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ORIGINAL RESEARCH

A Novel Founder Mutation in the SGCB Gene (Exon 5) Causes Limb Girdle Muscular Dystrophy (LGMDR4) in the Sathwara Community, Gujarat, India

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ABSTRACT

Objectives: Limb-girdle muscular dystrophy autosomal recessive type 4 (LGMDR4) earlier designated as LGMD2E is a rare genetic disease due to the mutation in the sarcoglycan gene (SG). To identify the mutation type in LGDMR4 in Sathwara community, India.

Methods: Molecular diagnosis involving NGS was carried out on blood samples using Illumina MiSeq. STRAND NGS software was used for the alignment and variant calling and StrandOmics variant annotation engine was used for the variant reporting. SGCB protein structure model was based on comparison of primary sequences with nearest available known structure. The converted amino acid sequence of NM_000232.5 in Fasta format was submitted to YASARA structure software.

Results: We report here the clinical and genetic data from children of three unrelated families belonging to the Sathwara community of Gujarat, India diagnosed with a relatively rare subtype of LGMDR4 (β-sarcoglycan, *SGCB*) with the same homozygous missense mutation (Chr 4:52894204C>T; c.683G>A; p.Gly228Glu) in the *SGCB* gene (exon 5).

Conclusion: LGMDR4 maybe a common muscular dystrophy among the Sathwara community, and further studies on this population is suggested for determining the basis of natural history of this disease. The identified novel missense mutation present may result in a conformational change in the extracellular domain of the SGCB protein leading to a modification of sarcoglycan complex. These results indicate high prevalence of this mutation in this community with founder effect.

Keywords: muscular dystrophy, LGMDR, SGCB gene, Founder mutation, India

INTRODUCTION

Most Limb-girdle muscular dystrophy (LGMD) are rare with an estimated prevalence quoted by various studies between 0.07 to 0.43 per 100,000. LGMDs are further divided into autosomal dominant inherited Type 1 and the more common autosomal recessively inherited Type 2. Further, LGMD2 comprises of various subtypes like LGMD2A, 2B, 2C, 2D, etc. based on the presence of mutation in genes of *CAPN*3, *DYSF*, *SGCG*, *SGCA*, *SGCB*, *SGCD*, etc. Calpainopathy, Dysferlinopathy, Sarcoglycanopathy, and Anoctaminopathy are regarded as the more common LGMDs. Recently, the classification was revised and it was suggested to assign the letter "D" for dominant or "R" for recessive after LGMD. This will be followed by a number based on order of discovery of the affected protein and also include the name of the affected protein[1]. As of today, more than 30 different genetic subtypes of LGMD have been identified and their number will increase with the developments in gene sequencing technology. The prevalence of LGMDs varies both regionally and ethnically[2]. A review on LGMDs which are commonly found in India has been reviewed, suggesting the importance of multicenter studies for documenting the incidence and prevalence of this neuromuscular disorder[3-5]. Sarcoglycanopathy is caused by a mutation in the gene (SGCB) that produces β-Sarcoglycan, a 43kDa glycoprotein present in the dystrophin-glycoprotein complex. It is

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the most common type of LGMDR with symptoms observed in the first ten years of childhood. Diagnosis of the LGMD subtype can be suspected using: clinical features recognized to be associated with some of the subtypes; muscle biopsy and immunostaining. Further diagnosis depends upon genetic testing using either a panel of genes or a wider exome-based study. The gene encoding for LGMDR4 (earlier designated as LGMD2E) consists of six exons and is located on chromosome 4q12. Frameshift, nonsense, and missense mutations as well as deletions are known to cause the LGMDR4 subtypes[6-8]. Missense mutations in exon 3 (coding for immediate extracellular domain) have been reported in Brazilian LGMD patients[6]. The Sathwara is a Hindu caste found in Gujarat, India. They are found mainly in North Gujarat, Saurashtra, and Kutch region. They are further divided and subdivided socially into various branches. There are exogamous clans, the main ones namely Parmar, Songara, Nakum, Kacchatiya, Chopda, Khandar, Kadiya, Bedia, Mori and Dabhi. In the present study, the clinical and genetic data of six (6) children from Sathwara families of Gujarat was investigated at the neuromuscular clinic, namely Royal Institute of Child Neurosciences, Ahmedabad, and genetic testing laboratory, namely Genexplore Diagnostics and Research Centre Pvt. Limited, Ahmedabad for LGMD. A severe form of the LGMDR4 subtype was identified due to a novel founder mutation.

METHODOLOGY

Standard protocol approvals and patient consent: This study involved an observational, cross-sectional study of LGMDR4 in children. All procedures were performed in compliance with relevant laws and institutional guidelines that have been approved by the institutional committee(s) and ethical approvals were obtained. The study was conducted after informed and written consent from the participants (parents) and utmost care has been taken for protection of the privacy rights of the human subjects.

Patient cohort: This study involved six (6) children in the age group between 5 to 13 years from three unrelated Sathwara families (Families 1, 2, and 3). The affected children visited the Royal Institute of Child Neurosciences, Ahmedabad with primary complaints of difficulties in walking. There was no prior data available for illness, genetic study, or counselling (see Figure 1(A), 1(B) and 1(C) and Table 1) for details.

Genome library construction and sequence analysis: Genomic DNA extracted from EDTA blood was quantified using Fluorometer (Qubit 3.0, Thermofisher Scientific, USA) and 50 ng taken for the library preparation. TruSight One library was prepared by transposon-based shearing of the genomic DNA. The protocol allows the DNA to be tagmented (Fragmented and tagged simultaneously in the same tube). A limited cycle PCR step allows the incorporation of adaptors, platform-specific tags, and barcodes to prepare the DNA sequencing libraries. The tagged and amplified sample libraries are checked for quality and quantity using Agilent Bioanalyzer (2100 Bioanalyzer, Agilent, USA). Target specific probes used to pull down the region of interest. Target libraries were amplified using limited PCR steps and loaded for sequencing on MiSeq. Sequencing performed using a standard kit on Illumina MiSeq with the expected data output of ~3GB per sample. The trimmed FASTQ files were generated using MiSeq reporter from Illumina. STRAND NGS software was used for the alignment and variant calling. StrandOmics variant annotation engine was used for the variant reporting.

Validation of SGCB variant: The identified variant in SGCB gene was validated using the mutation specific test. Bidirectional Sanger sequencing was performed in parents and other family members including suspected asymptomatic carriers. Briefly, genomic DNA was extracted from venous blood taken from patients, parents and other family members using blood and tissue DNA extraction kit (Qiagen 951336). Primer sequence to exon 5 of SGCB gene was designed, Forward:5'-Catttgactttcatctctcatgact-3';
Reverse:5'tggatttatgtacccaagaacct-3') using Primer-3 plus program (www-genome.wi.mit.edu/cgibin/primer/primer3_www.cgi). Target region was amplified by PCR. Amplified PCR amplicons were sequenced using bidirectional fluorescent sequencing on an ABI 3500 eight capillary sequencer, with Big Dye Version 3.1.

Structure prediction: The 3D structure of SGCB wild type and mutant proteins were prepared. Resolved crystalized structures of these proteins were searched in PDB, however no data for this protein was available. 3D models of the SGCB wild type and mutant protein were generated with comparative (Homology) modelling approach [9]. The Nucleotide sequence of *Homo sapiens* beta-sarcoglycan (SGCB), mRNA was retrieved from NCBI GenBank with the accession no NM_000232.5 and converted into amino acids using ExPASy tool (SIB Swiss Institute of Bioinformatics). This amino acid sequences of SGCB protein were used for the homology modelling.

Homology modelling: Absence of resolved crystalized structure prompted to build models of SGCB protein based on comparison of primary sequences with nearest available known structure. The converted amino acid sequence of NM_000232.5 in Fasta format was submitted to YASARA structure software. Based on the software parameters the model was generated based on: Modelling speed, PSI-BLAST iterations, E-value, Maximum number of

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templates, OligoState, alignment variations, LoopSamples and TermExtension[9-10]. The model was validated using the Z-score. The superposition of both structures was carried out in the UCSF Chimera software with 0.679 Å.

RESULTS

Clinical examination of all cases

Family 1 (Madhavpur area, Morbi, Dalwadi nav gam, Gotra: Kalsar)

Case-1 (Family 1, III-1: 11y): A female had no significant manifestations in birth and past medical history. The parents had noticed increasing difficulty in walking from the age of 8y; however, the symptoms prevailed earlier. She had difficulty in climbing the stairs, required support for the same and frequently fell while walking. There was no evidence of proximal upper limb involvement. There was no swallowing difficulties observed. Further follow up period revealed inability to stand up from sitting position on the floor since age 10y 6 months.

Case-2 (Family 1, III-4: 12y): This male child from a normal delivery weighed 2.45kg. at birth. The first symptom of LGMD namely, inability to crawl was observed by the parents at the age of 1.5y. He started walking at the age of 5y, but at the age of 7y developed difficulty in walking. Since then the patient was not able to get up from the floor without support. At age of 11y 6 months symptoms of bilateral shoulder weakness was observed. He had also lost ambulated subsequently at age 12y. There was no evidence of cardiac or respiratory involvement. Investigation for a possible mutation in the DMD gene was found to be negative.

Case 3 (Family 1, III-5: 13y): This male child from a normal delivery weighed 2.5kg. had difficulty in walking. During the follow up period the parents complained of frequent falls while walking and difficulty in climbing up the stairs. At the age of 11y he was unable to stand up from the floor. There are no other muscle groups involved.

Family 2 (Ahmedabad, Gujarat)

Case 4 (Family 2, III-1: 11y): A female was evaluated in the clinic for neuromuscular problems. She was born via normal delivery with birth weight of 2.7kg. Clinical history revealed that she had a delay in reaching the developmental milestones. At the age of 8y the first symptom of difficulty in walking was noticed. She had difficulty in climbing stairs and frequent falls whilst walking. Since the age 11y asymmetrical shoulder weakness was observed.

Case 5 (Family 2, III-2: 5y): A female patient (Family 2, III-1: 11y) did not have any obvious motor difficulties according to her parents. At age 5y she was able to walk, run and climb stairs. However, on clinical examination she did have mild difficulty in standing up from the floor.

Family 3 (Dwarka, Gujarat)

Case 6 (Family 3, III-1: 11y): This female had difficulty in walking with delay in achieving independent walking. First concerns regarding difficulty in climbing stairs were noticed around 2y of age. Since age 11y 6 months she was unable to get up from the floor and had developed mild bilateral shoulder weakness. Her 10y old brother and 5y old sister had no phenotypic characters of LGMD. Pedigree charts of the Families involved in the study are shown in Figures 1(A), (B) and (C).

Mutation analysis

An eleven 11y old female (Family 1; III-1) with complains of difficulties in walking and climbing stairs visited the Royal Institute of Child Neurosciences, Ahmedabad. She was examined for the muscle strength, other clinical manifestations and serum Creatinine Kinase (CPK) levels. She was found to have asymmetric shoulder muscle weakness and increased CPK values (9245.0 IU/ml). Her clinical representation was suggestive of limb-girdle muscular dystrophy (LGMD). A complete family history of the patient, her affected relatives and where possible, first degree relatives were taken by clinical geneticist. Clinical examination and pedigree analysis were suggestive of LGMDR.

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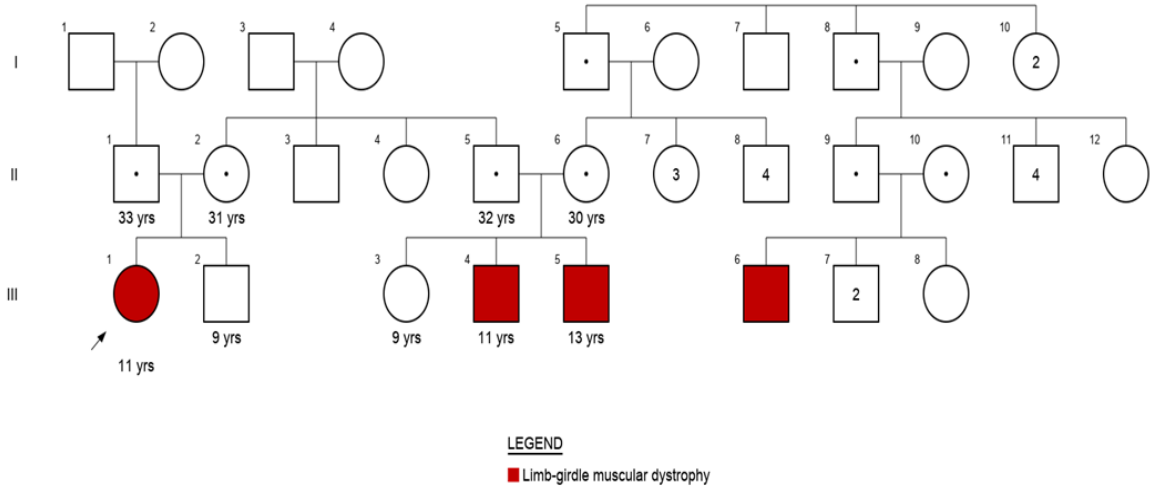
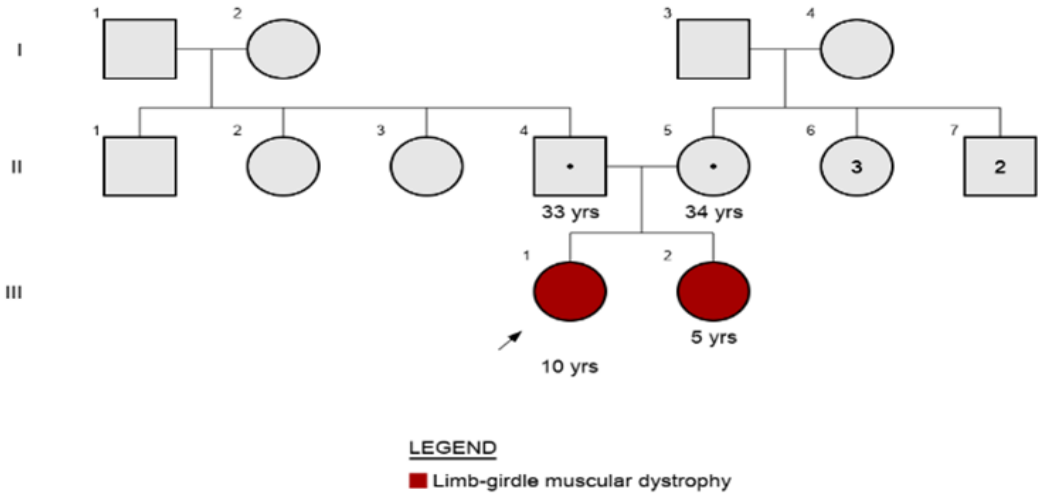


Family 1



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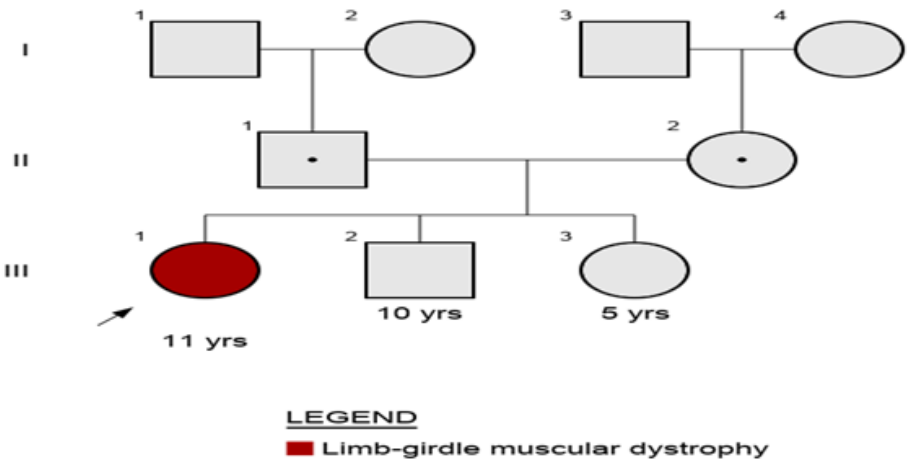

Family 3

Figure 1. Pedigree chart of families involved in the study for Limb-girdle muscular dystrophy LGMDR4.

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Table 1. Details of cases studied in three families in which LGMDR4.

Character	Family-1			Family-2		Family-3
	Case-1	Case-2	Case-3	Case-4	Case-5	Case-6
Age (y)	11	12	13	11	5	12
Serum CPK levels (IU/ml)	9245.0	6286.0	8420.0	8300.0	10200.0	7340.0
Age (y) when first symptom appeared	8 walking difficulties	1.5 delayed motor milestones	6 difficulty in walking	8 difficulty in walking	2 slight difficulty	2.5 developmental delay and walking difficulties
Lower limb Involvement	Yes					
Upper limb involvement	No	Yes (Mild bilateral shoulder weakness from the age of 11 years 6 months)	No	Yes (Asymmetrical shoulder weakness from the age of 11 years)	No	Yes (bilateral shoulder weakness from the age of 11 years 6 months)
Motor abilities	Walk without support unable to get up without support (from the age of 10 years 6 months)	Unable to walk unsupported (at the age of 12 years)	Walking with frequent falls Unable to get up from the floor (11 years)	Walking with frequent falls Unable to get up from the floor	Able to walk unsupported	Yes without support
Cardiac or respiratory involvement	No					
Swallowing difficulties	No					
SGCB gene mutation Exon Involved Variant identified Change in Amino Acid	Chr4:52894204C>TExon 5c.683G>A;p.Gly228Glu					
Zygocity	Homozygous					

The different forms of limb girdle muscular dystrophy (LGMD) are genetically heterogenous. Therefore, initially a Next generation sequencing (NGS) of proband (Family 1; III-1: 11y) was performed targeting muscular dystrophy genes (78 genes). This analysis revealed a Homozygous variant (Chr 4:52894204C>T; c.683G>A; p.Gly228Glu) GenBank Accession No. PP480237.1 (Figure 2c) in Exon 5 of SGCB gene. The identified homozygous missense substitution (p.Gly228Glu) alters a conserved residue in the protein. The variant was predicted to be damaging (SIFT, LRT, Mutation Taster, PolyPhen-r, Mutation Assessor and FATHMM) by using 6 *in silico* Missense prediction tools. The identified variant seems to be novel as it has not been reported previously in literature. The clinical significance of the identified variant was unknown, however, other missense mutation in SGCB gene have been reported in patients affected with LGMDR[11-12]. Germline pathogenic variation in the SGCB gene have been shown to be associated with LGMDR4, which manifests as weakness and wasting of skeletal muscle in the limb. Further, LGMDR4 caused due to mutation in SGCB gene, is also inherited in an

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autosomal recessive manner[12]. This supports our clinical and pedigree analysis of this patient.

Validation of SGCB variant

Results of variant SGCB validation revealed that parents (Family 1; II-1: 33y; II-2: 31y) of the proband (Family 1; III-1: 11y) of Family 1 are heterozygous for variant (Chr 4:52894204C>T; c.683G>A; p.Gly228Glu) GenBank Accession No. PP480236.1 identified in proband (Figure 2b). Maternal cousins (Family 1; III-4: 11y; III-5: 13y) of the proband showed this variant in homozygous condition (Figure 2c) and both were showing the clinical manifestation similar to the proband. Parents (Family 1; II-5: 32y; II-6: 30y) of these individuals were also found carrier for the novel mutation identified. Maternal brother (Family 1; II-5: 32y) of the proband was likely to be heterozygous (Figure 2b) as per the pedigree analysis of Family 1. However, he was non-consanguineously married to his wife (Family 1; II-6: 30y) who was also carrier for the same variant. This indicates that probably this mutation is likely to be common in this community.

Further analysis revealed similar clinical manifestation with increased CPK levels (8300 and 7340 IU/ml, respectively) in two more females (Family 2; III-1: 10y; Family 3; III-1: 11y) and pedigree analysis suggested autosomal recessive trait (Figure 1b). Surprisingly, after analysis of pedigree, it was identified that, both these families were unrelated to each other and also unrelated to 1st studied family, but belonged to the same "Sathwara" community. Hence, both patients and their first-degree relatives were directly investigated for the SGCB novel variant identified in Family 1. To our astonishment we found both affected females were homozygous (Figure 2c) for the same novel variant of SGCB gene identified in Family 1. This homozygous mutation was also detected in one of the siblings of Family 2, III-2: 5y. On clinical examination mild difficulty in standing up from the floor was observed. Currently, she is able to walk, run and climb stairs. Parents of these affected individuals were heterozygous (Figure 2b) and asymptomatic carriers.

Structural analysis

The three-dimensional structure of the SGCB protein was developed using YASARA software and 4C79-A was considered as template which possessed 2.31 total score, 0.21 E-value, 23% coverage. The three-dimensional structures of the homology modelled SGCB wild type as well as mutant protein is shown in Figure 3. Superposition of both structures was carried out in the UCSF Chimera software with 0.679 Å. This revealed only one (1) change at the p.Gly228Glu position, which is highlighted with red and green color.

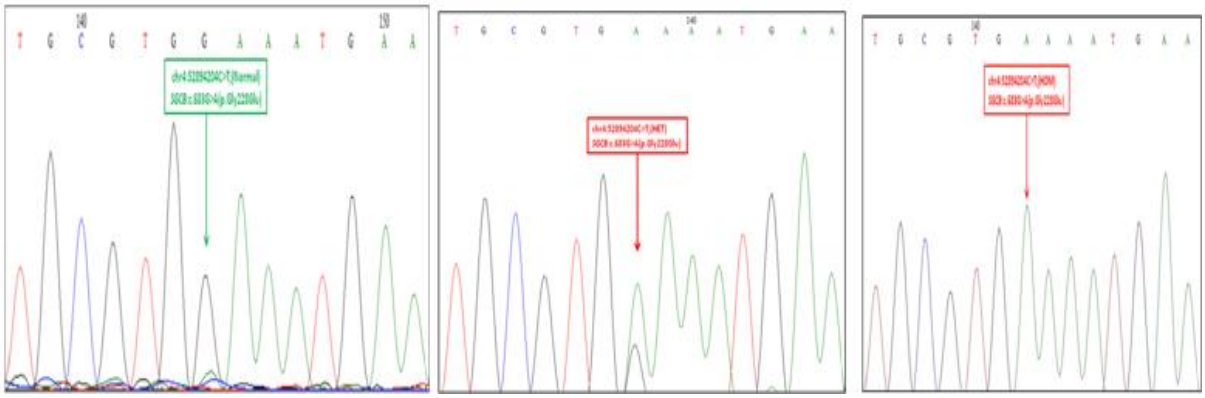

Figure 2. Electropherograms of (A) Normal, (B) Heterozygous (HET) and (C) Homozygous (HOM) mutations identified in the study samples for the LGMDR4 condition.

Protein homology modeling and evaluated the bioinformatics of the proteins as shown in Figure 3. Further the molecular docking analysis to determine the binding affinity of beta-sarcoglycan (SGCB) wild type and mutant type protein with its targeted binding proteins like F-Actin was not possible due to unavailability of sequence homology for SGCB using either of PHYRE2, MODELLER, or SWISS-MODEL bioinformatics tools. Therefore, model of SGCB has not been predicted. Due to point mutation in SGCB it may be possible that the mutant protein forms a weak interaction to its targeted proteins like alpha-, gama- or delta- sarcoglycan (SGCA, SGCG or SGCD respectively), dystrophin or F-Actin as compared to wild type protein and may have clinical correlation with patients' pathophysiological condition.

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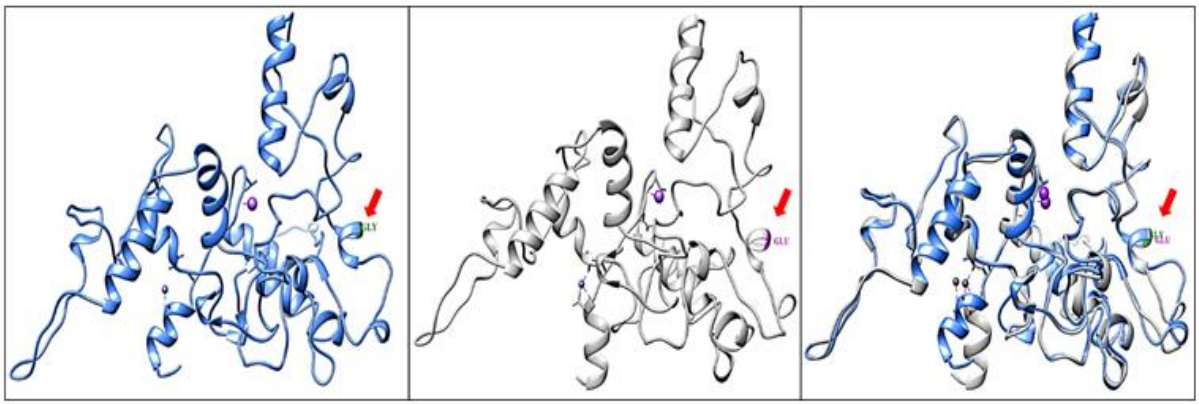

Figure 3. () Wild type protein (B) Mutant protein, and (C) Superposed image of wild type and mutant type protein. The mutation of the p.Gly228Glu was shown by the red arrow in all figures.

Loss of sarcoglycan in the dystrophin-glycoprotein complex is the specific cause of sarcoglycanopathy. Amongst the four sarcoglycans (alpha-, beta-, delta- and gamma-) two of them namely, beta- and gamma- have an intracellular tail which associates directly with the C terminus of dystrophin. In LGMDR4 defects in the gene encoding beta-sarcoglycan leads to absence of the four sarcoglycans and reduction in dystrophin and dystroglycans. Removal of glycosylation sites in beta-sarcoglycan is known to affect assembly of the complex. Missense mutations reside in the beta-sarcoglycan tail, which might lead to either loss-of-function or perturb the assembly of the sarcoglycan complex or simply generate processing mutants. In the large extracellular domain, the portion of beta-sarcoglycan proximal to the transmembrane domain is thought to be required for the binding to delta-sarcoglycan. Missense mutations also reside in apparently noncritical residues of the beta-sarcoglycan cysteine-rich domain[12-13].

Among the muscular dystrophies seen in childhood, Duchenne muscular dystrophy is the most common affecting one in 3500-5000 live male births (https://nyulangone.org/conditions/muscular-dystrophy/types). After DMD, Limb girdle muscular dystrophy (LGMD) subtypes and congenital myasthenic syndromes (CMS) can be present in childhood with lower limb progressive weakness with or without a proximal weakness in the upper limbs. In general, LGMD's are considered as rare disorders. The estimated prevalence of LGMDR3 and R4 is 0.07 per 100,000, and LGMDR6 around 0.43 per 100,000[14]. The frequency of LGMD's varies based on ethnicity. The presentation is of a slowly progressive limb weakness, usually involving proximal muscles of arms and legs. However other patterns those involving distal muscles is also possible. The age of presentation ranges between early childhood to late adult life. In clinics when it comes to making a diagnosis the hereditary pattern provides the most important clue, being X linked, Autosomal recessive (AR) or Dominant (AD). Amongst the AR (LGMDR) disorders ethnicity is the second clue followed by any pathognomonic features. If there are no classical features a muscle ultrasound or MRI and muscle biopsy can provide important information.

In India studies have suggested that LGMDR1 calpain3-related (earlier LGMD2A) is the most common subtype, with 43% patients with LGMD confirmed from a single study. Half of these patients presented in early childhood with an average age of presentation of 24 y.[3,15]. In a study from western India 26.4% patients were detected to have a sarcoglycanopathy (SG), out of this delta-SG was common and beta-SG was the rarest[16]. The most recognized ethnicity based founder mutation in India based on LGMD diagnosis has been found in the Agarwal community from Northern India, with a mutation in exon 22 of the *CAPN3* gene leading to LGMD2A[17]. Two (2) founder mutations were identified in this gene, a missense (c.2338G>C;p.D780H) and a splice-site (c.2099-1G>T) mutation, on 2 different haplotype backgrounds. Diagnosis of the patients revealed that they were: either homozygous for anyone or heterozygous for both of these mutations[17].

LGMDR4 β-sarcoglycan-related is considered to be most common in the Iranian population. In a study of Chinese patients in which 35 patients had a genetic mutation identified, seven (7) were diagnosed to have a SGCB mutation. In a study on eleven (11) families from Southern Indiana Amish population a common missense mutation (T151R) in the SGCB gene on chromosome 4q12 caused a mild type of LGMDR4. They also reported a second missense mutation (R91C) in the Amish family[18]. There are no large-scale genetic studies of sarcoglycanopathies carried out in India. Systematic review by Audhya et al. (2022) revealed that patients with LGMDR3-6 showed early onset of loss of ambulation (LOA) and faster progression as compared to late childhood or adult-onset of the disease. Large registries such a s Global FKRP Registry or clinical observations of patients over a period of time would be useful for characterizing basis for the natural history of LGDMR. It can also help to design clinical trials for this purpose[19].

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In all the cases there was non-consanguinity. A very identical phenotype of delayed motor milestones, followed by a period of stable walking leading to a rapid worsening of lower limb weakness after 10y of age was noticed. Age for loss of ambulation is around 12y. During last follow up there was no cardiac or respiratory involvement. There are no swallowing difficulties or specific clues to differentiate from other LGMD subtypes.

CONCLUSION

Next-generation sequencing (NGS) based gene panels can be an effective tool for the diagnosis of muscular dystrophies in children and others with atypical symptoms. Molecular diagnostics helps to offer families a chance to contribute to further research, take part in clinical trials and patient registries. Currently there are a few or no established patient registries or nationwide genetic databases in India to help accurate diagnosis for neuromuscular conditions in India. There are a few centers which offer immunostaining and have expertise in reporting muscle biopsies. Identifying ethnicity or region based common genetic mutations and conditions can cut both cost and time to reach a confirmed diagnosis. The present study on Sathwara community of Gujarat, India revealed the presence of LGMDR4 in the third-generation children. The homology model of the wild type SGCB protein revealed the 23% similarity with 4C79-A template. The homozygous substitution p.Gly228Glu was due to missense mutation Chr 4:52894204C>T; c.683G>A, which alters a wild type SGCB protein residue structure marked in homology modelling and superimposition study. Further research and histological data would be useful for understanding the effect of this mutation. Results of this study opens direction for future research like screening of population of Sathwara and other minor communities in India and correlate it with Iranian population where LGMDR4 is considered to be most common LGMD. Further studies involving larger population should be carried out for a more scientific approach for the diagnosis and ultimately cost-effective management of this disorder.

Conflict of Interest: The authors declare no conflict of interest.

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