Childhood onset limb-girdle muscular dystrophies in the Aegean part of Turkey

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The aim of this study is to analyze the epidemiology of the clinical and genetic features of childhood-onset limb-girdle muscular dystrophies (LGMD) in the Aegean part of Turkey. In total fifty-six pediatric cases with LGMD followed in four different pediatric neurology departments in the Aegean region of Turkey were evaluated. Among them, LGMD2C was the most common followed by LGMD2A, LGMD2D, and LGMD2F with equal frequencies. In twenty-eight patients (50%) the diagnosis could be confirmed by genetic analysis, where *SGCG* proved to be disease-causing in most of the cases. About half of the patients were diagnosed with whole exome or targeted gene sequencing. A positive correlation between muscle biopsy and genetic findings were observed in 11% of the patients. We report one novel frameshifting mutation in *TTN*. Knowledge on frequencies of childhood-onset limb-girdle muscular dystrophies and related genes in Turkey will lead to a prompt diagnosis of these neuromuscular disorders.

Key words: limb-girdle muscular dystrophy, genetic diagnosis, childhood, Turkey

Introduction

Limb-girdle muscular dystrophies (LGMD) are clinically and genetically heterogeneous muscle disorders. They are inherited in an autosomal recessive or dominant manner. Thirty-three recessively and dominantly inherited forms have currently been identified. The prevalence is about 4-7 per 100.000. They may have childhood, teenage or adulthood onsets. They are clinically characterized by symmetrical weakness of pelvic, scapular and trunk muscles, raised serum creatine kinase levels and dystrophic changes on muscle biopsy (1).

Autosomal recessive muscle disorders like LGMD and congenital muscular dystrophies are common in Turkey due to high rates of consanguineous marriages. Unfortunately, the distribution of subgroups is not well known. The Aegean region is one of seven regions found in Turkey. It is situated in the western part of the country. The region includes 8 provinces and over nine million inhabitants. Having knowledge of specific subgroups of a muscle disease, their related genes and clinical findings in a specific geographic region should alert physicians for prompt disease identification. The aim of this study is to document the clinical findings and related mutations in proven cases of childhood-onset LGMD in the Aegean part of Turkey.

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Material and methods

Patients

Patients with childhood onset LGMD clinical features plus confirmed histologic, genetic or histologic plus genetic diagnosis of LGMD of four different child neurology departments (Dokuz Eylül University, Ege University, Izmir Dr. Behçet Uz Children's Hospital, Tepecik Research, and Education Hospital) were included in the study. The individual database was reviewed in all cases. Clinical information including age, gender, family history and consanguinity was recorded. Serum creatine kinase levels, nerve conduction times and electromyographic examinations were also evaluated.

Muscle biopsy

Most of the patients underwent a diagnostic muscle biopsy after written informed consent was obtained. Muscle biopsies were obtained from gastrocnemius or vastus lateralis muscles. The morphological and immunohistochemistry analyses were performed in accordance with standard procedures (Supplement 1).

Genetic analysis

Genomic DNAs were extracted from the remnant muscle tissues or blood samples using available DNA extraction kits (Qiagen, US) following the manufacturer's standard protocol. The exon regions and flanking short intronic sequences of the 4 SGCs genes were amplified using the polymerase chain reaction (PCR), followed by direct sequencing of the PCR products (ABI, US). In addition, the multiplex ligation-dependent probe amplification (MLPA) technique was used for deletion and duplication analysis for patient 13 (2). Patients with nonspecific muscle biopsy findings underwent either whole exome sequencing (WES) (Table 1: patient 7, 8, 11, 12, 14, 21, and 26) or for patient 6, 9, 20, 22-25, 27 and 28 Mendeliome sequencing, which solved 54% of the genetically confirmed cases. For WES, we used the Nimblegen enrichment (SeqCap EZ Human Exome Library v2.00) kit and sequenced on the Illumina HiSeq 2000 with an average mean coverage of 103 fold or we used Agilent SureSelect V6 enrichment kit with an average mean coverage of 82 fold. For the other 10% of the patients, we used targeted gene sequencing on the Illumina TruSight One panel (Mendeliome, Illumina, San Diego, CA, USA) with an average mean coverage of 97 fold. The Cologne Center for Genomics VARBANK pipeline v.2.12 (https://varbank.ccg.uni-koeln.de/) was used for genomic data analysis (3-5). All patients were also investigated for dystrophin deletion/duplications by MLPA analysis. If parental or family members were available, we performed co-segregation studies by using Sanger Sequencing applying standard techniques.

Frequency and descriptive analyses were performed using the statistical software SPSS 9.05 for Windows.

Results

Clinical findings

The study was approved by Dokuz Eylül University School of Medicine Institute's Ethics Committee (No: 24/05/2018; 2018/13-38). Fifty-six patients were evaluated in total. The mean age of patients was 7.92 years (1 to 17). There were 31 boys (55%) and 25 girls (45%). The consanguinity rate was 38%. Patients with *LMNA* and *MYOT* mutations showed the lowest and patients with *SGCG* mutations the highest serum creatine kinase levels. Our patient collective was drawn up out of patients with the following mutations: 24 LGMD2C (43%), 7 LGMD2A (13%), 7 LGMD2E (13%), 6 LGMD2D (11%), 3 LGMD2J (5%), 3 LGMD2K (5%), 1 LGMD1B (2%), 1 LGMD1A (2%), 1 LGMD2B (2%), and 3 unclassified (4%). Difficulty in rising from the floor, delay in motor milestones, muscle weakness and HyperCKemia detected in routine laboratory tests were notable findings. Patients with LGMD2C had a similar disease course as Duchenne muscular dystrophy and all of them had calf hypertrophy. Most of the patients with LGMD2C were non-ambulatory after age 15. HyperCKemia detected in routine laboratory tests and mild proximal muscle weakness were the most common findings in LGMD2A, LGMD2D, and LGMD2E groups. Scapular winging, atrophy in hamstring muscles and Achilles contractures were important clinical clues in advanced stages of LGMD2A patients. Patients with LGMD2K had mild microcephaly, intellectual disability, and generalized muscle hypertrophy. They were slower than their peers. Brain magnetic resonance imaging of these patients was normal. Patients with LGMD2J and LGMD2B presented with HyperCKemia identified during routine laboratory examinations. A fifteen-year-old girl (patient no: 21) with LGMD1B presented with a new onset generalized weakness and she had frequent syncope attacks since early childhood. She had a positive Gower's sign and proximal muscle strength was 4/5 in lower extremities. She had mild pes cavus deformity and deep tendon reflexes were negative. Her serum creatine kinase level was normal but the electromyography recording was myopathic. A first degree atrioventricular block was determined as a cause of syncope attacks. Her mother and aunt were also treated for cardiac arrhythmia. A patient with LGMD1A (patient no: 22) presented mild increased serum creatine kinase level with no clinical findings. However, her mother and uncle had scapulo-

humeral weakness and a Gowers sign. They had a distinctive nasal, dysarthric pattern of speech. They also had reduced deep tendon reflexes. Their electromyography recording showed signs of myopathy. We could not make a subgroup classification in two patients due to lack of all sarcoglycans in one patient and combined deficiency of gamma and alpha deficiency in the other patient. Nerve conduction velocities were normal and electromyography showed myopathic motor unit potentials in all tested patients.

Muscle biopsy findings

52 patients (93%) had a muscle biopsy. 48 of these biopsies (92%) showed dystrophic findings including alteration of myofiber size and shape, splitting, increase in the number of internal nuclei, fiber type disproportions, regeneration and fibrosis. In the remaining four patients, muscle biopsy findings were unremarkable. The diagnosis of these patients with unremarkable biopsy finding were LGMD2C (two patients), LGMD2D (one patient) and LGMD1B (one patient). Three of these patients (LGMD2C and 2D) were asymptomatic at the time of muscle biopsy and muscle biopsy was done due to HyperCKemia (Fig. 1). However, the patient with LGMD1B was symptomatic at the time of muscle biopsy. Genetically, these patients were diagnosed with whole exome sequencing. Expression defects of sarcoglycan staining were as follows: 21 gamma (40%), 6 combined alpha and gamma (12%), 5 calpain (10%), 4 beta (8%), 3 all sarcoglycans (6%), 2 dysferlin (4%) and 1 alpha-dystroglycan (2%). Ten patients (20%) showed normal staining for sarcoglycans. We could compare muscle biopsy and genetic findings in 27 patients. In terms of dystrophic changes without immunohistochemistry, genetic diagnosis was obtained in 23 of 27 patients. However, the correlation between immunohistochemistry and genetic diagnosis was poor. There was a correlation between genetic diagnosis and immunohistochemistry findings in only 5/27 patients (patient numbers: 1-5). All of these patients had a diagnosis of LGMD2C.

Genetic analysis

In twenty-eight patients (50%) the clinical diagnosis could be confirmed by genetic analysis. 15 of these patients were diagnosed by whole exome sequencing (WES) or targeted gene sequencing. Results of genetic analysis were as follows, given the number of patients: 9 *SGCG* homozygous, 6 *SGCA* (5 homozygous missense mutation and 1 homozygous 7bp-deletion of exon 3), 3 *SGCB* (2 homozygous and 1 whole gene deletion), 3 sibling with a novel *TTN* homozygous, 3 *POMT1* homozygous, 1 *LMNA* heterozygous, 2 *CAPN3* homozygous and 1 *MYOT* heterozygous (Table 1). The mutation c.107163_107167delTACTT (NM_001267550.1) in *TTN* was listed in ClinVar, however, detailed clinical descriptions were not given (www.ncbi.nlm.nih.gov/clinvar/variation/196657/) and there are no scientific publications describing this mutation in *TTN* before, this will be the first report to be published patients with this *TTN* mutation (Fig. 2). MLPA analysis for the detection of deletions, duplications and complex rearrangements in the dystrophin gene were performed and reported as normal for each case.
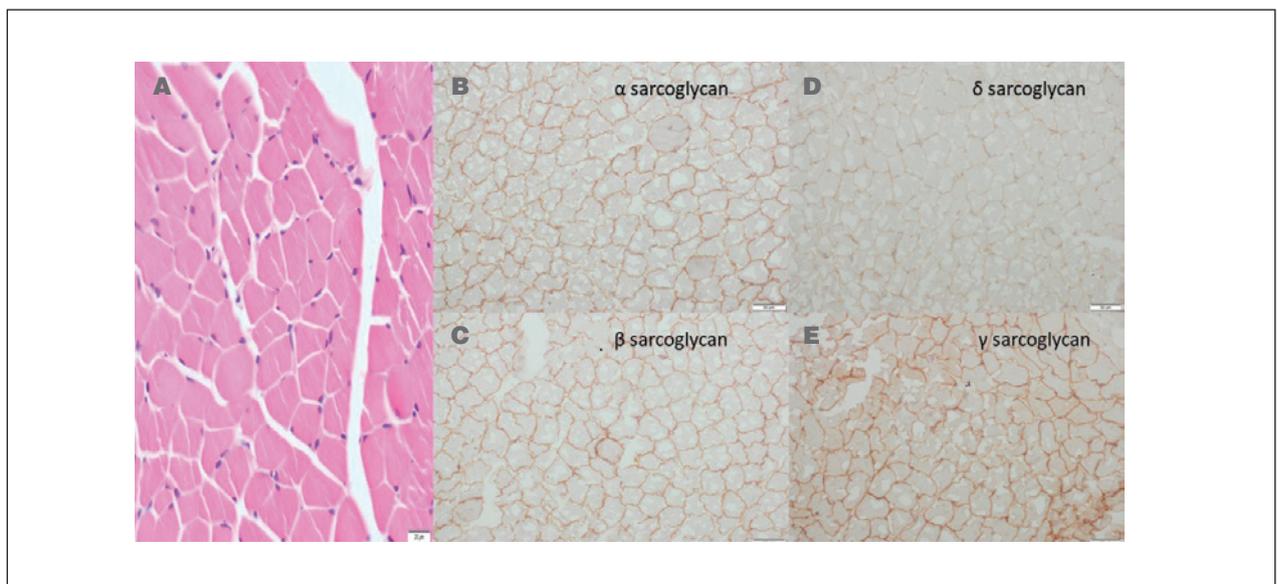

Figure 1. The muscle biopsy of the patient with the genetically confirmed LGMD2D showing no sign of dystrophy **(A)** and normal expression of all sarcoglycans **(B, C, D, E)**. The biopsy was taken at the age of 8 years.

Discussion

LGMDs are non-syndromic hereditary muscle disorders limited to skeletal muscle. Protein testing by immunostaining or immunoblotting on a muscle biopsy or demonstration of complete or partial deficiencies for a particular protein followed by mutation studies of the corresponding gene(s) may establish the diagnosis in most of the cases (1). However, about 50% of currently identified LGMD would have no molecular diagnosis although all known genes of LGMD were sequenced by traditionally Sanger Sequencing (1). Turkey is a rich genetic pool for neuromuscular disorders. In Turkey, 50% of pediatric muscular disorders consist of patients with Duchenne/Becker muscular dystrophy, which is followed by LGMD2 (20%) and congenital muscular dystrophy (15%) (6). In a large adult neurology clinic in Istanbul, 46% of the patients are diagnosed with Duchenne/Becker muscular dystrophy, followed by facioscapulohumeral dystrophy (FSHD) (18%), *myot*onic dystrophy (16%) and LGMD2 (14%) (6). Evaluation of 38 LGMD2 families from Turkey showed that calpainopathy and beta-sarcoglycanopathy were the most common subtypes. Gamma sarcoglycanopathy was the third common (7). In our cohort, gamma-sarcoglycanopathy was the most common (44%) followed by calpainopathy, beta- and alpha-sarcoglycanopathy. In this cohort, gamma-sarcoglycanopathy was clinically the most severe group as a whole, whereas dysferlinopathy was the mildest.

Patients with gamma-sarcoglycanopathy had early onset of symptoms resembling Duchenne muscular dystrophy and a progressive decline in motor functions as becoming wheelchair-bound after age 15. All of them also had gastrocnemius hypertrophy as in Duchenne muscular dystrophy.

Mild proximal muscle weakness and increased serum creatine kinase levels incidentally detected during routine laboratory tests were the main presenting findings in calpainopathy, alpha- and beta- sarcoglycanopathy patients. A typical appearance for calpainopathy including generalized atrophy, scapular winging and selective involvement of hamstring muscles were observed in one of the seven patients in our cohort. This was related to his advanced age (17 years old). Patients with dysferlinopathy and LGMD2J were also diagnosed by idiopathic HyperCKemia because symptoms dominantly involving distal muscles occur after adolescence. Mutations of *POMT1* gene typically lead to Walker Warburg syndrome characterized by severe muscle, brain and eye involvement. In 2005, Balci et al. described a form of LGMD2 due to common mutation of *POMT1* gene (p.A200P) characterized by mild mental retardation, microcephaly, and normal brain magnetic resonance imaging, highly elevated serum creatine kinase levels and reduced glycosylation of alpha-dystroglycan in muscle biopsy (8). Our patients with LGMD2K (patient numbers: 26-28) also

had the same clinical findings but we could not show an alpha-dystroglycan deficiency in muscle biopsy due to the lack of antibody at the time of diagnosis. The patients were diagnosed by whole exome sequencing.

We only had two patients in the autosomal dominant group. The patients were clinically asymptomatic only with mildly increased serum creatine kinase levels. They also had a family history of cardiac arrhythmia and cardiomyopathy as expected in LGMD1B. The mother and uncle of the LGMD1A patient had typical scapulohumeral weakness and myopathic electromyography. The cases with LGMD1A and LGMD1B show the importance of family history and examination of other family members in neuromuscular disorders.

Reduced or absent sarcolemmal expression of one of the four sarcoglycans can be found in patients with any type of sarcoglycanopathies and dystrophinopathies. Different patterns of sarcoglycan expression could predict the primary defect but residual sarcoglycan expression could be highly variable and accurate prediction of the genotype could not be achieved in most of the cases. Our study also showed that some patients had combined or total deficiencies of sarcoglycans in muscle biopsies. We reached a certain diagnosis in these patients (patient numbers: 10, 13, 15, 16, 17, 18, 19) with genetic studies but could not classify two patients in a particular group due to lack of genetic studies. Although there was a good correlation between dystrophic findings and genetic analysis, this was not valid for immunohistochemistry findings. All patients with genetic-histologic correlations were in the gamma-sarcoglycanopathy group (patient numbers: 1-5). However, there were also patients with normal immunostaining for gamma-sarcoglycan, but mutations in the *SGCG* gene (patient numbers: 6, 7, 9). On the other hand, there were also patients with all defective sarcoglycans in muscle biopsy and single gene mutation (patient numbers: 8, 10, 13, 15-19). This finding suggests that muscle biopsy is helpful in terms of dystrophy but defective sarcoglycan in muscle biopsy should be confirmed genetically. Interestingly, the muscle biopsy of the index patient with LGMD2J showed dysferlin deficiency. Histologically, the muscle biopsy of this patient only revealed a focus with degenerative and regenerative fibers. In this focus there were macrophages showing myophagia (Fig. 3) However, in the rest of the biopsy there was no prominent difference between the sizes of myofibers. Although the absence of calpain-3 has been documented in association with C-terminal titin mutations due to the loss of calpain-3 binding site in the titin C-terminus, dysferlin deficiency has not been reported before (9). This novel finding reminds us that patients with tibial muscular dystrophy who have dysferlin deficiency in their muscle biopsy but negative genetic analysis results for dysferlin gene should be checked for *TTN* mutations. However, this novel finding should be supported with additional

Table 1. Pathologic findings and mutations of patients with a genetic diagnosis. Following reference sequences have been used for the mutation nomenclature: SGCG NM_000231.2, SGCB NM_000232.4, CAPN3 NM_000070.2; LMNA NM_170707.3, MYOT NM_006790.2, POMT1 NM_007171,3, TTN NM_001267550.1; NA = not applicable, ROH = Regions of Homozygosity are calculated via data analysis pipeline from next-generation sequencing data (https://varbank. ccg.uni-koeln.de/), ROH > 200 indicated certain consanguinity of parents.

Patient n.	Dystrophy in muscle biopsy	Defective sarcoglycan in muscle biopsy	Gene	Mutation	Final diagnosis	
1/2	Yes	Gamma	SGCG	c.525delT (p.F175Lfs*20)	LGMD2C	
3	Yes	Gamma	SGCG	c.848G > A (p.C283Y)	LGMD2C	
4/5	Yes	Gamma	SGCG	c.800_801delGT (p.Cys267Serfs*51)	LGMD2C	
6	No	Normal	SGCG	c.525delT (p.F175Lfs*20)	LGMD2C	
7	Yes	Normal	SGCG	c.525delT (p.F175Lfs*20)	LGMD2C	
8	Yes	Normal	SGCG	c.525delT (p.F175Lfs*20)	LGMD2C	
9	No	Normal	SGCG	c.101G > A (p.R34H)	LGMD2C	
10	Yes	All sarcoglycans	SGCB	c.265G > A (p.V89M)	LGMD2E	
11/12	Yes	Normal	CAPN3	c.2243G > A (p.R748Q)	LGMD2A	
13	Yes	All sarcoglycans	SGCB	Homozygous whole gene deletion	LGMD2E	
14	NA	NA	SGCB	c.919T > C (p.C307R)	LGMD2E	
15	Yes	Alpha and gamma	SGCA	c.232_238delTACACCC	LGMD2D	
16/17	Yes	Alpha and gamma	SGCA	c.226C > T (p.L76F)	LGMD2D	
18/19	Yes	Alpha and gamma	SGCA	c.101G > A (p.R34H)	LGMD2D	
20	No	Normal	SGCA	c.850C > T (p.R284C)	LGMD2D	
21	No	Normal	LMNA	c.734T > C (p.L245P)	LGMD1B	
22	NA	NA	MYOT	c.163T > C (p.S55P)	LGMD1A	
23/24/25	Yes	Normal	TTN	c.107163_107167delTACTT (p.F35721Lfs*)	LGMD2J	
26	Yes	Normal	POMT1	c.1939G > A (p.A647T)	LGMD2K	
27/28	Yes	Normal	POMT1	c.1939G > A (p.A647T)	LGMD2K	

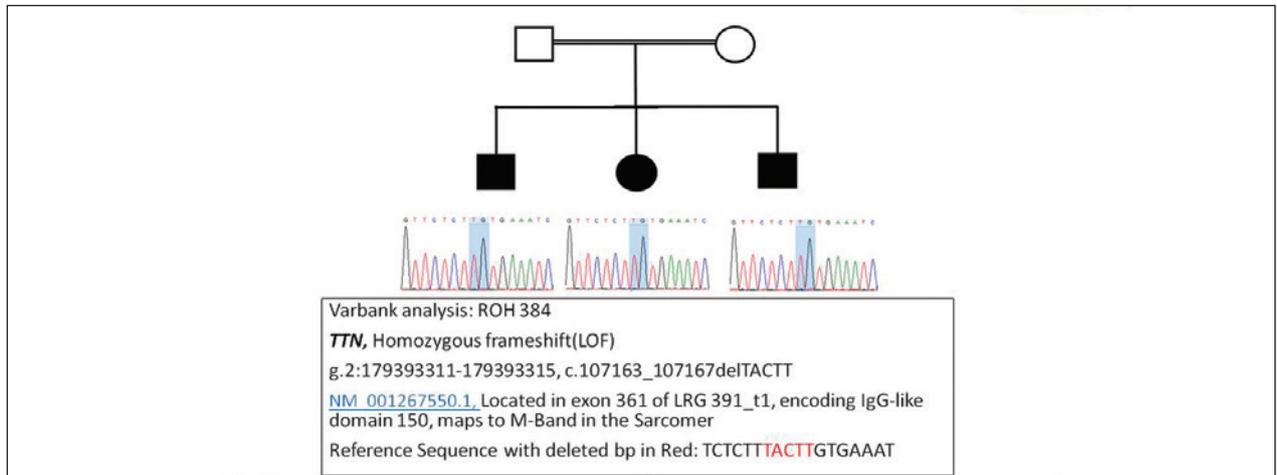|	Correlation between gene defect and muscle biopsy	Previously described mutation	Genetic methodology	ClinVar	Allele status of parents (ROH)
	Yes	Yes, reference 10	Sanger sequencing	189243 (Pathogenic)	NA
	Yes	Yes, reference 16	Sanger sequencing	2006 (Pathogenic)	NA
	Yes	Yes, reference 2	Sanger sequencing	Not included	NA
	No	Yes, reference 10	Mendeliome	189243 (Pathogenic)	NA (115)
	No	Yes, reference 10	Whole exome sequencing (Agilent V6r2)	189243 (Pathogenic)	NA (356)
	No	Yes, reference 10	Whole exome sequencing (NimbleGenV2)	189243 (Pathogenic)	Heterozygous (270)
	No	Yes, reference 27	Mendeliome	Not included	Heterozygous (430)
	No	Yes, reference 23	Sanger sequencing	Not included	NA
	No	Yes,references 11,12	Whole exome sequencing (Illumina Nextera)	128670 (Pathogenic-likely pathogenic)	NA
	No	Yes, reference 24	MLPA	NA	NA
	NA	Yes, reference 25	Whole Exome Sequencing (NimbleGenV2)	Not included	Heterozygous (502)
	No	Yes, reference 20	Sanger sequencing	NA	NA
	No	Yes, reference 19	Sanger sequencing	432007 (Pathogenic)	NA
	No	Yes, reference 17	Sanger sequencing	92301 (Likely pathogenic)	NA
	No	Yes, reference 20	Mendeliome	9439 (Pathogenic/likely pathogenic)	Heterozygous (184)
	No	Yes, reference 41	Whole exome sequencing (Agilent V6r2)	Not included	NA (95)
	NA	Yes, reference 43	Mendeliome	Not included	Co-segregation (56)
	No	Novel	Mendeliome	196657 (Pathogenic/likely pathogenic)	NA (385)
	No	Yes, reference 32	Whole exome sequencing (NimbleGenV2)	Not included	Heterozygous (350)
	No	Yes, reference 32	Mendeliome	Not included	Heterozygous (592)

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Figure 2. Pedigree of the family with TTN mutation. Homozygous frameshift mutation, g.2:179393311-179393315, c.107163_107167delTACTT, NM_001267550.1, Located in exon 361 of LRG 391_t1, encoding IgG-like domain 150, maps to M-Band in the Sarcomere, reference Sequence with deleted bp kursiv: TCTCTT*TACTT*GTGAAAT.
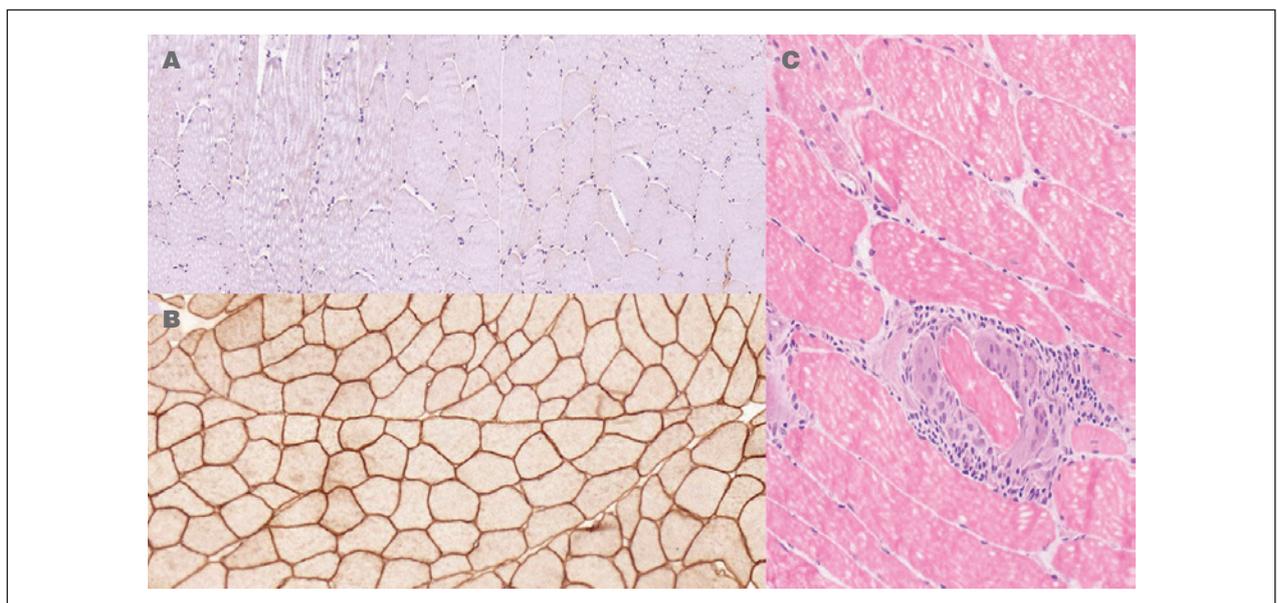

Figure 3. The muscle biopsy of the index patient showed a complete deficiency of dysferlin immunohistochemically **(A)**. The positive simultaneously stained control slide of another patient's biopsy reveals a complete sarcolemmal staining of the dysferlin antibody **(B)**. There was only one focus with degenerative and regenerative fibers. In this focus, there were also macrophages showing myophagia **(C)**. The muscle biopsy was taken at the age of 17 years from the left quadriceps femoris.

cases and functional studies in muscle biopsy. So it remains speculative if this is correct or not.

The autosomal recessive forms represent more than 90% of all LGMDs. LGMD2A or calpainopathy is considered to be the most common form of recessive LGMD in most populations, accounting for about 30-40% of the patients (10). The c.550delA is the most common mutation among patients from different European populations (11). A founder effect for the c.550delA has been reported in some populations like Russia, Croatia, Bulgaria, Northern Italy and Turkey (10, 12). In 1997, the *CAPN3* mutation was detected in 10 families from Turkey with LGMD2A. The c.550delA mutation was detected in the half of the families (13). In 2006, Balci et al. revealed 93 Turkish LGMD2

families and detected *CAPN3* mutations in 29 patients from 21 families (10). In this study, c.550delA mutation was the most common followed by c.1469G>A (p.R490Q) mutation. In our study, we detected c.2243G>A (p.R748Q) mutation in two siblings. This mutation had been reported in several Spanish patients and also previously in one patient from Turkey (13, 14).

The four common types of LGMD are called sarcoglycanopathies; LGMD2C (*SGCG*), LGMD2D (*SGCA*), LGMD2E (*SGCB*) and LGMD2F (*SGCD*) (15). The frequencies of mutations in *SGCA* and *SGCD* are respectively the highest and lowest in most populations. A clinical study from Iran evaluating 25 sarcoglycanopathy patients showed that more than the half of the patients had *SGCB* mutations followed by *SGCG* (28%), *SGCD* (12%) and *SGCA* (4%) mutations (16). In India, mutation frequencies regarding sarcoglycanopathies were reported as *SGCG* 44.4%, *SGCD* 27.8% and *SGCB* 5.6% (12). In our study *SGCG* mutation (9 patients, 31%) was the most common. The frequencies of *SGCA* and *SGCB* mutations were 20% (6 patients) and 10% (3 patients), respectively. Mutations in sarcoglycan genes are commonly small defects, such as short deletions/insertions or single nucleotide substitutions (17). The majority of sarcoglycanopathies are associated with missense mutations but large deletions had also been reported (18, 19). More than 70 pathogenic mutations had been described in *SGCA* (20). The most common mutation of this gene is p.R77C which has a founder effect in Finland and Magdalen (12). This mutation was also reported as a founder mutation in several LGMD2D patients from Europe and also Brazil (20). Other founder mutations are p.R34L in Taiwan and p.R192* in Egypt (10). In our LGMD2D patients, we found five different mutations in 6 patients. These mutations had previously been described (2, 19, 21-23). LGMD2E is a clinically heterogeneous disorder associated with missense, truncating and deletion mutations in *SGCB* located on the 4q12 chromosomal region. Large homozygous microdeletion of chromosome 4q11-q12 and isolated exon deletion had also been reported (24). The founder mutations of *SGCB* are c.377_384dup in Northern Italy and p.T151R in Amish population. The p.M1L, p.Q11*, p.V89M, p.I92T, p.I92S and c.739insA mutations had been reported from Turkey (25). In this study, the patient with whole gene deletion (patient no: 13) had previously been reported by Diniz at al. (26). The p.V89M mutation (1 patient) had been reported in the Turkish population and p.C307R mutation (1 patient) had been presented as a poster presentation in 1st National Neuromuscular Disease Congress in Ankara, 2017 (25, 27). The reported founder mutations of *SGCG* are c.525delT in North Africans, p.C283Y in Roma Gypsies and c.87insT in Northern Italy. Especially the high prevalence of the c.525delT mutation, which is also called the Maghrebian form (formerly name delta521T) present as a founder

mutation in North Africa and Spain, can be an indicator of the migratory movement in Mediterranean Sea. (6). The frameshift mutation c.800_801GT (formerly 923-924TG) in the (TG) 4 repeat in the 3' coding area and deletion of exon 5 of the *SGCG* gene (formerly described as 510del120) had been reported from Turkey (13, 28). Another study evaluating 20 LGMD2C patients from Turkey showed most patients had silent homozygous or heterozygous mutations (2). In our study, 5 mutations were found in 9 patients. The most common mutation was c.525delT which was found in 5/9 patients. Patients with c.800_801delGT mutations had been reported previously (2). Additionally, p.C283Y (1 patient) and p.R34H (1 patient) mutations had also been described previously (18, 29).

Titin is a giant muscle protein expressed in cardiac and striated muscles. It spans half of the sarcomere from Z line to M line. It plays major functions in muscle assembly, force transmission and maintenance of resting tension. Its gene is located on 2q31.2 and encodes the largest protein of the human genome (15). More than 230 mutations of *TTN* have been reported in human gene mutation database (HGMD) (30). Mutations of this gene cause tibial muscular dystrophy, dilated cardiomyopathy 1G, familial hypertrophic cardiomyopathy 9, early onset myopathy with fatal cardiomyopathy, proximal myopathy with early respiratory muscle involvement and LGMD2J (15). Symptoms in LGMD2J start in the first or second decade and progresses over the next 20 years to wheelchair confinement. We detected a novel homozygous mutation c.107163_107167delTACTT (NM_ 001267550.2) in three siblings (patient number: 23-25). The index patient was diagnosed due to idiopathic HyperCKemia. She is currently 17 years old. The symptoms started at the age of 14 years. She has symmetric weakness prominent in distal lower extremity muscle and she has difficulty while walking on heels. Upper extremity, axial and facial muscles are not involved. There are no signs of cardiac and respiratory dysfunctions. Serum creatine kinase level is 2939 IU/L. Her younger siblings are asymptomatic.

Mutations in *POMT1*, which mapped to chromosome 9q34.13, are related to autosomal recessive muscular dystrophies which include three subgroups as MDDGA1, MDDGB1 and MDDGC1 (also known as LGMD2K) (31). The Walker-Warburg syndrome (WWS) is the most severe phenotype but patients with milder mutations in *POMT1* might present with milder forms of LGMD (31). While there is not any clear genotype-phenotype correlation, the variable phenotypic severity can be related to the type and location of the mutations (31). The POMT1 protein has loops within the lumen (loops 1, 3 and 5) and within the cytoplasm (loops 2, 4 and 6). Patients with mutations only affecting the loops within the cytoplasm are more likely to show the milder phenotype of LGMD. Mutations affecting the transmembrane domains, loop 1 and loop 5 within the lumen of the

endoplasmic reticulum are associated with the more severe clinical presentation of congenital muscular dystrophy (32). Two *POMT1* mutations had been reported from Turkey. One of them was a p.R514* mutation in a patient with Walker-Warburg syndrome. This mutation affects the loop of protein within the lumen of the endoplasmic reticulum (33). The other reported mutation was p.A200P mutation leading to a significantly milder clinical phenotype with predominant muscular dystrophy and mild intellectual disability, but without obvious brain malformations (8). The second reported mutation affects the part of protein within the cytoplasm. Herein we reported three patients (patient number: 26-28) with a previously reported homozygous p.A647T mutation which affects loop 6 (34). They shared a similar phenotype with patients reported by Balci et al. in 2005 (8).

LGMD1B is caused by *LMNA* which is located on 1q22.15. Mutations of this gene are associated with a wide range of disease phenotypes, ranging from cardiac, neuromuscular and metabolic disorders to premature aging syndromes. Muscular dystrophies associated with *LMNA* are autosomal dominant Emery-Dreifuss muscular dystrophy, a form of congenital muscular dystrophy and LGMD1B. It is difficult to establish a correlation between phenotype and genotype (35). However, these clinical entities can be determined by the same *LMNA* mutation and coexist in the context of the same family (36). Some *LMNA* mutations had been reported from Turkey before. The p.Glu31del mutation had been reported in a patient with *LMNA*-CMD and others including p.D47N, p.R349W, p.R482W, p.A527H, and p.A529V had been reported in patients with lipodystrophy mandibuloacral dysplasia (37-42). Herein we reported *LMNA* mutations in a patient with LGMD1B for the first time from Turkey (patient number: 21). The mutations p.L245P been reported as a poster presentation in 12th European Pediatric Neurology Congress in Lyon/France. The phenotype of this patient included proximal muscle weakness with normal serum creatine kinase, pes cavus, foot-drop gait, absent deep tendon reflexes and first degree A-V block (43).

LGMD1A is caused by mutations in *MYOT* gene localized on the 5q31.2 chromosomal region. Mutation of *MYOT* also causes myofibrillar myopathy (44). We detected p.S55F mutation in one patient (patient number: 22). This young girl had mild serum creatine kinase elevation but had no clinical findings of a muscle disease yet. However, her mother and uncle had typical findings of LGMD1A. This mutation had also previously been reported in an Argentinian family with LGMD1A (45). The clinical findings in the Argentinian family started by 42-58 years of age with proximal leg and arm weakness which later progressed to distal weak-

ness. Half of the patients had dysarthric speech and serum CK levels were elevated to 5-fold to 15- fold. The clinical findings of the index case's mother and uncle were similar in this family, however, symptoms started in the second decade. Their serum CK never exceeded 1000 IU/l.

Gamma sarcoglycanopathies were the most common LGMD in Aegean part of Turkey like Spain and North Africa. For example in Tunisia, the frequency of LGMD2 is higher than for dystrophinopathies and LGMD2C is the most common type (6). About 90% of the cases with LGMD2C carry c.525delT exon 6 founder mutation. In the Catalan part of Spain, LGMD2C is the most common type of LGMD2 and interestingly 50% of their gamma-sarcoglycan mutations is the Maghrebian form as c.525delT (6). In our study, 55% of our cases with LGMD2C also carried c.525delT mutation. These findings suggest that the Aegean part of the Turkey has probably been influenced by migrations in the Mediterranean see from a genetic epidemiologic point of view.

SGCA, SGCB, SGCD, SGCG, CAPN3, LMNA, and *POMT1* mutations have been reported in LGMD patients from Turkey (2, 7, 8, 10, 13, 20, 21, 26-28). Rare types of childhood-onset LGMD2 due to mutations of *TRAPPC11, POMK,* and *PLEC* were also reported from Turkey (46-48). Molecular genetic diagnosis of LGMD remains challenging. Recently, clinicians consider multi-gene panels or whole exome sequencing (WES) for genetic diagnosis of LGMD. The diagnostic rate of WES ranges between 34% and 45% (49). Next-generation sequencing has the advantage of detecting a mutation in a very large gene like *TTN* (50). In our cohort, more than half of the cases with the genetic diagnosis had been solved by WES.

In conclusion, LGMD2C is the most common LGMD2 in the Aegean part of Turkey. Most of the childhood cases present with mild proximal muscle weakness and increased serum creatine kinase levels detected during routine laboratory examinations. Results of immunostaining of muscle biopsies should be confirmed with molecular genetic testing because there is a very limited correlation between immunohistochemical and genetic findings. Whole exome sequencing confirms the diagnosis in most of the cases with nonspecific clinical and muscle biopsy findings.

Acknowledgments

Supplement 1
Details of muscle biopsy

Samples were frozen in isopentane cooled in liquid nitrogen and 8 to 12 micron sections were cut using the cryostat. Slides were stained with hematoxylin&eosin, Gomori's trichrome, modified Gomori's trichrome (Engel-Cunningham modification), oil red-O, Periodic Acid Shiff (PAS), d-PAS, crystal violet stains. For enzyme histochemical techniques we used nicotinamide adenine dinucleotide tetrazolium reductase (NADH-TR), succinate dehydrogenase (SDH), cytochrome oxidase (COX) and combined COX- SDH stains. Spectrin (Novo-castra, UK, NCL-spec1), dystrophin N-terminus (Novo-castra, UK, NCL-dys3), adhalin (Novocastra, UK, NCL-a-sarc), other sarcoglycans (beta, delta, gamma; Novo-castra, UK, NCL-b-d-g-sarc), laminin alpha-2 chain (Novocastra, UK, NCL-merosin), myotilin (Novo-castra, UK, NCL-myotilin), collagen VI (Novocastra, UK, NCL-

COLL-vI), β-dystroglycan (Novocastra, UK, NCL-b-DG), HLA Class 1 (Novo-castra, UK, NCL-HLA-ABC), NCAM (ThermoScientific, CA, USA, CD56), nitric oxide synthase-1 (Novo-castra, UK, NCLNOS-1), emerin (Novo-castra, UK, NCL-emerin), caveolin 3 (Novus Biologicals, CA, USA, NB110-5029), calpain 3 (Abcam, Cambridge, UK, ab103250) and dysferlin (Novo-castra, UK, NCL-Hamlet-2) antibodies were used by standard techniques for immunohistochemical analyses. Myosin heavy chain fast (Novo-castra, UK, NCL-MHCf) antibody was used for discriminating between fiber types, and myosin heavy chain neonatal (Novo-castra, UK, NCL-MHCn) antibody was used for identification of immature fibers. Monoclonal antibodies against T-cells (CD3), B-cells (CD 20), macrophages (CD68), suppressor T cells (CD8) and helper T cells (CD4) were used in selected cases for the identification of the inflammatory cell spectrum (2).

References

1. Vissing J. Limb girdle muscular dystrophies: classification, clinical spectrum and emerging therapies. Curr Opin Neurol 2016;29:635-41.

2. Diniz G, Hazan F, Yildirim HT, et al. Histopathological and genetic features of patients with limb girdle muscular dystrophy type 2C. Turk Patoloji Derg 2014;30:111-7.

3. Yis U, Baydan F, Karakaya M, et al. Importance of skin changes in the differential diagnosis of congenital muscular dystrophies. Biomed Res Int 2016;2016:3128735.

4. Bamborschke D, Pergande M, Becker K, et al. A novel mutation in sphingosine-1-phosphate lyase causing congenital brain malformation. Brain Dev 2018;40:480-3.

5. Wunderlich G, Brunn A, Daimagüler HS, et al. Long term history of a congenital core-rod myopathy with compound heterozygous mutations in Nebulin gene. Acta Myol 2018;37:121-7.

6. Topaloglu H. Epidemiology of muscular dystrophies in the Mediterranean area. Acta Myol 2013;32:138-41.

7. Dincer P, Akcoren Z, Demir E, et al. A cross section of autosomal recessive limb-girdle muscular dystrophies in 38 families. J Med Genet 2000;37:361-7.

8. Balci B, Uyanik G, Dincer P, et al. An autosomal recessive limb girdle muscular dystrophy (LGMD2) with mild mental retardation is allelic to Walker-Warburg syndrome (WWS) caused by a mutation in the POMT1 gene. Neuromuscl Disord 2005;15:271-5.

9. Harris E, Topf A, Vihola A, et al. A 'second truncation' in TTN causes early onset recessive muscular dystrophy. Neuromuscl Disord 2017;27:1009-17.

10. Balci B, Aurino S, Haliloglu G, et al. Calpain-3 mutations in Turkey. Eur J Pediatr 2006;165:293-8.

11. Angelini C, Fanin M. Calpainopathy. In: Adam MP, Ardinger HH, Pagon RA, et al., Eds. GeneReviews(R). Seattle (WA) 1993-2018. 2005 May 10 [updated 2017 Aug 3]

12. Khadilkar SV, Faldu HD, Patil SB, et al. Limb-girdle muscular dystrophies in India: a review. Ann Indian Acad Neurol 2017;20:87-95.

13. Dincer P, Leturcq F, Richard I, et al. A biochemical, genetic, and clinical survey of autosomal recessive limb girdle muscular dystrophies in Turkey. Ann Neurol 1997;42:222-9.

14. Saenz A, Leturcq F, Cobo AM, et al. LGMD2A: genotype-phenotype correlations based on a large mutational survey on the calpain 3 gene. Brain 2005;128:732-42.

15. Nigro V, Savarese M. Genetic basis of limb-girdle muscular dystrophies: the 2014 update. Acta Myol 2014;33:1-12.

16. Alavi A, Esmaeili S, Nilipour Y, et al. LGMD2E is the most common type of sarcoglycanopathies in the Iranian population. J Neurogenet 2017;31:161-9.

17. Giugliano T, Fanin M, Savarese M, et al. Identification of an intragenic deletion in the SGCB gene through a re-evaluation of negative next generation sequencing results. Neuromuscl Disord 2016;26:367-9.

18. Sandona D, Betto R. Sarcoglycanopathies: molecular pathogenesis and therapeutic prospects. Expert Rev Mol Med 2009;11:e28.

19. Trabelsi M, Kavian N, Daoud F, et al. Revised spectrum of mutations in sarcoglycanopathies. Eur J Hum Genet 2008;16:793-803.

20. Reddy HM, Hamed SA, Lek M, et al. Homozygous nonsense mutation

in *SGCA* is a common cause of limb-girdle muscular dystrophy in Assiut, Egypt. Muscle Nerve 2016;54:690-5.

21. Diniz G, Tosun Yildirim H, Akinci G, et al. Sarcolemmal alpha and gamma sarcoglycan protein deficiencies in Turkish siblings with a novel missense mutation in the alpha sarcoglycan gene. Pediatr Neurol 2014;50:640-7.

22. Diniz G, Tosun Yildirim H, Gokben S, et al. Concomitant alpha- and gamma-sarcoglycan deficiencies in a Turkish boy with a novel deletion in the alpha-sarcoglycan gene. Case Rep Genet 2014;2014:248561.

23. Stehlikova K, Skalova D, Zidkova J, et al. Autosomal recessive limb-girdle muscular dystrophies in the Czech Republic. BMC Neurol 2014;14:154.

24. Ghafouri-Fard S, Hashemi-Gorji F, Fardaei M, et al. Limb girdle muscular dystrophy type 2E due to a novel large deletion in *SGCB* gene. Iran J Child Neurol 2017;11:57-60.

25. Balci B, Wilichowski E, Haliloglu G, et al. Beta-sarcoglycan gene mutations in Turkey. Acta Myol 2004;23:154-8.

26. Diniz G, Tekgul H, Hazan F, et al. Sarcolemmal deficiency of sarcoglycan complex in an 18-month-old Turkish boy with a large deletion in the beta sarcoglycan gene. Balkan J Med Genet 2015;18:71-6.

27. Kirik S, Ozgur B, Isikay S, et al. Limb girdle 2E tanılı hastada *SGCB* geninde tanımlanan yeni mutasyon. 1.Ulusal Nöromuskuler Hastalıklar Kongresi Bildiri Kitab, 2017, s28.

28. Dincer P, Piccolo F, Leturcq F, et al. Prenatal diagnosis of limb-girdle muscular dystrophy type 2C. Prenat Diagn 1998;18:1300-3.

29. Piccolo F, Jeanpierre M, Leturcq F, et al. A founder mutation in the gamma-sarcoglycan gene of gypsies possibly predating their migration out of India. Hum Mol Genet 1996;5:2019-22.

30. Wu L, Xiang B, Zhang H, et al. Three novel recessive mutations in LAMA2, SYNE1, and *TTN* are identified in a single case with congenital muscular dystrophy. Neuromuscul Disord 2017;27:1018-22.

31. Hu P, Wu S, Yuan L, et al. Compound heterozygous *POMT1* mutations in a Chinese family with autosomal recessive muscular dystrophy-dystroglycanopathy C1. J Cell Mol Med 2017;21:1388-93.

32. Wallace SE, Conta JH, Winder TL, et al. A novel missense mutation in *POMT1* modulates the severe congenital muscular dystrophy phenotype associated with *POMT1* nonsense mutations. Neuromuscul Disord 2014;24:312-20.

33. Yis U, Uyanik G, Kurul S, et al. A case of Walker-Warburg syndrome resulting from a homozygous *POMT1* mutation. Eur J Paediatr Neurol 2007;11:46-9.

34. Endo T, Manya H, Seta N, et al. POMGnT1, *POMT1*, and *POMT2* mutations in congenital muscular dystrophies. Methods Enzymol 2010;479:343-52.

35. Madej-Pilarczyk A, Niezgoda A, Janus M, et al. Limb-girdle muscular dystrophy with severe heart failure overlapping with lipodystrophy in a patient with *LMNA* mutation p.Ser334del. J Appl Genet 2017;58:87-91.

36. Maggi L, Carboni N, Bernasconi P. Skeletal muscle laminopathies: a review of clinical and molecular features. Cells 2016;5.

37. Garg A, Cogulu O, Ozkinay F, et al. A novel homozygous Ala529Val *LMNA* mutation in Turkish patients with mandibuloacral dysplasia. J Clin Endocrinol Metab 2005;90:5259-64.

38. Demir T, Onay H, Savage DB, et al. Familial partial lipodystrophy linked to a novel peroxisome proliferator activator receptor -gamma (PPARG) mutation, H449L: a comparison of people with this mutation and those with classic codon 482 Lamin A/C (*LMNA*) mutations. Diabet Med 2016;33:1445-50.

39. Kutbay NO, Yurekli BS, Onay H, et al. A case of familial partial lipodystrophy caused by a novel lamin A/C (*LMNA*) mutation in exon 1 (D47N). Eur J Intern Med 2016;29:37-9.

40. Ozer L, Unsal E, Aktuna S, et al. Mandibuloacral dysplasia and *LMNA* A529V mutation in Turkish patients with severe skeletal changes and absent breast development. Clin Dysmorphol 2016;25:91-7.

41. Karaoglu P, Quizon N, Pergande M, et al. Dropped head congenital muscular dystrophy caused by de novo mutations in *LMNA*. Brain Dev 2017;39:361-4.

42. Akinci B, Onay H, Demir T, et al. Clinical presentations, metabolic abnormalities and end-organ complications in patients with familial partial lipodystrophy. Metabolism 2017;72:109-19.

43. Celebi HBG, Ayca S, Polat M, et al. A novel de nova missense *LMNA* mutation in a Turkish patient with laminopathies. Eur J Paediatr Neurol 2017;21:227.

44. Selcen D, Engel AG. Myofibrillar Myopathy. In: Adam MP, Ardinger HH, Pagon RA, et al., Eds. GeneReviews (R). Seattle (WA)1993-2018. 2005 Jan 28 [updated 2012 Oct 29].

45. Hauser MA, Conde CB, Kowaljow V, et al. Myotilin mutation found in second pedigree with LGMD1A. Am J Hum Genet 2002;71:1428-32.

46. Gundesli H, Talim B, Korkusuz P, et al. Mutation in exon 1f of PLEC, leading to disruption of plectin isoform 1f, causes autosomal-recessive limb-girdle muscular dystrophy. Am J Hum Genet 2010;87:834-41.

47. Ardicli D, Gocmen R, Talim B, et al. Congenital mirror movements in a patient with alpha-dystroglycanopathy due to a novel POMK mutation. Neuromuscul Disord 2017;27:239-42.

48. Koehler K, Milev MP, Prematilake K, et al. A novel TRAPPC11 mutation in two Turkish families associated with cerebral atrophy, global retardation, scoliosis, achalasia and alacrima. J Med Genet 2017;54:176-85.

49. Ghaoui R, Cooper ST, Lek M, et al. Use of whole-exome sequencing for diagnosis of limb-girdle muscular dystrophy: outcomes and lessons learned. JAMA Neurol 2015;72:1424-32.

50. Harris E, Topf A, Barresi R, et al. Exome sequences versus sequential gene testing in the UK highly specialised Service for Limb Girdle Muscular Dystrophy. Orphanet J Rare Dis 2017;12:151.