듀센형 근이영양증 환자의 엑솜 시퀀싱

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Whole Exome Sequencing of a Patient with Duchenne Muscular Dystrophy

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Duchenne muscular dystrophy (DMD) is the most common and lethal dystrophy in childhood, caused by mutations in the dystrophin (DMD) gene. Multiplex ligation dependent probe amplification (MLPA) or array comparative genome hybridization (aCGH) is widely used as an initial molecular diagnostic tool. If no deletions or duplications are found in MLPA or aCGH, the samples must be subjected to a second test of direct sequencing. Direct sequencing of the DMD gene, however, is time-consuming, high-cost, and can be inconclusive. Here, we performed whole exome sequencing on a patient with progressive muscle weakness whose MLPA result was negative; the result revealed a rare frame shift mutation. Direct sequencing on the patient's mother showed the same mutation. Whole exome sequencing can be a new diagnostic routine for DMD patients with negative MLPA3

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Key Words: Duchenne muscular dystrophy, dystrophin, High-Throughput DNA Sequencing, genetics, mutation

Introduction

DMD is the most common and lethal dystrophy in childhood, with an incidence of 1 in 3,500 newborns¹⁾. DMD is a genetic disorder characterized by progressive muscle tissue degeneration, onset before 3 years of age, independent ambulation loss between the ages of 10 and 14 years, and death between the ages of 20 and 30 years²⁾. The disease is caused by mutations in the dystrophin (DMD) gene which is the largest known gene, with 79 exons spanning 2.4 Mb of Deoxyribonucleic acid (DNA)³⁾. More than 4,700 mutations have been identified in DMD and are subdivided into deletions (65.8 %), duplications (13.6 %), and point mutations (micro-insertions, microdeletions, and nonsense, missense, and splicing mutations; 20.6%)⁴⁾. Because of the enormous size of the gene and wide range of mutation spectrum, fast & accurate diagnosis cannot be achieved easily⁵⁾. Because over 2/3 of DMD patients have large deletions or duplications in DMD gene, MLPA or aCGH is widely used as an initial molecular diagnostic tool. MLPA has enabled more reliable and faster quantitative detection of the entire dystrophin

gene containing 79 exons to study the deletions and duplications⁶⁻⁹⁾. However,

Submitted: 7 March, 2014
Revised: 31 March, 2014
Accepted: 31 March 2014

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1/3 of the remaining patients with negative MLPA should be performed direct sequencing or considered to have muscle biopsy. Despite time-consuming, high-cost, and pain of the entire process, the results can be inconclusive⁵⁾. Here, we performed whole exome sequencing on a patient with progressive muscle weakness whose MLPA result was negative.

Case report

A 10 year old male patient visited our neurology clinic for evaluation of progressive muscular weakness. The patient was born at 34 weeks gestation by spontaneous vaginal delivery with birth weight of 2.1 kg. After birth, his developmental delay became more obvious as time went by, he started to walk alone at 3 years of age and speech was delayed and could say only “mama” and “this” until 4 years of age. He showed lordotic posture, proximal muscle wasting, Gowers’ sign, tip toeing, and waddling gait. There was no family history of muscular dystrophy. Initial laboratory findings showed increased levels of serum creatine kinase, lactate dehydrogenase, aldolase, myoglobin, and aminotransferase. Psychometric assessment identified the patient in moderate mental retardation; Full-scale IQ of 45 by Wechsler Intelligence Scale for Children, low level of social maturity; Social quotient of 40.46 by Social Maturity Scale, and having autistic features in child behaviour checklist (CBCL). Blood samples with Ethylenediaminetetraacetic acid (EDTA, 3 ml quantities) were collected from the patient and his mother; informed consent forms were collected from the patient and parents towards genetic analysis and utilization of the results for diagnosis and research purpose. Genomic DNA was extracted by QIAamp DNA Mini kit (Qiagen). Quality and quantity was assessed by Qubit Fluorometer (Invitrogen) and Quant-iT BR assay kit (Q32850, Invitrogen). We first analyzed chromosomes from the patient using MLPA. MLPA analysis was carried out using PO34 and PO35 probes purchased commercially from MRC, Holland (Amsterdam, The Netherlands). The procedures were carried out according to the manufacturer’s protocols⁷⁾. 100 ng DNA was denatured and hybridized overnight at 60°C with 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). Samples were then treated with Ligase 65 for 15 min at 54°C. The reactions were stopped by incubation at 98°C for 5 min. Finally, Polymerase chain reaction (PCR) amplification was carried out with the specific SALSA FAM PCR primers. As patient’s sample showed no deletion or duplication by MLPA, we next analyzed chromosomes using whole exome sequencing. Targeted exonic

regions were captured with Agilent SureSelect Human All Exon Kit v4. Exome sequencing was performed on Illumina HiSeq2000 platform with 100bp paired-end reads at a minimum of 50x coverage. Whole exome sequencing revealed 5 exonic variants; 1 of the variants was rare frame shift mutation (NM_004009.3:exon65:c.9439_9442del:p.3147_3148del) and the others were polymorphisms (Fig 1A). We analyzed chromosomes from the mother using direct sequencing to find out whether she inherited the mutation. Exon 65 and flanking regions (–200 intronic bases in 5’ and 3’ direction) were amplified by PCR, using a primer pair specific for DMD (primer sequences available upon request). Screening for genetic abnormalities was performed by direct sequencing methods using dye terminator chemistry (Big-Dye) on an automated DNA sequencer (ABI3130, Applied Biosystems, Foster City, CA). Reference sequences for DMD were based on NCBI records NG_012232.1, NM_004009.3 and NP_004000.1. Direct sequencing revealed that the same mutation was in mother’s DNA (Fig 1B).

Discussion

Before DNA technology developed, diagnosis of Duchenne and its milder form Becker muscular dystrophy (BMD) was done by various biochemical markers, electromyogram, and invasive procedures like muscle biopsy¹⁰⁾. Molecular genetic testing is now the mainstay of diagnosis of DMD¹¹⁾. Chamberlain et al. first designed an assay using multiplex PCR and electrophoresis to detect several deletion-prone exons in 1988¹²⁾. Multiplex PCR covers exons at the deletion hotspots and detects 90–98% of all deletions, but duplications are not identified by this method. Currently, MLPA and aCGH are the most widely applied methods worldwide because they can detect large deletions and duplications together¹³⁾. The development of MLPA has provided a more sensitive technique for detecting deletions. All 79 exons are covered by two sets of probes, with individual exons appeared as a single peak. By the size of the single peak, gene dosage can be determined, allowing detection of duplications or deletions^{6–8,14)}. A targeted high-density aCGH that allows high-resolution analysis of the DMD gene has also been developed recently, allowing identification of deletions and duplications but also previously unidentified deep intronic mutations¹⁵⁾. However, none of these techniques can detect exon-level copy number variations (CNVs) and single nucleotide polymorphisms (SNPs). Thus, if no deletions or duplications are found in MLPA or aCGH, the samples must be analyzed further for a second test of direct sequencing. Direct sequencing of the DMD gene, however, is

Chromosome X DMD gene

EXON65

gagggcataaa	aataaaacac	ctttcaaaga	gagtgtggtt	cacgtttggt	31227927
gtttattaaa	gggtatgaga	gagtcctagc	taggattctc	agaggaaaaa	31227877
ggacactgaa	aggaaggttt	tactctttga	gtcattttgtg	attttatttg	31227827
ttttttgcag	TGGATCTCTT	GAGCCTGTCA	GCTGCAATG	ATGCCTTGGA	31227777
CCAGCACAAC	CTCAAGCAAA	ATGACCAGCC	CATGGATATC	CTGCAGATT	31227727
TTAATTGTTT	GACCACTATT	TATGACCGCC	TGGAGCAAGA	GCACAACAAT	31227677
TTGGTCAACG	TCCCTCTCTG	CGTGGATATG	TGTCTGAAC	GGCTGCTGAA	31227627
TGTTTATGAT	ACgtacgtat	ggcatgtttt	tatttccggg	gctctgtcac	31227577
aggaggctta	gcgtacagaa	tggatgaagt	taattagcat	tacaacaggg	31227527
ccctggaagc	tgtggaccta	atgctcttcc	tgtgataaat	catttttgca	31227477
tttatgactt	gaaaaatatt	ttgataagct	aggagttaag	agccagaatg	31227427

(A) • NM_004009.3:exon65:c.9439_9442del:p.3147_3148del

(B)

Fig. 1. Microscopic appearance of *Strongyloides stercoralis* from duodenal biopsy specimen. A filariform larva (arrow) is seen in the lumen of an intestinal gland (H&E stain, $\times 200$).

not commonly performed due to its time consuming, labor-intensive, and high cost of the test nature. The high demand for low-cost and high-throughput sequencing has led to the development of next generation sequencing (NGS)¹⁶, and recent studies applying NGS have proven its ability in detecting exon-level CNVs and SNPs^{17,18}. Moreover, the cost is lower and turn-around time is less than using MLPA plus direct sequencing. In conclusion, NGS can be a new diagnostic routine for DMD

patients with negative MLPA.

요약

듀센형 근이영양증은 소아에서 가장 흔하고 치명적인 유전성 근육 질환으로, 디스트로핀 유전자의 변이로 인해 발생한다. 디스트로핀 유전자의 결실이나 중복에 의해 생기는 경우가 많으므로 MLPA나

aCGH가 진단을 위해 많이 쓰인다. 그러나 환자의 1/3에서는 변이가 발견되지 않는데, 이럴 경우 direct sequencing을 통해 MLPA나 aCGH에서 확인되지 않는 미세한 결실, 중복, 또는 point mutation이 있는지 확인하게 된다. 그러나 DMD 유전자는 크기가 매우 크기 때문에 direct sequencing을 하기에는 시간과 비용이 엄청나게 소모되며 최종 결과도 확정적이지 않을 가능성이 있다. 우리는 MLPA에서 변이가 발견되지 않은 진행성 근이영양증 환자를 대상으로 whole exome sequencing을 시행하였는데, 디스트로핀 유전자의 frame shift mutation이 발견되었다. 환자의 어머니를 대상으로 시행한 direct sequencing에서도 환자와 같은 변이가 발견되었다. Whole exome sequencing은 MLPA에서 변이가 발견되지 않는 듀센형 근이영양증 환자의 새로운 진단 도구로 훌륭한 역할을 할 수 있을 것으로 생각된다.

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