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Identification of *DMD* Mutation in Korean Siblings Using Full Gene Sequencing

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ABSTRACT Duchenne Muscular dystrophy (DMD) is an X-linked recessive disorder caused by mutations in the dystrophin gene, which is located in Xp21. The majority of the identified mutations are large deletions and duplications, and gene dosage assays were developed for quantitative genomic screening of copy number variations. However, remaining 25% of the DMD are due to point mutations and require direct full gene sequencing. We report Korean siblings with novel small intragenic duplication in an exon 41 (c.5756dupT) which was detected by direct sequencing of whole dystrophin (*DMD)* gene exons. This 1-bp duplication is a novel frameshift mutation and induces premature termination (p.Leu1919Phefs*13). Gene therapy in DMD has been developed and it is important to know the exact mutation site and type to predict prognosis and to prepare further therapy. Therefore, in DMD patients with normal *DMD* gene dosage, direct sequencing of *DMD* gene is essential to detect small intragenic deletions/insertions and missense, nonsense, and splicing mutations.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is the most common inherited muscle disease, characterized by dystrophin deficiency caused by mutations in the dystrophin (*DMD*) gene (Davies et al. 1988). The *DMD* gene is located at Xp21 with 2.2 million base pairs, with 79 exons and several promotors, comprising 0.6% of entire human genome. Dystrophin is 427-kDa cytoskeletal protein that is a member of the β-spectrin/α-actinin protein family. This protein is important for the strength and flexibility of the muscle fiber menbranes. Dystrophin can be organized into four separate regions. These are the actin-binding domain at the NH_2 terminus, the central rod domain, the cysteine-rich domain, and the COOH-terminal domain. (Blake et al. 2002). In previous studies, Multiplex ligation-dependent probe amplification (MLPA) has been shown as a useful method for quantitatively detecting mutations in the *DMD* gene, not only for identifying deletions but also for duplications and female carriers (Yang et al. 2013). However, about 25% of the DMD are due to point mutations and require direct full gene sequencing. In this study, the researchers report Korean male siblings with novel small intragenic duplication which was detected by direct sequencing of whole *DMD* gene. Like our case, in patient with negative MLPA, Sanger direct sequencing is essential for the diagnosis of the DMD. The role of direct sequencing cannot be over-emphasized in DMD studies.

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CASE REPORT

A 2 year-old Korean male was referred for evaluation of gating disturbance and general weakness. Physical examination revealed Gower sign and calf muscular hypertrophy. According to the family history, his 8 year-old brother has been suffered by similar muscular dystrophy since 2 years ago. Blood examination revealed elevated serum levels of creatine kinase (CK) 19,470 U/L (normal: 26-200U/L), aspartate aminotransferase (AST) 241 U/L (normal: 14-40 U/L), and alanine aminotransferase (ALT) 408 U/L (normal: 9-45 U/L). Chromosomal analysis revealed a normal male karyotype (46, XY). His brother showed similar clinical and laboratory findings (Table1). The needle electromyography findings in brother showed abnormal spontaneous activities (positive sharp waves and fibrillation potentials) at rest, and small amplitude, short duration and polyphasic motor unit action potentials with early recruitment patterns on volition. Also, turn-amplitude

analysis revealed a pattern that was compatible with myopathy.

Table1: Comparison of clinical findings between proband and his elder brother

Parameter	*Proband*	*Brother*
Sex	Male	Male
Age	2	8
Symptom	Gait disturbance	Gait disturbance
Physical examination	Gower sign(+), Calf hypertrophy (+/+)	Gower sign(+), Calf hypertrophy (+/+)
Serum creatine kinase (U/L)	19,470	>20,000
AST/ALT (U/L)	241/408	219/357
LDH (U/L)	4,105	2,843
Electromyography	ND	Compatible with myopathy
Chromosomal analysis	46,XY	46,XY
Muscle biopsy	ND	ND
Radiologic findings	ND	ND

ND, not done

The researchers firstly screened *DMD* gene dosage using SALSA® MLPA® Kit P034-A2/P035-A2 *DMD*/Becker (MRC-Holland, Amsterdam, Netherlands) because partial gene deletions or duplications accounts for the majority of DMD cases. The gene dosage assay was normal, therefore direct sequencing for all 79 coding exons of *DMD* with their immediate flanking intronic regions were performed using the previously reported primer sequences (Roberts et al. 1991).

Direct sequencing analysis for all 79 coding exons of *DMD* revealed a novel single nucleotide insertion in exon 41 (c.5756dupT) (Fig. 1). This mutation was found in siblings. This 1-bp duplication produced a frameshift mutation at codon 1919 and resulted in a stop codon at codon 1931 (p.Leu1919Phefs*13) in a rod domain and a loss of function of dystrophin.

DISCUSSION

Diagnosis of DMD is done by measuring CK, muscle biopsy and electromyography, but gold standard for diagnosis relies on the molecular diagnostic method. In the DMD patients, large deletion accounts for 65% and large duplication accounts for 10% of the total mutations (Cutis and Haggerty 2001), while the most of the remaining cases are due to point mutation,

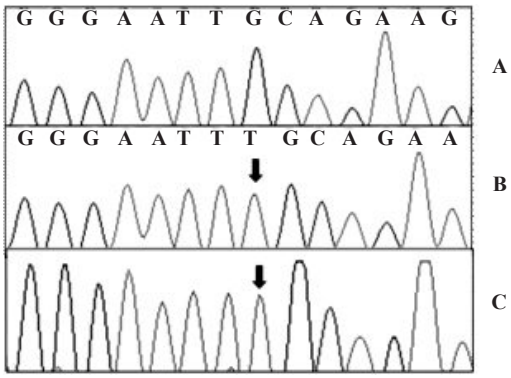

Fig. 1. Direct sequencing results *DMD* gene in normal control (A), proband (B) and his brother(C). This 1-bp duplication of thymine (c.5756dupT) at exon41 produced a frameshift mutation at codon 1919 creating a premature termination codon at codon 1931 (p.Leu1919 Phefs*13).

small portion of them are insertions/deletions or splice site changes. Complex rearrangements and deep intronic changes account for approximately 2% of the DMD cases (Laing et al. 2011). About 25% of the cases require molecular diagnostic method at the nucleotide level. However, the direct sequencing analysis is considered to be laborious, expensive and time consuming. As many recent studies reported DMD patients with negative MLPA, point mutation detected by Sanger direct full gene sequencing, the role of direct sequencing in diagnosis of DMD is increased. The novel point mutations in *DMD* gene are also revealed continually (Chen et al.2013, Yang et al.2013).

There have been a few reported cases of genetic analysis of the *DMD* gene in Korean population. Lee et al. reported 16 cases of DMD patients with exon deletions or duplications by multiplex PCR and MLPA assay. They also identified a novel nonsense mutation (c.4558G>T; Glu1520*) confirmed by direct sequencing (Lee et al. 2012). Song et al. reported 2 cases of female DMD carriers evaluated by MLPA (Song et al. 2011). Therefore, about 90% (18/20) of genetically defined Korea DMD probands was detected by MLPA with 70% of exon deletion and 20% of duplication. Remaining 10% (2/20) including our siblings revealed negative *DMD* gene dosage assay, and direct sequencing was required to defined molecular change of the *DMD* gene.

The majority of large deletions detected in DMD cluster around two mutation “hot spots”.

Deletion cluster region I spans exons 45–53 and removes part of the rod domain, while deletion cluster region II spans exons 2–20 and removes some or all of the actin-binding sites together with part of the rod domain. Unlike the large deletions, small deletions and point mutations appear to be evenly distributed throughout the gene (Curtis and Haggerty 2001; Murugan S et al. 2010).

In this case, the mutation occurred in exon 41. Duplication of 5756th thymine resulted in frameshift mutation and truncation of dystrophin protein. This novel mutation was not listed either in *UMD-DMD* Database or in previous publications (Tuffery-Giraud et al. 2009). Many mutations in the *DMD* gene disrupt the open reading frame and thus cause the premature abortion of the synthesis of the dystrophin, leading the severe DMD phenotype as like our siblings. In the other hand, in-frame mutations conserve the reading frame and reveal less severe symptoms and much longer to normal life expectancies.

Over the last 10 to 15 years several investigators has made much effort to develop an efficient gene therapy for DMD. Small synthetic antisense oligonucleotides (AOs) against splicing regulatory sequences have been proposed to produce in-frame dystrophin mRNA from the out-of frame mRNA by inducing exon skipping during splicing (Takeshima et al. 1995). AOs have been designed to target pre-mRNA exons at or in close proximity to the site of mutation such as exon/intron boundary, exon splice enhancer element or branch point (Aartsma-Rus et al. 2009). This is an appealing approach for conversion of the severe DMD disease into a condition analogue to the substantially milder BMD. The main targets for exon skipping therapy are exons 44, 45, 51, and 53 (Takeshima et al. 2010) and AOs against exon 51 are now in phase II or III clinical trials (Aartsma-Rus 2010; Lu et al. 2011). Recently, Malueka RG et al. categorized of *DMD* exons into five groups (A-E) by indexes of splicing regulatory factors (Malueka et al. 2012). Group A includes exon 45, 51 and 53 and is characterized by a high density of exon splicing enhancers (ESE) which can be a good target of AOs. Exon 41 belongs to group A, so we expect that our patients will be a candidate for gene therapy in near future.

Full sequence analysis of the *DMD* gene requires a high level of laboratory efforts because of the large number of separate amplicons required to cover all 79 exons. However, it is more important to know the exact mutation site and type to predict prognosis and to prepare further therapeutic approach. *DMD* full gene sequence analysis should be undertaken especially in patients with negative gene dosage assay.

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