

MOLECULAR GENETIC ANALYSIS OF LIMB GIRDLE MUSCULAR DYSTROPHY 2A (LGMD2A) IN TWO CONSANGUINEOUS PAKISTANI FAMILIES

Muhammad Jaseem Khan^{1✉}

✉ Institute of Paramedical Sciences, Khyber Medical University, Peshawar, Pakistan
E-mail: jaseem.ipms@kmu.edu.pk
Cell number: +923315296282
Date Submitted: May 02, 2015
Date Revised: December 07, 2015
Date Accepted: December 09, 2015

ABSTRACT

OBJECTIVES: To investigate molecular pathogenesis of the disease and to significantly improve diagnosis and understanding of muscular dystrophy in Pakistani population.

METHODS: This study was carried out at the department of Biochemistry, Quaid-i-Azam University Islamabad on two families affected with limb girdle muscular dystrophies (LGMD). Duration of the study was one year. Blood samples were collected from three normal and three affected individuals of the family A, eight affected individuals and four normal individuals of the family B for genetic analysis. Technique of homozygosity mapping was used to track the gene responsible for autosomal recessive LGMD2A in two families. The gene *CAPN3* was sequenced in two families using Sanger sequencing approach.

RESULTS: Genotyping data and haplotypes analysis showed that all affected individuals of family A (V-4, V-6, V-7) and family B (IV-1, IV-2, IV-4, IV-5, IV-6, IV-7, IV-10, IV-11) were homozygous while all normal individuals of family A (III-1, IV-1, V-5) and family B (III-4, III-5, IV-8, IV-12) were heterozygous, thus establishing linkage of the family A to *CAPN3* gene on chromosome 15q15.1-q21.1. Subsequently, sequencing of all the 24 exons and exon-introns boundaries of *CAPN3* genes showed no disease causing DNA sequence variant.

CONCLUSION: No disease causing DNA sequence variant was found in these exons suggesting the presence of mutation in the regulatory sequences or any other genes present in this region.

KEY WORDS: Cytogenetic Analysis (MeSH), Limb-girdle muscular dystrophy type 2A (MeSH), LGMD2A (MeSH), Consanguinity (MeSH), Pakistan (MeSH).

THIS ARTICLE MAY BE CITED AS: Khan MJ. Molecular genetic analysis of limb girdle muscular dystrophy 2A (LGMD2A) in two consanguineous Pakistani families. *Khyber Med Univ J* 2015; 7(4): 152-156.

INTRODUCTION

Gradual degeneration and weakness of muscles is called Muscular dystrophies (MD). The onset of MD may be observed in early age or in some cases in middle age. Phenotype varies according to distribution and level of muscle weakness. To date, more than 30 different type of MD have been identified and classified that affect skeletal muscles with variable frequencies. Nine major forms of MD have

been described on the basis of mode of inheritance. MD may inherit in autosomal recessive (Limb Girdle, Congenital and Distal), autosomal dominant (Myotonic, Facioscapulohumeral and Oculopharyngeal) or X-linked (Duchenne, Becker and Emery Dreifuss) fashion.¹

In Limb girdle muscular dystrophies (LGMD) due to genetic defect, progressive weakness is seen in the muscles of proximal limb. LGMD has two modes of

inheritance; LGMD1 which is dominant and LGMD2 which is recessive. The autosomal recessive forms are then further classified into 16 different genetic types from LGMD2A to LGMD2P.²

LGMD2A is the most commonly occurring type of the LGMDs. LGMD2A in different ethnic groups is about 20% to 40 % of all LGMDs while in United States about 10 % is reported.³

The onset of the disease is in 2nd decade of life. However, the onset may be in the age of 2.5 to 49 years.⁴ Creatine phosphokinase (CPK) level ranges from normal to 50 times higher than normal in calpainopathy. Some patients develop severe contractures.⁵ The progression of the disease is slow and takes 1 to 2 decades to become unable to walk after diagnosis. There is no winging of scapula and abductor of hip involvement. Glutei and hip adductors are primarily involved. The phenotypes are highly variable with respect to hypertrophy of calf muscles among the patients.⁶ The Magnetic Resonance Imaging (MRI) shows that adductors and semimembranosus muscles are predominantly affected in ambulatory patients.⁵ Lateral and posterior muscles of thigh reduced movement of the patients may also be affected. In calf, Lateral heads of gastrocnemius is spared while the medial head of gastrocnemius and soleus are affected. In calpainopathy, facial and cardiac disorders are not present. There is no sign of any intellectual impairment. In calpainopathy, selective skeletal muscles may also be involved.⁷

Calpainopathy is caused by mutation in *CAPN3*, a non-lysosomal cysteine protease.⁸ *CAPN3* gene is present on chromosome 15q15.1-q21.1. To date, 214 mutations have been reported in *CAPN3* as an underlying cause of LGMD 2A.²

Homozygosity mapping is the common method used to screen consanguineous families for any DNA sequence variation that might be associated with a disease phenotype. Homozygosity mapping, a genetic analysis technique, is based on the principle that a particular portion of genome of siblings from consanguineous marriages would be homozygous because of identity by descent (IBD). One-sixteenth part of genome of offspring from first cousin marriages is expected to be homozygous.⁹ That particular region of homozygosity is random between different off springs of these cousin marriages except for a definite disease locus that is exclusively shared and common between all affected siblings.

Objectives of the present study were to investigate molecular pathogenesis of the disease in two families (A and B). This study aimed to significantly improve diagnosis and understanding of muscular dystrophy in Pakistani population.

METHODS

This study was carried out at the department of Biochemistry, Quaid-i-Azam University Islamabad. Duration of the study was one year. Families affected with limb girdle muscular dystrophies (LGMD) were visited personally at their residences to collect information about family history and to construct pedigree for which elders of the families were interviewed. The case history, number of affected individuals, number of generations involved, the associated anomaly if any and onset of the disorder were carefully recorded. For genetic inference, family pedigrees were constructed by the standard methods described by Bennett et al.¹⁰

The blood samples were collected in EDTA tubes (BD Vacutainer® K3 EDTA, Franklin Lakes NJ, USA) and the samples were stored at 4°C.

Blood samples were collected from three normal (III-1, IV-1, V-5), and three affected individuals (V-4, V-6, V-7) of the family A, eight affected individuals (IV-1, IV-2, IV-4, IV-5, IV-6, IV-7, IV-10, IV-11) and four normal individuals (III-4, III-5, IV-8, IV-12) of the family B for the present study (Figure 1 and 2).

DNA was extracted from 11 affected and 7 normal individuals of both the family A & B using the standard Phenol-Chloroform method.¹¹

Genetic Analysis

Genomic DNA of the families A & B were polymerase chain reaction (PCR) amplified using Thermocycler T3000 (Biometra, Gottingen, Germany).

The two families (A, B) were subjected to genetic analysis in order to identify the suspected pathogenic DNA variation that causes the disease. To search for the candidate locus/gene responsible for LGMD2A, highly polymorphic microsatellite markers (average heterozygosity > 75%) spanning the linkage interval of CAPN3 gene on chromosome 15q15.1-q21.1 were genotyped. Microsatellite markers used for linkage mapping are given in Table 1. After obtaining the positive and convincing linkage to a known gene in both families, corresponding gene was sequenced to identify any functional variant causing the disease phenotype in that family.

After amplification, 2% agarose gel was used to analyze PCR products. Ethid-

ium bromide (Sigma-Aldrich, St Louis, MO, USA) was added in gel to visualize the DNA under UV trans illuminator. Equal quantity of loading dye (Bromophenol Blue) and PCR product were loaded on 2% agarose gel. To resolve amplified PCR products of microsatellite markers, 8% non denaturing polyacrylamide gel was used. Microsatellite markers have been taken from UCSC human genome browser while primers for CAPN3 gene were synthesized by primer3 software.

RESULTS

Clinical Presentation

A five generation consanguineous family (A) segregating autosomal recessive form of LGMD2A was recruited from Khyber Pakhtunkhwa province of Pakistan (Figure 1). Three individuals were affected in family, including one male (V-4) and two females (V-6, V-7). No respiratory or cardiac abnormalities were found. Parents of affected individuals were normal.

Blood samples were collected from both the parents (III-1, IV-1), one normal (V-5) and three affected individuals (V-4,

TABLE 1: LIST OF MICROSATELLITE MARKERS USED TO TEST LINKAGE TO CAPN3 GENE

S. No.	Gene	Chromosome	Markers	cM*
I	CAPN3	15q15.1-q21.1	D15S1232	30.15
			D15S971	30.88
			D15S1042	32.39
			D15S118	32.39
			D15S221	36.78
			D15S994	41.37
			D15S784	42.12
			D15S537	42.58
			D15S222	42.81
			D15S172	42.98
			D15S659	43.74
			D15S1039	45.72
			D15S1028	46.89
D15S982	48.57			
D15S170	50.28			

*Genetic distance (centi Morgan) and +physical distance (mega base pairs) are according to the second-generation combined linkage physical map of the human genome (Matise et al., 2007)¹²

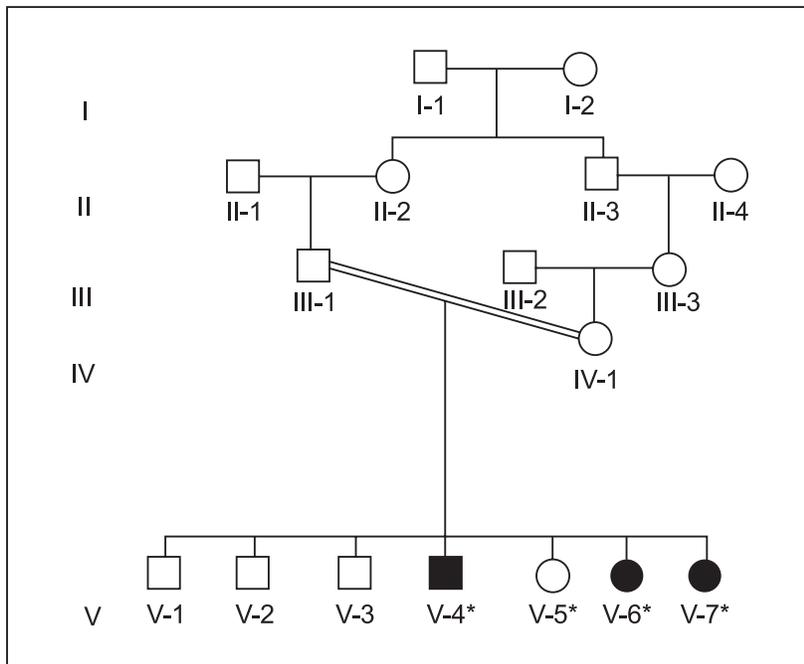


Figure 1: Pedigree drawing of the family A segregating autosomal recessive LGMD 2A. Males affected are indicated by filled squares and females affected are indicated by filled circles. Double lines between individuals represented consanguineous union. The individual members labeled with asterisks indicate the samples available for this study.

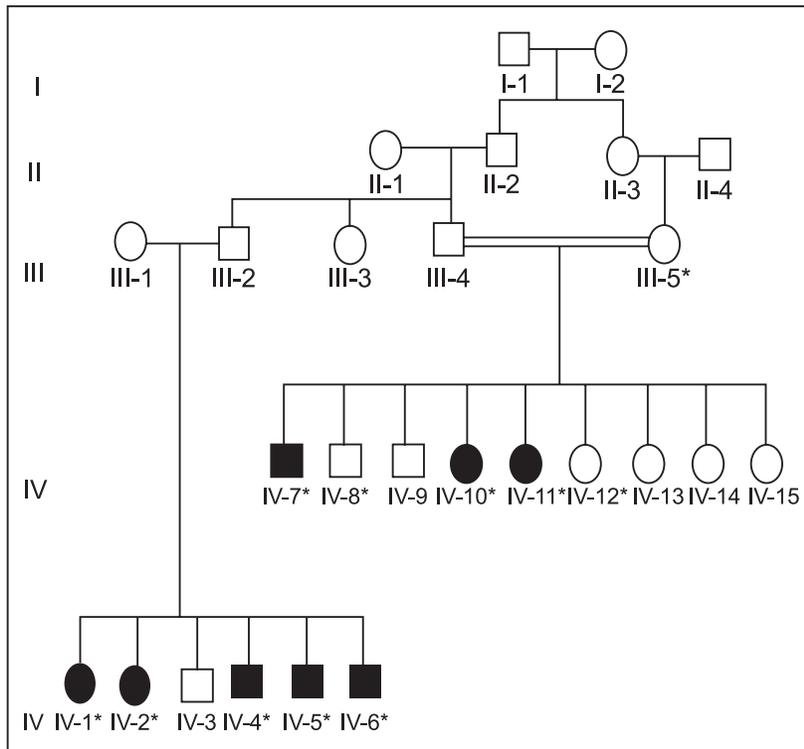


Figure 2: Pedigree drawing of the family B segregating autosomal recessive LGMD 2A. Males affected are indicated by filled squares and females affected are indicated by filled circles. Double lines between individuals represented consanguineous union. The individual members labeled with asterisks indicate the samples available for this study.

V-6, V-7) of the family A for the present study.

Family B was also recruited from Khyber Pakhtunkhwa province of Pakistan (Figure 2). Eight individuals were affected in family B, including four males (IV-4, IV-5, IV-6, IV-7) and four females (IV-1, IV-2, IV-10, IV-11). All eight affected individuals showed LGMD type 2A. No respiratory or cardiac abnormalities were found. Pedigree analysis showed that the parents of affected individuals were normal.

Blood samples were collected from eight affected individuals (IV-1, IV-2, IV-4, IV-5, IV-6, IV-7, IV-10, IV-11) and four normal individuals (III-4, III-5, IV-8, IV-12) of the family B for the present study.

Genetic Mapping of Candidate Gene Involved in Hereditary Muscular Dystrophy

The gene involved in hereditary muscular dystrophy was tested for genetic analysis in the two families showing autosomal recessive muscular dystrophy. Genotyping was carried out using 5 to 10 microsatellite markers linked to candidate gene. Analysis of the microsatellite markers was performed using standard PCR reaction and electrophoresis on 8% non-denaturing polyacrylamide gel as discussed in materials and methods. Amplified PCR products were visualized by staining the gel with ethidium bromide and genotypes were assigned by visual inspection.

Mapping of a Gene Involved in LGMD type 2A

Family A

In family A six members including three normal (III-1, IV-1, V-5) and three affected (V-4, V-6, V-7) were tested for genetic analysis by typing microsatellite markers for respective candidate gene. DNA analysis with polymorphic microsatellite markers (D15S1232, D15S994, D15S537, D15S659, D15S1028, D15S170) (Table 1) linked to CAPN3 gene on chromosome 15q15.1-q21.1 were studied. Genotyping data and haplotypes analysis (Figure 3) showed that all affected individuals (V-4, V-6, V-7) were homozygous while all normal individuals (III-1, IV-1, V-5) were heterozygous, thus establishing linkage of the family A to CAPN3 gene on chromosome 15q15.1-q21.1.

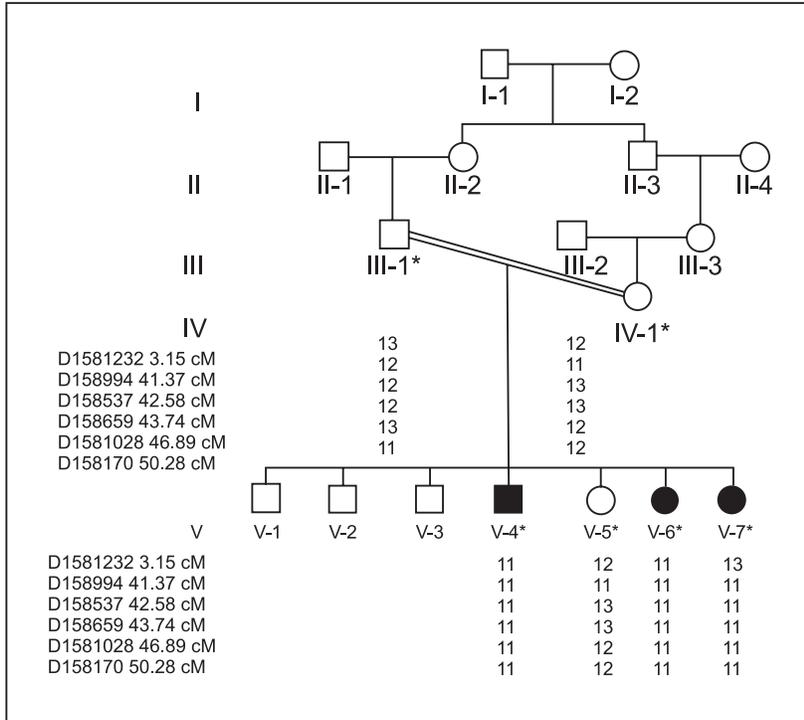


Figure 3: Haplotype analysis of the family A segregating LGMD2A. For each individual, haplotypes of the most closely linked microsatellite markers are shown below the symbol.

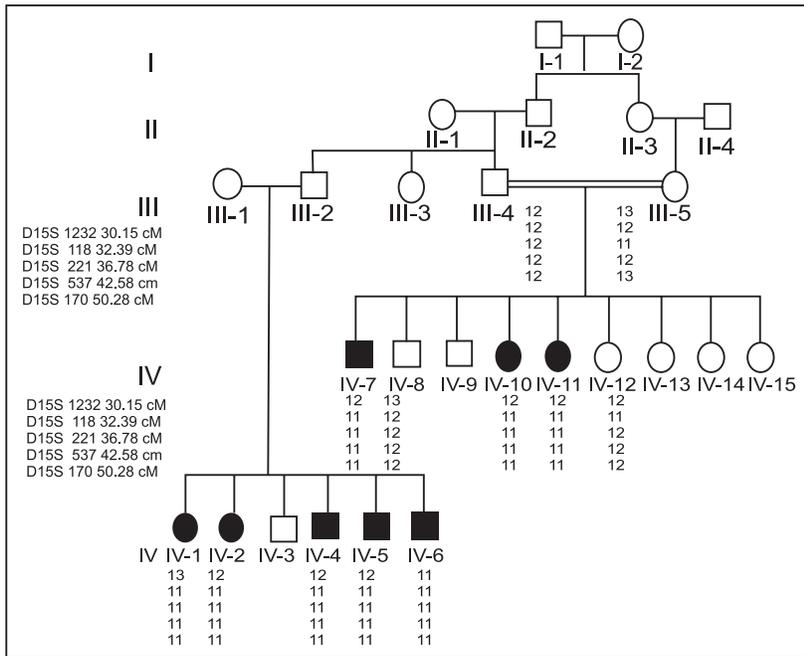


Figure 4: Haplotype analysis of the family B segregating LGMD2A. For each individual, haplotypes of the most closely linked microsatellite markers are shown below the symbol.

Family B

In family B, DNA of four normal (III-4, III-5, IV-8, IV-12) and eight affected individuals (IV-1, IV-2, IV-4, IV-5, IV-6, IV-7, IV-10, IV-11) were tested for genetic analysis by typing microsatel-

lite markers for respective candidate gene. Polymorphic microsatellite markers (D15S1232, D15S118, D15S221, D15S537, D15S170) linked the family on chromosome 15q15.1-q21.1 containing CAPN3 gene. Genotyping data and

haplotypes analysis (Figure 4) showed that all affected individuals (IV-1, IV-2, IV-4, IV-5, IV-6, IV-7, IV-10, IV-11) were homozygous while all normal individuals (III-4, III-5, IV-8, IV-12) were heterozygous, thus establishing linkage of the family B to CAPN3 gene on chromosome 15q15.1-q21.1.

Sequencing of all coding exons of CAPN3 gene in family A and B

After establishing genetic analysis of family A and B to CAPN3 locus, sequencing of all the 24 exons and exon-introns boundaries of CAPN3 genes was carried out to identify a pathogenic variant. However, no disease causing DNA sequence variant was found in these exons suggesting the presence of mutation in the regulatory sequences or any other genes present in this region.

DISCUSSION

The present investigation describes the segregation of a severe form of calpainopathy in an autosomal recessive inheritance pattern in two consanguineous families (families A and B) of North-Western Pakistani origin. All the patients in family A were confined to wheel chairs. Patients of the family B showed variable phenotypes, males were severely affected as compared to females. Three males (IV-5, IV-6, IV-7) and three females (IV-2, IV-10, IV-11) are confined to wheel chair. A male (IV-4) 15 years of age, show toe walking while one of female is ambulant. Affected individuals of the families showed features of LGMD2A similar to those reported earlier. The severity of disease was variable among the affected.

Homozygosity mapping of both the families (A and B) using multiple microsatellite markers established linkage to the region containing CAPN3 gene on chromosome 15q15-q21.1. All the exons and intron-exon boundaries of the CAPN3 gene of the patients as well as parents of both the families A and B were sequenced by Sanger sequencing. Analysis of the sequencing data of the CAPN3 gene failed to identify any functional sequence variation in the patients of both the families (A & B). A diverse spectrum of CAPN3 mutations was reported. Pathogenic variants are reported in all 24 exons of

CAPN3 gene. Exon 22, 21, 13, 11, 10, 7, 5, 4 and 1 reported 85% mutation of CAPN3. Some variants frequently reported in a particular population and about 70% mutations were private variants.¹³

Since only coding sequences have been sequenced, a functional sequence variation in the regulatory sequences of the CAPN3 gene cannot be ruled out.

The findings that the previously identified gene is not involved in causing the calpainopathy phenotype in these families suggests for the further genetic heterogeneity in the disease. Whole genome single nucleotide polymorphism (SNP) and copy number variations (CNVs) analysis, the next step, should be considered for these families to identify the underlying cause.

To date, 214 mutations including 47 splicing, 17 small insertion, 53 small deletion, 5 small indels, 8 gross deletions and 1 gross insertion mutations have been reported in the gene CAPN3.² In Pakistan, no prevalence study is conducted for calpainopathy but in Brazilian, Italian, British, Australian, American and Dutch (Netherlands) populations, the calpainopathies observed were 32%, 28.4%, 26.5%, 8%, 12% and 21% respectively.¹⁴⁻¹⁸

CONCLUSION

The exclusion of the pathogenic sequence variation in the coding sequences and the intron-exon boundaries of CAPN3 gene in the families A and B having clinical phenotypes similar to calpainopathy suggest for the genetic heterogeneity of the disease especially in Pakistani population. Since only coding region of the gene has been sequenced, there might be a possibility of a functional sequence

variation in the regulatory sequences of the CAPN3 gene.

To further elucidate the underlying causes of the disease, whole genome single nucleotide polymorphism (SNP) and copy number variations (CNVs) analysis may be performed in these families.

REFERENCES

- Zatz M, de Paula F, Starling A, Vainzof M. The 10 autosomal recessive limb girdle muscular dystrophies. *Neuromuscul Disord* 2003; 13: 532-44.
- Nigro V, Aurino S, Piluso G. Limb girdle muscular dystrophies: update on genetic diagnosis and therapeutic approaches. *Curr Opin neurol* 2011; 24(5): 429-36.
- Moore SA, Shilling CJ, Westra S, Wall C, Wicklund MP, Stolle C, et al. Limb girdle muscular dystrophy in the United States. *J Neuropathol Exp Neurol* 2006; 65(10): 995-1003.
- Fanin M, Nascimbeni AC, Fulizio L, Trevisan CP, Meznaric-Petrusa M, Angelini C. Loss of calpain-3 autocatalytic activity in LGMD2A patients with normal protein expression. *Am J Pathol* 2003; 163(5): 1929-36.
- Mercuri E, Bushby K, Ricci E, Birchall D, Pane M, Kinali M, et al. Muscle MRI findings in patients with limb girdle muscular dystrophy with calpain 3 deficiency (LGMD2A) and early contractures. *Neuromuscul Disord* 2005; 15(2): 164-71.
- Zatz M, Vainzof M, Passos-Bueno MR. Limb-girdle muscular dystrophy: one gene with different phenotypes, one phenotype with different genes. *Curr Opin Neurol* 2000; 13: 511-17.
- M Vainzof, F de Paula, A M Tsanaclis, M Zatz, The effect of calpain 3 deficiency on the pattern of muscle degeneration in the earliest stages of LGMD2A. *J Clin Pathol* 2003; 56: 624-26.
- Piluso G, Politano L, Aurino S, Fanin M, Ricci E, Ventriglia VM, et al. Extensive scanning of the calpain-3 gene broadens the spectrum of LGMD2A phenotypes. *J Med Genet* 2005; 42(9): 686-93.
- Kramerova I, Beckmann JS, Spencer MJ. Molecular and cellular basis of calpainopathy (limb girdle muscular dystrophy type 2A). *Biochim Biophys Acta* 2007; 1772: 128-44.
- Richard I, Broux O, Allamand V, Fougereousse F, Chiannikulchai N, Bourg N, et al. Mutations in the proteolytic enzyme calpain 3 cause limb girdle muscular dystrophy type 2A. *Cell* 1995; 81: 27-40.
- Bennett RL, Steinhaus KA, Uhrich SB, O'Sullivan CK, Resta RG, Lochner-Doyle D et al. Recommendations for standardized human pedigree nomenclature. Pedigree standardization task force of the national society of genetic counselors. *Am J Hum Genet* 1995; 56: 745-52.
- Matisse TC, Chen F, Chen W, De La Vega FM, Hansen M, He C, et al. A second-generation combined linkage physical map of the human genome. *Genome Res.* 2007 Dec; 17(12): 1783-6. Epub 2007 Nov 7.
- Milic A, Daniele N, Lochmuller H, Mora M, Comi GP, Moggio M, et al. A third of LGMD2A biopsies have normal calpain 3 proteolytic activity as determined by an in vitro assay. *Neuromuscul Disord* 2007; 17: 148-56.
- Zatz M, de Paula F, Starling A, Vainzof M. The 10 autosomal recessive limb-girdle muscular dystrophies. *Neuromuscul Disord* 2003; 13: 532-44.
- Guglieri M, Magri F, D'Angelo MG, Prella A, Morandi L, Rodolico C, et al. Clinical, molecular, and protein correlations in a large sample of genetically diagnosed Italian limb girdle muscular dystrophy patients. *Hum Mutat* 2008; 29: 258-66.
- Lo HP, Cooper ST, Evesson FJ, Seto JT, Chiotis M, Tay V, et al. Limb-girdle muscular dystrophy: Diagnostic evaluation, frequency and clues to pathogenesis. *Neuromuscul Disord* 2008; 18: 34-44.
- Sveen ML, Schwartz M, Vissing J. High prevalence and phenotype-genotype correlations of limb girdle muscular dystrophy type 2I in Denmark. *Ann Neurol* 2006; 59: 808-15.
- Moore SA, Shilling CJ, Westra S, Wall C, Wicklund MP, Stolle C, et al. Limb-girdle muscular dystrophy in the United States. *J Neuropathol Exp Neurol* 2006; 65: 995-1003.

CONFLICT OF INTEREST

Authors declared no conflict of interest

GRANT SUPPORT AND FINANCIAL DISCLOSURE

NIL

AUTHOR'S CONTRIBUTION

Author **MJK** has made substantial contributions to the manuscript in conception, study design, acquisition, analysis & interpretation of data, drafting the manuscript and final approval of the version. Author agrees to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.