

Molecular Characteristics of Duchenne Muscular Dystrophy in a Lebanese Cohort

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Abstract

Background: Duchenne Muscular Dystrophy (DMD) is a progressive neuromuscular disorder characterized by a relentless clinical course with diagnosis usually established around three to four years of age. DMD is caused by mutations in the dystrophin gene, where deletions and duplications of one or more exons represent the bulk of related genetic aberrations.

Aims and methods: Our aim in the current study is to analyze the frequency and the distribution pattern of deletions/duplications associated with dystrophin gene exons and assess the mean diagnostic age of DMD in a small Lebanese group of dystrophic patients suspected with DMD/BMD based on observed clinical features.

Results and discussion: Among 52 samples analyzed, we identified 33 cases (63%) with deletions and two cases (4%) with duplications. Deletions were of variable sizes, ranging from 1 to 47 exons and occurred mostly (78%) in two deletion hotspots (HS), HS1 (18%) and HS2 (60%), covering exons 6-19 and 45-52 respectively. Single exon deletions were even further restricted (90%) to the deletion hotspots, mainly to HS2 (80%). The average age of DMD molecular diagnosis in our subject study was 7 years of age.

Conclusion: Molecular analyses were consistent with those obtained in previous studies, with however an average age of DMD diagnosis significantly later than what is usually reported. Our study illustrates the need to implement early molecular diagnosis in order to institute optimal care, including available targeted treatments, for our patients.

Keywords: DMD; Dystrophin; Muscular dystrophies; Deletions; Lebanese cohort

Introduction

Duchenne muscular dystrophy (DMD) is a devastating, progressive, X-linked disorder that affect the neuromuscular system in children. It is the most common muscular dystrophy to date, with an incidence of one in 3500–6000 male live births [1,2]. Clinical manifestations are most often absent at birth with normal height and weight [3]. However, the disease evolves quickly with marked muscle weakness and wasting, learning disabilities, loss of ambulation and cardiac complications by the age of 10. Assisted ventilation may become necessary by the age of 20, with death occurring between 20 and 40 years of age [4–6].

DMD is caused by mutations in the dystrophin gene (DMD), which encodes a structural rod-shaped sub-membrane sarcoplasmic protein, which is a major component of a vital protein complex, the dystrophin-associated protein complex (DAPC) [7,8]. Dystrophin is organized into four major domains extending from myofibrillar proteins to sarcolemmal-bound proteins, providing a vital role in force transduction during muscle contraction and a structural role by ensuring the stability of the sarcolemma [9]. Moreover, the multiple domains and binding sites implicate dystrophin in the regulation of different signaling pathways [10]. Dystrophin gene covers more than 2.2 million base pairs and consists of 79 exons [11]. Since its discovery in 1986, thousands of mutations scattered along the whole gene length have been reported [12]. Deletions and duplications of one or more exons constitute the vast majority (70%-80%) of detected genetic aberrations, whereas small rearrangements and point mutations account for the remaining mutations [13]. Sporadic *de novo* mutations arising either in the mother germ cells or even during the proband's embryonic development, account for one third of all reported cases [14,15]. In addition to triggering the severe clinicopathological form;

Duchenne muscular dystrophy, mutations in the DMD gene are also, at times, responsible for its allelic milder form known as Becker muscular dystrophy (BMD). Most importantly, the disease outcomes are associated, to a certain extent, with the type of the mutation, hence the relevance of early molecular diagnosis [6,16,17]. Moreover, a large number of DMD patients (83%) could theoretically benefit from antisense-mediated exon skipping as most identified deletions cluster to specific hotspots [18].

Detailed information about the nature of DMD associated mutations, their occurrences, and their associated phenotypic patterns will allow appropriate counselling, adequate prognosis, a deeper understanding of the complex genotype-phenotype relationship, an enhanced clinical care, and successful therapeutic trial planning. To our knowledge there is currently no studies performed in Lebanon on the molecular epidemiology of DMD. Here we report the frequency and the distribution pattern of deletions/duplications associated with dystrophin exons as well as the average diagnostic age of DMD in a small Lebanese group of dystrophic patients suspected with DMD/BMD based on clinical features.

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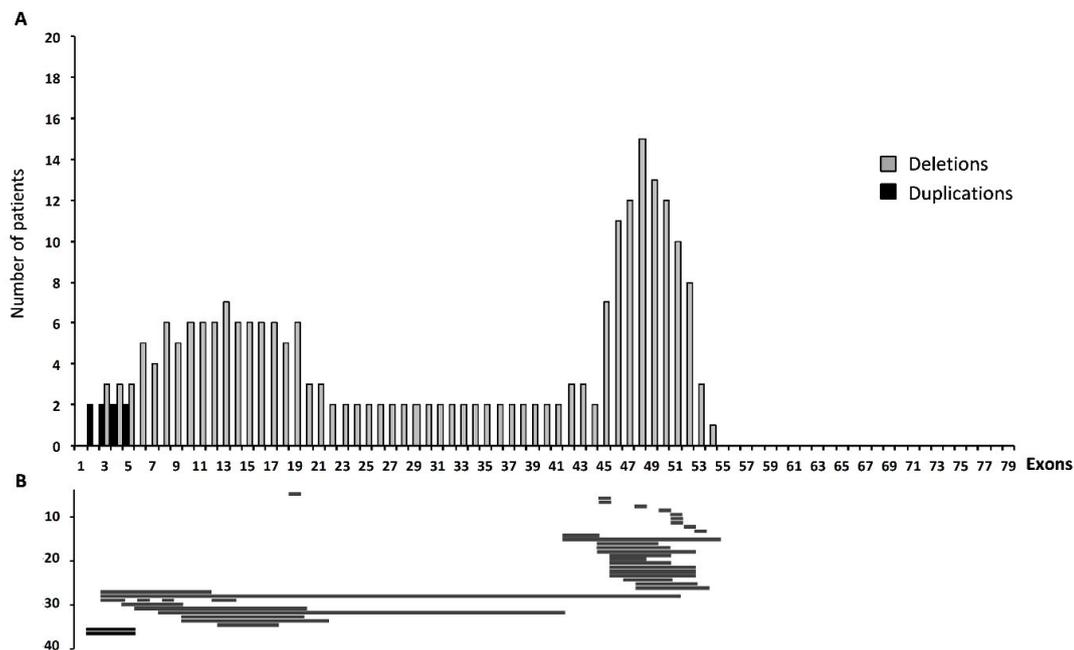


Figure 1: Frequencies and patterns of deletions and duplications of DMD gene exons. (A) Exons 6-19 and exons 45-52 represented two deletion hotspots harboring 90% of all single exon deletions and 75% of all deletions combined. (B) Each horizontal bar represents exons deletion/duplication pattern in a single patient.

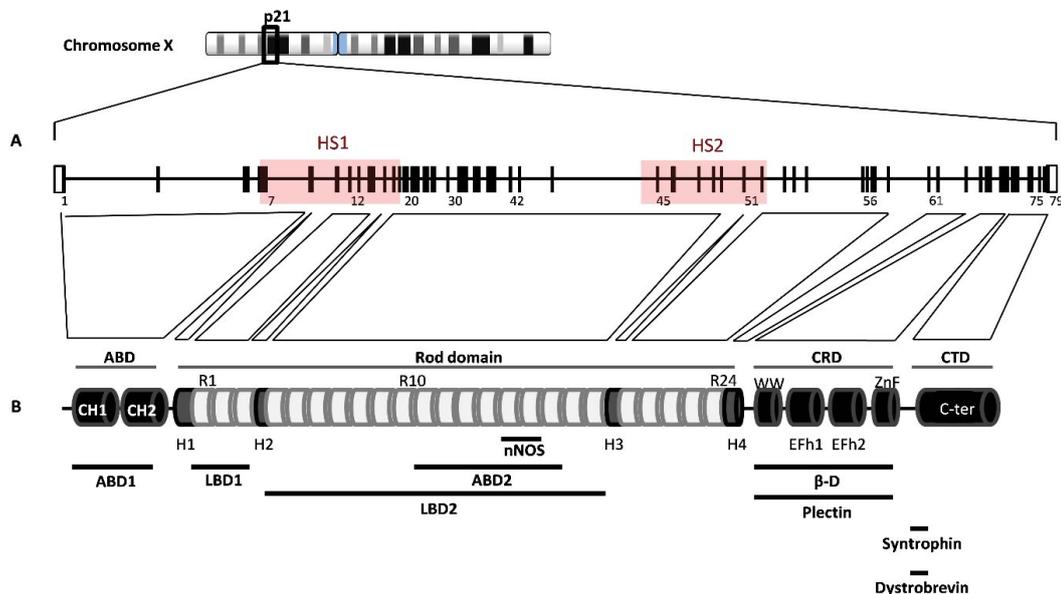


Figure 2: Schematic representation of the dystrophin gene lined up with its associated multi-domain protein and positioning of deletion hotspots. (A) DMD is made up of 79 exons (I) distributed over 2.2-2.5 Mb. Identified deletion hotspots HS1 and HS2 (red shade) cover exons 6-19 and 45-52 respectively, which particularly impinge on the Rod domain of DMD. The relative distribution of the exons throughout the gene is respected. (B) DMD protein (3685 amino acids) is organized into four distinct domains, 1) The N-terminal actin binding domain (ABD) comprising two calponin homology domains CH1 and CH2, 2) The rod domain, a large central section of 24 structurally similar spectrin-like repeats (R1-R24), interrupted by four proline rich hinges (H1-H4), binds to various partners e.g. nNOS, membrane lipids (LBD), filamentous actin (ABD2), 3) The cysteine rich domain (CRD), which includes two potential EF-hand Ca^{2+} -binding sites (Efh1-Efh2) that overlap with β -dystroglycan (β -D) and plectin binding sites, a WW domain characterized by the presence of two conserved tryptophan, and a zinc finger (Znf) domain, and 4) The C-terminal domain (CTD) that binds to dystrobrevin and syntrophins. Note that the few examples of DMD partners shown in this figure do not represent an exhaustive list of partners. Data used to construct this figure is from the Leiden Muscular Dystrophy Page (<http://www.dmd.nl/>) [10,24,25].

Materials and Methods

Patients

Our study included a small group of male patients (n=52) clinically suspected with a Duchenne's type of muscular dystrophy and referred directly to our Medical Genetics Unit for DMD molecular analysis. No prior diagnostic muscle biopsy has been performed on any of the patients. The study was approved by the Institutional Review Board of the American University of Beirut Medical Center. Oral and written consents were provided by the parents and patients to perform and report all needed tests for diagnostic evaluation. Upon their consent, blood was collected from all participants for genetic analysis.

Molecular analysis

Genetic analysis focused mainly on the identification of exon deletions and duplications in the DMD gene by Multiplex Ligation-Dependent Probe Amplification (MLPA) using the SALSA[®] MLPA[®] probemixes P034-B2 DMD-1 and P035-B1 DMD-2 from MRC-Holland, Amsterdam-Netherlands. Analysis was carried out according to the manufacturer's instructions. Fluorescent amplification products were visualized by capillary electrophoresis using an automated ABI 3500 genetic analyzer (Applied Biosystems, Foster City, CA). Single exon deletions were further confirmed by PCR and Sanger sequencing. Obtained data were analyzed using the Coffanalyzer. Net, an MLPA analysis software from MRC-Holland.

Results

Patients and molecular genetic findings

A small group of patients who were suspected to express a DMD/BMD pattern of muscular dystrophy on the basis of clinical diagnosis, were included in our investigations. Muscle biopsy and histological studies were not requested and therefore not conducted in neither patient. Genetic analyses were performed in our tertiary care medical genetic unit at the American University of Beirut Medical Center (AUBMC) and aimed mainly at confirming the suspected clinical diagnosis, while further characterizing the frequency and patterns of DMD exons deletions/duplications. Patients were between 3 and 26 years, they were referred from outpatient clinics and were all clinically assessed by referring physicians. All patients were seen for progressive proximal myopathy involving mostly quadriceps, with difficulty climbing stairs and typical Gower's maneuver sign.

Among the 52 cases analyzed molecularly, 35 cases (67%) had positive molecular findings with deletions/duplications covering a big portion of the coding sequence of the dystrophin gene, while 17 cases (33%) were devoid of any detectable large genetic modification (Table 1 and Supplementary Table 1). The vast majority of observed genetic aberrations were deletions (33 cases) with two hot spots identified. The first (HS1) is located N-terminally between exons 6 and 19 while the second (HS2) is located around the middle of the coding sequence between exons 45 and 52 (Figure 1). Both hot spots accounted for 78% of all deletions observed in our group of non-related patients (Figure 2). Deletions starting at HS1 represented 18% of all deletions combined while those starting at HS2 represented 60% of all cases. Single exon deletions were mainly restricted (90%) to identified hotspots, with HS1 harboring 10%, and HS2 80%, of all single exon deletions. Exon 48 was the most commonly deleted exon with an estimated incidence of 6.3%. One deletion spanned over the two hotspots and represented the largest deletion identified in our group of patients. It covered 47 exons that extended from exon 3 to 49. Two cases of duplication were

Patient #	Age	Genetic Modification
P1	11	Del exon 19
P2	9	Del exon 45
P3	8	Del exon 45
P4	4	Del exon 48
P5	12	Del exon 50
P6	7	Del exon 51
P7	6	Del exon 51
P8	7	Del exon 51
P9	8	Del exon 52
P10	6	Del exon 53
P11	7	Del exons 46 to 52
P12	6	Del exons 10 to 19
P13	2	Del exons 10 to 21
P14	4	Del exons 13 to 17
P15	7	Del exons 3 to 11
P16	5	Del exons 3 to 49
P17	13	Del exons 3-4-6-8-12-13
P18	7	Del exons 42 to 54
P19	7	Del exons 42-43
P20	9	Del exons 45 to 49
P21	7	Del exons 45 to 50
P22	17	Del exons 45 to 52
P23	6	Del exons 46 to 48
P24	18	Del exons 46 to 50
P25	5	Del exons 46 to 52
P26	9	Del exons 46 to 52
P27	6	Del exons 47 to 50
P28	6	Del exons 48 to 52
P29	4	Del exons 48 to 53
P30	13	Del exons 5 to 9
P31	7	Del exons 6 to 19
P32	3	Del exons 8 to 41
P33	5	Del exons 46 to 50
P34	23	Dup exons 2 to 5
P35	25	Dup exons 2 to 5

Table 1: Confirmed deletions/duplications identified and age distribution at diagnosis onset of male patients. Age is expressed in years.

identified in two unrelated patients and involved the same exons (2 to 5). Both duplications were localized outside deletion hotspots. No deletions/duplications were observed C-terminally beyond exon 53. Carrier testing was done in ten females. Six of them were tested because of an affected male sibling, and none of those women was a carrier; and four of them because of an affected male offspring, among which three were found to be carriers. Finally, in our group of male patients, the estimated age average at which the molecular diagnosis was obtained was 7 years (Table 1).

Discussion

The clinical course of Duchenne muscular dystrophy is commonly predictable, and the diagnosis is generally established around three to four years of age [4]. Parents however, often outline a diagnostic endeavor, with diagnosis confirmed more than one to two years from symptoms onset. Early molecular investigations of dystrophin mutations could potentially improve the disease outcomes as different types of mutations carry different phenotypic and prognostic features [6,17].

In the current study we aimed at analyzing the frequency and the distribution pattern of deletions/duplications associated with

dystrophin exons, and the mean diagnostic age of DMD in a small Lebanese group of patients suspected to have an evolving muscular dystrophy with clinical characteristics suggesting a Duchenne's type. Our data were consistent with previously reported findings with slight differences due mainly to the small number of patients [13,19,20].

Deletions were the most common genetic modification observed in our group of unrelated patients. Among 52 samples analyzed, 33 cases (63%) were identified with deletions of variable sizes ranging from 1 exon to 47 exons. Most deletion events (78%) occurred in two deletion hotspots, HS1 (18%) and HS2 (60%), covering exons 6-19 and 45-52 respectively. Single exon deletions were even further restricted (90%) to the deletion hotspots, mainly to HS2 (80%). A large number of our DMD patients may therefore theoretically benefit from antisense oligonucleotides (AON)-mediated exon skipping, which generate a more stable and partially functional dystrophin protein. Eteplirsen, a recent FDA approved AON that induces specifically exon 51 skipping, was tested in multiple clinical trials and was shown to slow the disease progression in patients [21]. Although facing many challenges, other AONs targeting additional exons are currently being developed, which will further increase the number of patients that fall in the category adapted to such therapeutic approach [18]. Restoring a reading frame by skipping one or multiple exons is not always of therapeutic significance as some domains represent a docking site for proteins implicated in various essential cellular processes (Figure 2). For instance, R16 and R17 of the rod domain represent the binding site of the neuronal nitric oxide synthase (nNOS), which plays a key role in the regulation of muscle blood flow through NO signaling. R16-R17 segment is encoded by exons 42-45 that are deleted in 24% of our patients, which results in excessive blood flow restriction known as functional ischemia. Combining exon skipping with treatments that boost NO signaling to bypass ischemia, such as Tadalafil (Cialis) and sildenafil (Viagra, Revatio) could represent the optimal approach in those cases.

In addition to deletions, two cases (4%) with duplications affecting exons 2-5 were identified. No large genetic aberrations were observed beyond exon 53. Such result does not exclude the occurrence of deletions/duplications in the C-terminal domain of the dystrophin gene; rather it potentially reflects the small group size and the low frequency of deletions observed worldwide in that particular domain. Furthermore, 17 cases (33%) were devoid of large genetic modifications as indicated by MLPA analysis. Those represent most likely cases of small insertions/deletions and point mutations or may represent cases that fall within a different group of muscular dystrophies harboring similar features to that observed in DMD/DMB such as Limb girdle muscular dystrophies and Emery-Dreifuss muscular dystrophies.

The estimated age average at which the molecular diagnosis was obtained in our group of patients was 7 years compared to 4-5 years worldwide [22]. Our data are presumably indicative of a lack of awareness of the disease by both parents, mostly coming from rural areas with low socioeconomic background, and the primary care physicians. This highlights the pressing need for an educative scheme by care facilities to enhance early diagnosis. Muscle quality was also shown to be an essential feature for successful therapeutic interventions aiming at restoring DMD expression [8]. Indeed, development of fibrosis and fat accumulation in DMD patients' skeletal muscles are early and progressive events that will impede treatment efficiencies. Enhancing early molecular diagnosis is therefore critical for an optimal outcome of the disease as it will allow appropriate counseling, and eventual targeted care and follow up.

Our data also laid stress on the relevance of a muscle biopsy for the evaluation, diagnosis and treatment of DMD patients. Histochemical and immunohistochemical analyses allow a comprehensive characterization of the impact of DMD mutations on the structure and function of skeletal muscles. Such impact can be highly variable, and mainly linked to the existence of genetic modifiers [23-25]. Investigations which would include a muscle biopsy, in parallel to molecular analysis, provide compelling evidence that allow differential diagnosis, optimization of therapeutic management, and establishment of appropriate preventive care.

Conclusion

The information obtained from the current work will represent an incentive, for us and others, to initiate additional exhaustive studies, including larger groups of dystrophic patients from our society. Such studies will allow the establishment of a comprehensive national database, which will be extremely beneficial for basic research, trial development, and clinical care.

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