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Validation of MLPA-detected Single Exon Deletion of the *DMD* Gene by Multiplex PCR

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Abstract

Multiplex ligation-dependent probe amplification (MLPA) is the most widely used technique to detect deletions and duplications of the *DMD* (dystrophin) gene that constitute two third of the cases of Duchenne (DMD)/ Becker muscular dystrophy (BMD). However, MLPA can yield false positive results for single exon deletions owing to the presence of single nucleotide polymorphisms (SNPs) at the ligation site. This study aimed to validate single exon deletions detected by MLPA in DMD/BMD individuals by multiplex polymerase chain reaction (PCR). The data of patients with DMD/BMD diagnosed by MLPA between January 2016 to February 2021 was collated and those with apparent single exon deletions were recruited. MLPA was performed using the P034/P035 DMD (MRC Holland) kit. Multiplex PCR was performed using Chamberlain and Beggs primer sets with appropriate controls. Sequencing of the relevant exon of the *DMD* gene was planned for discordant results between MLPA and multiplex PCR. SNPs within 8bp from ligation sites on MLPA probes and its frequency in the South Asian population was ascertained from appropriate databases. Single exon deletion was present in 166 (20.4%) of the affected individuals. Validation of MLPA results by multiplex PCR was performed in 135 affected individuals for the following exons- 4, 8, 43, 44, 45, 46, 48, 50, 51, 52 and 53. We obtained 100% concordant results for single exon deletions by MLPA and multiplex PCR probably due to zero frequency of alternate alleles for SNPs in the South Asian population. However, confirmation of MLPA-detected single exon deletions by an alternate technique is still essential due to emerging novel single nucleotide polymorphisms.

Keywords: Duchenne muscular dystrophy, Becker muscular dystrophy, Multiplex ligation-dependent probe amplification, multiplex polymerase chain reaction

Introduction

Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD) are X-linked neuromuscular disorders caused by mutations in the dystrophin gene. The *DMD* gene is the largest known human gene containing 79 exons and spans about 2.5 Mbp of genomic sequence at the Xp21 locus (Barzegar et al., 2015). Approximately, two thirds of variants are large gene deletions or duplications, and the remainder are sequence variations (Flanigan et al., 2009). Previous techniques used for analysis of copy number changes like Southern blotting and quantitative polymerase chain reaction (qPCR) can detect fewer regions in specific genomic areas. At present, Multiplex Ligation dependent Probe Amplification (MLPA) is the most widely used technique to detect deletions and duplications in disorders like DMD due to its high degree of multiplexing. However, the ability of MLPA to detect single exon deletions can be varyingly affected based on population-specific single nucleotide polymorphisms (SNP) especially the CA mismatch between the 3' end of the left probe oligonucleotide and the target and this in turn can result in false-positive exon deletion. This is considered as one of the major pitfalls of MLPA as highlighted by various studies (Kim et al., 2016; Wang et al., 2008). It is therefore suggested that employment of a different technique like multiplex PCR, array comparative genomic hybridisation (aCGH) or Southern blotting is required to confirm single exon deletions detected by MLPA.

In this study, we tried to validate the results of

single exon deletions in the *DMD* (dystrophin) gene detected by MLPA in Indian patients with DMD by utilising an alternative multiplex PCR technique.

Methodology

This is a retrospective analysis conducted in our institute. The Institutional Ethics Committee approval was obtained. The data of patients with DMD/BMD diagnosed by the MLPA technique between the period January 2016 to February 2021 was collated. A subset of patients with apparent single exon deletions were recruited for further evaluation. Female carriers with deletion or duplication of dystrophin gene were excluded from the study.

DNA extraction and MLPA analysis

Genomic DNA was extracted from peripheral blood samples using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). The DNA was quantified using a QIAxpert (Qiagen, Hilden, Germany) and stored at 4ºC. MLPA assay was performed using the P034/P035 DMD Kit with version B1 for P035 and B2 for P036 kit (MRC Holland, Amsterdam, Netherlands). Amplified products were analysed using an ABI 3100 analyser (Thermo Fisher Scientific, Massachusetts, USA). Peak heights were normalized, and a hemizygous deletion was confirmed when the normalized peak ratio value was 0 for male individuals.

Multiplex PCR analysis: DNA quality of all the stored samples was assessed by measuring its concentration in UV-Vis Spectrophotometer Q5000 (Quawell, USA). The exons tested by multiplex PCR included 4, 8, 43, 44, 45, 46, 48, 50, 51, 52 and 53 of the *DMD* gene. Chamberlain and Beggs primer sets were used. An external control and a single exon with different base pair size that was not deleted in the patient was used as an internal control in the patient's sample. The reaction mix contained 200 ng genomic DNA, 1µL of each primers, 10µL master mix and nuclease free water to make a final concentration of 20µL. Cycling conditions were 35 cycles of denaturation at 96°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 5 min. The PCR product (20µL) was electrophoresed in 2% agarose gel, stained with 2µL of ethidium bromide and run for 20-30 mins at 120V. The bands were visualized under an ultraviolet transilluminator.

Sequencing of the gene was planned if results were discordant between MLPA and multiplex PCR. National Center for Biotechnology Information dbSNP (https://www.ncbi.nlm.nih.gov/snp/) database was used to search for the single-nucleotide polymorphisms (SNP) within 8bp from the ligation sites on probes included in the P034/P035 DMD Kit. Allele frequency of these SNPs in the South Asian population was also ascertained from gnomAD (https://gnomad.broadinstitute.org), ALFA project (https://thealfaproject.com) and the recently published Indian database of 1455 individuals (Kausthubham et al., 2021)

Results

A total of 1051 patients were clinically diagnosed with DMD/BMD during the study period. The MLPA technique detected deletions and duplications in 813 (77%) individuals during the study period. Amongst them, single exon deletions were identified in 166 (20.4%) individuals. While single exon deletions identified in our cohort involved the following exons: 2, 4, 8, 9, 11, 13, 17, 18, 20, 43, 44, 45, 46, 48, 50, 51, 52, 53, 54, 55 and 57, we analysed only those individuals with 4, 8, 43, 44, 45, 46, 48, 50, 51, 52 and 53 exon deletion of the *DMD* gene. This was due to non-availability of few primers, insufficient number of patient samples in a single exon group and insufficient quantity and concentration of DNA in a few individuals. The distribution of single exon deletions of our cohort and those exon deletions analysed by multiplex PCR are depicted in **Figure 1**. Finally, DNA samples from a total of 135 patients with various single exon deletions were evaluated by the multiplex PCR technique. Amongst these, exon 45 followed by exon 51 were the commonest single exons found to be deleted in DMD/BMD patients.

Amongst the evaluated samples, we did not find any discrepancy between the results of MLPA and multiplex PCR for all the evaluated samples confirming the true positivity of MLPA results in our cohort (**Figure 2a & 2b**). The SNPs within 8bp from the ligation sites on probes of the exons and the frequency of alternate alleles in South Asian population were obtained from gnomAD, ALFA project and the recently published Indian database of 1455 individuals. These details are represented in **Table 1** for all the exons where single exon deletion was identified by MLPA. The alternate allele frequency of all these SNPs was zero in the South Asian population including the

Indian database.

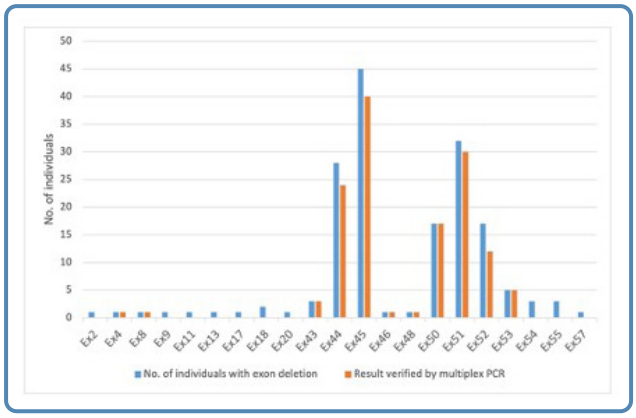

Figure 1 Total number of individuals with various single exon deletions identified by MLPA in our cohort versus those which were verified by multiplex PCR.

Discussion

Deletions and duplications of the dystrophin gene are the most frequently observed molecular events in DMD/BMD. In our study, single exon deletions constituted 20.4% of overall deletions/duplication events. Exon 45 followed by 51 was the frequent single exon deletion observed. These findings were similar to previous studies from India (Basumatary et al., 2013; Deepha et al., 2017) . In this study, we obtained 100% concordant results between MLPA and multiplex PCR technique for single exon deletion. Analysis of the alternate allele frequency for SNPs within 8bp of binding site on probes suggested absence of these alternate alleles in South Asian population.

Amongst various molecular techniques available to detect copy number variations (CNVs), MLPA offers important advantages as it can be used to identify female carriers, detect duplications and to map deletion and duplication borders which has important implications for prognosis and management. (Janssen et al., 2005). However, one of the frequent pitfalls of MLPA reported in literature (Kim et al., 2016) is false deletion results by sequence variations on probe-binding sites especially for single exon deletions. According to the MLPA design protocol, sequence variations within 8bp from ligation sites can affect the hybridization or ligation of the MLPA probe (MRC Holland, Amsterdam, Netherlands).

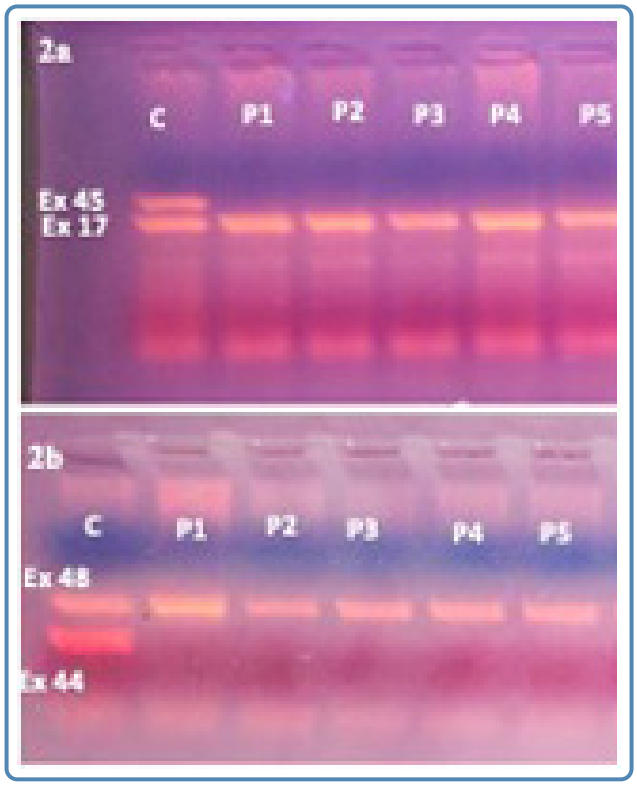

Figure 2 Multiplex PCR products run on gel electrophoresis.
2a: Top row showing band of exon 45 product and bottom row showing band of exon 17 product (internal control band).1st lane showing presence of both bands exon 45 and 17 in a control individual and rest of the lanes shows absence of band for exon 45 only, suggestive of exon 45 deletion in DMD patients.
2b: Top row showing band of exon 48 product (internal control band) and bottom row showing band of exon 44 product.1st lane showing presence of both bands exon 44 and 48 in a control individual and rest of the lanes shows absence of band for exon 44 only, suggestive of exon 44 deletion in DMD patients.

Also, CA mismatch between the 3' end of left probe oligonucleotide and the target is found to be associated with the lowest probe signal among single-nucleotide mismatches and studies have postulated the reason to presence of a single hydrogen bond for C and A nucleotide (Janssen et al., 2005; Aboul-ela et al., 1985). While efforts are

continuously being taken to prevent designing of probes in those regions, CA mismatch due to new mutagenesis cannot be prevented and hence interpretation of single exon deletions by MLPA have to be considered always with caution.

In our study, we did not observe any false positive results for single exon deletions detected by MLPA and multiplex PCR confirmed the presence of these single exon deletions. Next, we enlisted the SNPs especially those causing CA mismatches on binding regions of probes within 8bp from ligation sites by obtaining data from dbSNP. The databases like ALFA project, gnomAD and the recently published Indian database of 1455 samples were analysed for determining the frequency of alternate alleles. While considerable number of SNPs including those causing CA mismatches especially between the 3' end of left probe oligonucleotide and the target were identified, their allele frequency was zero in South Asian population. This probably explains the concordance of single exon deletion results by MLPA and multiplex PCR. A previous study in Korean patients has detected a false positive rate of 14.7% (11/75) for single exon deletions identified by MLPA and factors like CA mismatches in the binding region of the probe and probe melting temperature (Tm) ≤75°C were found to be causative for false positive MLPA results (Kim et al., 2016).

Various studies have proven that novel sequence variants (especially C to T transition) account for a considerable portion of point mutations for DMD gene and hence the probability of new CA mismatches can increase (Buzin et al., 2005). One solution to partially solve this issue is to upgrade the versions of MLPA regularly by designing new probes based on the occurrence of new SNPs. Another easier option is to cross check the results of the single exon deletion detected by MLPA with a simple alternate technique like multiplex PCR.

Conclusion

To conclude, this is the first study in literature that exclusively validated the results of MLPA-detected single exon deletions of the dystrophin gene by the multiplex PCR technique. Absence of false-positive results for single exon deletion by MLPA could probably be due to zero frequency of alternate alleles for SNPs. Confirmation of the results of single exon deletions obtained from MLPA by an alternate technique is however still warranted due to emerging novel single nucleotide polymorphisms.

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Table 1 SNPs within 8 bp from ligation sites on probes and their allele frequency in the South Asian population for the detected exons from gnomAD, ALFA project and in-house exome data of 727 samples.

S no.	Exon number	Nucleotide change (NM_004006.3)	SNPs	Partial probe sequence adjacent to ligation site	Alternate Allele Frequency in South Asia (gnomAD and ALFA project)	Alternate Allele Frequency in South Asia (Indian database [5])
1	Exon 2	c.62T>C	rs398124010	TTCAAAAGAAAA-CAT***T***CACAAAAT	NA	0.000
2	Exon 4#	c.230T>A	rs886042956	TGCCCTGAACAA-TG***T***CAACAAGGC	NA	0.000
3	Exon 8#	-	-	AGGTAAAGTGTG-TAAAGGACAGCT	-	-
4	Exon 9	c. 861G>A	rs1284806719	GCACAGGGATAT-GA***G***A***G***AACTTCT	0.00[a]	0.000
		c.863G>T/C	rs767304770		0.00[a] 0.000[b]	0.000
5	Exon 11	c.1173C>T/G	rs1057522737	ATTTGACAGC***C***C-A***T***CAGGGCCGGG	0.00[a] 0.000[b]	0.000
		c.1176T>C	rs1057521572		0.00[a]	0.000
6	Exon 13	c.1594C>T/G	rs763936813	AATAATCTG***A***C***C***-TTAA***G***TT***G***TTCT	NA	0.000
		c.1597C>T	rs1556834538		NA	0.000
		c.1604T>C	rs770149373		NA	0.000
		c.1602G>A	rs1556834513		NA	0.000
7	Exon 17	c.2084T>G	rs1556780761	GGAA***C***AG***A***TCC***T***-GGTAAAGCATGC	NA	0.000
		c.2080A>T	rs777042810		0.00[a]	0.000
		c.2077C>T	rs1060502661		NA	0.000
8	Exon 18	c.2226G>A	rs775246170	AGAAGCTG***T***GTT-***G***CAGAGTCCTGA	0.00[a] 0.000[b]	0.000
		c.2222T>C	rs886039135		0.00[a]	0.000
9	Exon 20	c.2436G>A	rs398123889	CGGTG***G***ATCGAA-TTCTG***C***CAGTTG	NA	0.000
		c.2448C>A	rs1603634746		NA	0.000
10	Exon 43#	c.6195T>C	rs398124006	GCATTGCAAA***GT***-***G***CAA***CG***CCTGTG	NA	0.000
		c.6194G>T	rs767872954		NA	0.000

		c.6196G>T	rs1468473458		0.00[a] 0.000[b]	0.000
		c.6200C>T	rs1405599353		0.00[a]	0.000
		c.6201G>A	rs969925680		0.00[a] 0.000[b]	0.000
11	Exon 44[#]	c.6373C>T	rs128626251	GAA***C***AGTTTCTC-AGAAAGACACAA	NA	0.000
12	Exon 45[#]	c.6535A>T/G/C	rs1361261157	ACAGATGCC***A***GT-ATTCTA***CA***GGAA	NA	0.000
		c.6545A>G	rs749804804		0.00[a] 0.000[b]	0.000
		c.6544C>T	rs1557038061		NA	0.000
13	Exon 46[#]	c.6704C>G/T	rs1316893006	AACATTGCTA***G***T-ATCCCACTTGAA	0.00[a] 0.000[b]	0.000
14	Exon 48[#]	c.7024C>T	rs749996618	CAGTTAAATC***A***T-C***T***GCT***GC***TGTGG	0.00[a] 0.000[b]	0.000
		c.7016A>G	rs398124041		0.00[a] 0.000[b]	0.000
		c.7019T>C	rs1569492076		NA	0.000
		c.7023G>T	rs886042910		NA	0.000
15	Exon 50[#]	-	-	AGAGAATGGGAT-CCAGTATACTTA	-	-
16	Exon 51[#]	c.7427A>G	rs757394130	GCTCTGGCAGAT-TTCA***A***C***C***GGGCT	0.00[a] 0.000[b]	0.000
		c.7429C>T	rs759274835		0.00[a] 0.000[b]	0.000
17	Exon 52[#]	-	-	CTAGCCTCTTGA-TTGCTGGTCTTG	-	-
18	Exon 53[#]	c.7781A>G	rs777178221	CTGAGC***A***G***G***TCT-TAGGACAGGCCA	0.00[a] 0.000[b]	0.000
		c.7783G>T	rs1254164861		NA	0.000
19	Exon 54	c.7914T>A	rs754997935	TGGCAGACAAAT-GTAGA***T***GTGGCA	0.00[a] 0.000[b]	0.000
20	Exon 55	c.8069T>G	rs398124061	AAACTCATAGAT-***T***ACTGCA***A***CAGT	NA	0.000
		c.8076A>G	rs144518527		0.00[a] 0.000[b]	0.000
21	Exon 57	-	-	CTGAAAGATGAT-GAATTAAGCCGG	-	-

a: ALFA project, b: gnomAD, NA: frequency data not available, - indicates absence of SNPs within 8bp from ligation sites on MLPA probes, # indicates those MLPA-detected exon deletions that have been confirmed by multiplex PCR.