted for 6 of 25 (24.0%) male fetuses, which is somewhat lower compared to 34 of 91 (37.4%) male fetuses in the previous study [13]. This may be due to the variance in patient group and race, the percentage of patients without proband results, and the diagnostic method used for prenatal diagnosis.

Among 30 fetuses analyzed by Multiplex PCR and MLPA, the percentage of patients without proband results was 6 of 30 (20.0%) fetuses. In particular, there was not much genetic information about probands in the initial cases during study periods, because genetic counselling for DMD in the early stages of the study was relatively uncommon compared to the present, and there might be possibilities of early death of proband prior to molecular diagnosis. For another reason, it is considered that muscle biopsies were common in the early 2000s accounting for more than two thirds among various diagnostic tools for DMD.

When analyzed chronologically over 10 years, the rate of muscle biopsies in total diagnosed cases has gradually fallen from 96.8% in 1990s to 19.3% in 2010s as molecular diagnosis techniques improved [14]. Because of the improvement of molecular diagnosis techniques, most DMD patients are diagnosed genetically without muscle biopsies recently.

As MLPA was first described by Schouten et al. [15] in 2002, multiplex PCR was used for detection of deletions in dystrophin gene in this study without MLPA. Multiplex PCR is a method to test the presence or absence of exon deletion of the DMD gene dystrophin; Xp21.2 and the test confirmed the exon deletion of 26 sites. About two thirds of DMD patients are caused by exon deletion and multiplex PCR can diagnose more than 99% of exon deletion patients. Therefore, some laboratories still use this method because of its convenience and cost-efficiency to perform [12].

Multiplex PCR is used to identify deletions at a hemizygous locus but it is impossible to completely describe deletions because of its limitation to cover the whole gene and incapacity to detect duplications or female carriers. These are the critical drawbacks of Multiplex PCR [16]. It also has difficulties to differentiate whether the deletion detected is in-frame or out-of-frame, and to apply exon skipping therapy consequently. Additionally, it is impossible to determine boundaries of the deletion [12]. In present study, no duplication was found in the mutation of the proband when multiplex PCR was used but the duplication was found after MLPA introduction. In 24 total cases in which proband results exist, 19 (79.2%) cases were exon deletion mutation and 5 (20.8%) cases were duplication mutation. This rate is similar to the results of previous studies reporting mutation spectrum in the dystrophin gene of 507 Korean boys [14].

When the DMD gene variant observed in an affected male,

carrier diagnosis could be applicable to the mother and female relatives at risk, who are heterozygotes for the pathogenic gene variant. In this study, carrier test was performed in 21 cases except 3 cases among 24 cases in which DMD gene variants of affected males were identified. Carrier test results provide information in deciding whether to perform a prenatal diagnosis. Also these results are clinically important as abnormal cardiac monitoring is essential for asymptomatic heterozygous females and a cardiac evaluation should be considered prior to conception or during pregnancy [12].

In the present cohort, the results revealed that 9 of 21 (42.9%) mothers were carriers. Of the 21 cases with both proband and carrier results, 12 of 21 (57.1%) were carrier negative. The *de novo* mutation of the DMD gene and germline mosaicism can be considered for this discrepancy. If the mother was not a carrier, her son with DMD mostly had a *de novo* mutation. However, germline mosaicism in DMD should not be ignored as described in the 1980s, conferring a recurrence risk for the next son [17,18].

Although 7 male fetuses of carrier negative patients were all negative in the prenatal diagnosis and the 7 male fetuses who were born healthily in this study, the possibility of germline mosaicism cannot be completely excluded. Wang et al. reported 3 cases of germline mosaicism in 50 mothers with no pathogenic mutations [19]. Furthermore, Bermúdez-López et al. [20] verified that germinal mosaicism deletions have been reported in at least 11.6% of families of DMD patients, suggesting that mosaicism should be considered in the genetic counseling. In our center, prenatal diagnosis was suggested to at-risk pregnant women in DMD families regardless of their carrier status.

Because recent prenatal diagnosis is offered based upon the presence of an affected boy, it is insufficient to detect fetuses with *de novo* mutations. *De novo* mutations represents approximately one third of DMD patients. This issue can be resolved by offering prenatal diagnostic test for the dystrophin gene to every pregnant woman. Advancing in noninvasive prenatal diagnosis will possibly improve the current conditions despite there are still many challenges to overcome [9,21]. Moreover, cost-effectiveness should be considered.

Point mutations, microdeletions, microinsertions, or splicing mutations are known as the cause for 30% of all DMD gene mutations [5]. These mutations can be detected by dystrophin gene sequencing performed at the genomic or complementary DNA level. In the present study, multiplex PCR and MLPA are employed for the detection of large and small mutations. However, our study does not use sequencing to detect small mutations because of high costs [22]. Limitation of this study is that some

variants such as deep intronic mutations or complex rearrangements may not be identified through traditional DNA-based mutation screening techniques [7]. In the future, studies using Array CGH, PCR-based Sanger sequencing in prenatal diagnosis that can detect point mutations are expected.

PGD is another option for carrier women. With PGD, either unaffected male embryos or females can be selected and implanted into the uterus. Additionally, germline mosaicism is an issue and in order to find it developing new diagnostic methods such as measuring creatinine phosphokinase level in amniotic fluid and fetal blood or establishing a direct diagnostic tool through fetal muscle biopsy are essential. Chorionic villus sampling or amniocentesis used for sample collection in this study are invasive methods carrying associated risks. In the future, research and development of noninvasive prenatal diagnostic tests will be required providing a basis for prenatal diagnosis of DMD such as the discovery of cell-free fetal DNA in maternal plasma [21].

In conclusion, this study showed prenatal diagnosis and carrier detection using multiplex PCR, MLPA, and linkage analysis were effective measures to detect DMD.

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