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Comparison of multiplex ligation-dependent probe amplification (MLPA) analysis versus multiplex PCR assays in the detection of dystrophin gene

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ABSTRACT

The Duchenne muscular dystrophy (DMD) gene is located in the short arm of the X chromosome (Xp21). It spans 2.4 Mb of the human genomic DNA and is composed of 79 exons. Mutations in the dystrophin gene results in DMD and Beckers muscular dystrophy. In this study, the efficiency of MLPA over multiplex PCR assays in an Iranian population was investigated.Multiplex PCR assays and multiplex ligation-dependent probe amplification (MLPA) analysis were carried out in 65 patients affected with DMD. Multiplex PCR detected deletions in 51% of the patients with DMD. MLPA analysis could determine all the deletions detected by the multiplex PCR. Additionally, MLPA was able to identify one more deletion and duplication in patients without detectable mutations by multiplex PCR. Moreover, MLPA precisely determined the exact size of the deletions.Although MLPA analysis is more sensitive for detection of deletions and duplications in the dystrophin gene, multiplex PCR might be used for the initial analysis of the boys affected with DMD in the Iranian population as it was able to detect 95% of the rearrangements in patients with DMD.

KEY WORDS: DELETION, DUCHENE MUSCULAR DYSTROPHY, DUPLICATION, MLPA, MULTIPLEX PCR

INTRODUCTION

The Duchenne muscular dystrophy (*DMD*) gene (Medelian Inheritance in Man MIM 300377) located at Xp21 spans 2.4 Mb and contains 79 exons. The *DMD* gene is the largest known human gene, and its product is called dystrophin, which is mainly expressed in the skeletal and cardiac muscles, with small amounts expressed in the brain. Dystrophin is comprised of an N-terminal actin-binding domain, 24 spectrin-like repeat units interspersed by four hinge regions, a cysteine-rich domain and a C-terminal domain. It is involved in the linkage between the cytoskeletal actin and the extracellular matrix (Blake *et al.*, 2012; Ehmsen *et al.*, 2013, Roberts *et al.*, 2014 and Hoffman *et al.*, 2015).

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Various mutations in *DMD* gene result in Duchenne muscular dystrophy and Becker muscular dystrophy (DMD/BMD) (Prior & Bridgeman, 2015). DMD (MIM 310200) is the most common X-linked recessive lethal disease affecting one in 3500 newborn males. Affected boys are characterized by progressive dystrophy of the skeletal muscles and are usually wheelchair-bound before the age of12 years (Emrey, 2002). BMD (MIM 300376) is a milder form of the disease, with an incidence of around one in 12,000 live born males (Bushby, 2012). Patients with BMD have a later age of onset (some as late as 40-50 years of age) and a slower clinical progression. In boys affected by DMD, the prematurely truncated, unstable dystrophins lacking cystein-rich and C-terminal domains are generated. These proteins have completely lost their function as a bridge due to mutations disrupting the open reading frame. In patients with BMD, low levels of full length dystrophin or internally deleted dystrophin resulting from in-frame mutations are detected (Aartsma-Rus, 2006). Deletions or duplications are found in about 60-65% and 5-10% of patients with muscular dystrophy, respectively (Den Dunnen *et al*., 1989 and White *et al.,* 2015).

Although deletions encompass all 79 exons, two deletion hotspots – one including exons 45-55 and the other including exons 2-19 – are recognized. Approximately 30-35% of the cases with BMD/DMD are caused by point mutations (nonsense or splice sites) or small rearrangements (Muntoni, 2013).Multiplex ligation-dependent probe amplification (MLPA) analysis has proven to be a method of choice for the detection of deletions and duplications (Sellner *et al*., 2004; Schouten *et al*., 2012; Schwartz and Dunø, 2015).

Before development of MLPA, detection of deletions and duplications in the *DMD* gene was mostly carried out by applying Southern blot (Puget, 1999) and multiplex polymerase chain reaction (PCR). Because Southernblotting was a labor-intensive and time-consuming method, it was replaced by the multiplex PCR (Prior & Bridgeman, 2015). The most commonly used multiplex PCR protocols cover 18 exons at the deletion hot spots and detect 90-98% of all deletions (Beggs *et al.,* 1990; Chamberlain et al., 1988). Multiplex PCR is proven to be a method for the rapid detection of deletions in boys affected with DMD; however, it is not efficient for the precise identification of *DMD* gene duplications in males and heterozygous deletions and duplications in females. The aim of this study was to prove the efficiency of the MLPA analysis in a subset of Iranian patients. Therefore, we used multiplex PCR assays followed by MLPA analysis in 65 cases with DMD.

MATERIAL AND METHODS

PATIENT SAMPLES

This study was performed in a total of 65 boys affected with DMD from the central provinces of Iran referred by the neurology specialists to Genetic Lab, AlhadiHospital, for genetic analysis of their *DMD* gene. The diagnosis of DMD was according to the clinical features, electromyography, an elevated serum creatinekinase activity and an X-linked family history. After obtaining informed consent, genomic DNA was extracted from blood leukocytes using a QIAampDNA mini kit (Qiagen, Hilden, Germany). The exact DNA concentration was determined using a NanoDrop instrument (Thermo 2000c) after dilution to 50 ng/ml.

MULTIPLEX PCR

Two multiplex PCR assays covering exons 1, 3, 4, 6, 8, 12, 13, 17, 19, 43, 44, 45, 46, 47, 48, 50, 51, 52, and 51 in the hot spot regions of the *DMD* gene were performed in a total volume 50 µl according to the technique described by Chamberleidon and Beggs. [16,17] Multiplex PCR products were visualized on 2% agarose gel.

MLPA ANALYSIS

MLPA analysis was carried out using the SALSA probe mix 034 (*DMD* exons 1-10, 21-30, 41-50, and 61-70) and 035 (*DMD* exons 11-20, 31-40, 51-60, and 71-79) according to the manufacturer's instructions. In brief, the ligation reaction was performed using 200 ng of target DNA in the following steps: Denaturation at 98°C for 5 min, hybridization with the SALSA probe mix 034 and 035 at 60°C for 16 h and ligation by Ligase-65 mix at 54°C for 15 min, and then ligase inactivation by incubation at 98°C for 5 min. Finally, multiplex PCR was performed using the specific SALSA FAM PCR primers, dNTPs, PCR buffer and polymerase for 30 cycles (95°C for 30 s, 60°C for 30 s and 72°C for 1 min). The fragments were analyzed on a 3100 capillary sequencer (Applied Biosystems, UK) with a 36-cm capillary array and POP-4TM polymer (Applied Biosystems, UK) by mixing with 0.2 µl of the GeneScanTM-500 ROXTM size standard (Applied Biosystems, UK) and 10 µl of HiDiFormamide (Applied Biosystems, UK). The results (size and peak area) were analyzed using GeneMarkersoftware.

RESULTS AND DISCUSSION

Multiplex PCR assays indicated that 38 of 65 boys affected with DMD have exon deletions. However, MLPA

analysis indicated one more deletion and duplication. These new mutations identified by MLPA were confirmed by direct sequencing of the relevant exons using primers described earlier (Sedlácková *et al.*, 2015). Furthermore, two deletions with different sizes were identified than what was detected by multiplex PCR. In male patients, total absence of MLPA peak areas indicated deletions of one or more exons, and multiplication of MLPA amplification products indicated duplications. Among the 40 identified rearrangements, 36 were located in the central hot spot region, including exons 44-55. Four deletions were identified in the 5 hot spot region.

In this study, we performed multiplex PCR assays and MLPA analysis to identify deletions and duplications in the *DMD* gene. We used multiplex PCR as the first step for identification of deletions in male patients. Then, MLPA analysis was performed to confirm the results obtained by the multiplex PCR.Because the *DMD* gene is X-linked, deletions are easily identified by conventional multiplex PCR in male patients. However, this method cannot be used for carrier analysis in women and detection of duplications and uncommon deletions in men with DMD. In addition, multiplex PCR is more difficult to be performed because there are different primer pairs for exons in the hot spot regions, and it is not possible to amplify these exons in a single reaction. However, MLPA can be used to detect rearrangements in all exons of dystrophin gene in both females and males by only two reaction sets (Yau *et al.*, 1996; Abbs and Bobrow, 1992).

In our study, two multiplex assays covered 19 exons of the *DMD* gene, and in 51% of the cases deletions were identified. However, because all 79 exons of the *DMD* gene are included in two separate MLPA kits, MLPA analysis of subjects revealed the exact range of deletion in two patients and two more rearrangements. In two patients, multiplex PCR assays detected deletion of exon 50; however, deletion of exons 49-50 was detected by MLPA analysis. In addition, duplication of exon 51 and deletion of exon 53 were detected by MLPA analysis. Single duplication of exon 51 is not identified in other populations. However; duplication of exons 51-55 was reported in a Taiwanese patient (Hwa *et al.*, 2015).

Exon 51 duplication was identified in an 11-year-old boy whose mother did not show any mutation with MLPA analysis. The identified duplication in the boy might be a *de novo* mutation, or it could have resulted from maternal germ line mosaicism. These findings indicated that MLPA analysis is more sensitive and reliable than conventional multiplex PCR for the detection of exon deletions and duplications. Although deletions and duplications can occur in any exon of the *DMD* gene, their frequency is higher in two regions – one located in the 5 region (called minor hot spot) and the other located in the central part (called major hot spot). In the present study, 90% of the rearrangements were located in the central hot spot region between exons 44 and 55. Eight of the 36 deletions located in central hotspot region were deletion of exons 45-51. In another study performed by Khordadpoor- Deilamani *et al.* (2015) 52 of 53 deletions in the dystrophin gene were detected by multiplex PCR. This indicates that 98.11% deletions in dystrophin gene are located in hot spot regions, which is in agreement with our findings.

In the remaining cases without detectable deletion or duplication, after confirmation of the diagnosis by the neurology specialists, DNA sequencing can be used for identification of point mutations, small insertion or deletion that are scatteredthroughout the dystrophin gene. Alternatively, a dense set of polymorphic and dymorphic markers can be used to track mutation in patients with a family history. Identification of deletions and duplications in the DMD gene is important for prenatal diagnosis and determination of genotype–phenotype correlation. In recent years, MLPA is recommended to be used as the method of choice for detection of deletions and duplications in the DMD gene. In this study, we detected 95% of the deletions by multiplex PCR, which shows that most of the deletions in our population are located in the hot spot regions. These findings indicate that an initial analysis of patients with DMD using multiplex PCR and then MLPA analysis of patients without detectable mutation is more practical in our population. This approach is described to be a precise and cost-effective tool for DMD diagnosis in developing countries as described by Murugan et al. (2015) in the Indian population.

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