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Congenital Hypotonia: Cracking a SAGA of consanguineous kindred harboring four genetic variants

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Abstract

Background: We aimed to determine the molecular and biochemical basis of an extended highly consanguineous family with multiple children presenting severe congenital hypotonia.

Methods: Clinical investigations, homozygosity mapping, linkage analyses and whole exome sequencing, were performed. mRNA and protein levels were determined. Population screening was followed.

Results: We have identified a novel nonsense variant in *NGLY1* in two affected siblings, and compound heterozygosity for three novel *RYR1* variants in two affected sisters from another nuclear family within the broad pedigree. Population screening revealed a high prevalence of carriers for both diseases. The genetic variants were proven to be pathogenic, as demonstrated by western blot analyses.

Conclusions: Revealing the genetic diagnosis enabled us to provide credible genetic counselling and pre-natal diagnosis to the extended family and genetic screening for this high-risk population. Whole exome/genome sequencing should be the first tier tool for accurate determination of the genetic basis of congenital

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Abbreviations: AR, autosomal recessive; CDDG, congenital disorder of deglycosylation; CH, Congenital hypotonia; MB, muscle biopsies; MMD, multiminicore disease; WES/WGS, whole exome/genome sequencing.

Limor Kalfon and Meirav Baydany contributed equally to this paper.

This paper is dedicated to the memory of Yishay Shoval PhD.

hypotonia. Two different genetic disorders within a large consanguineous pedigree should be always considered.

1 | INTRODUCTION

Congenital Hypotonia (CH) is a common symptom that encompasses clinically and genetically heterogeneous neuromuscular disorders including congenital myopathies, muscular dystrophies, neuropathies and inborn errors of metabolism (Sparks, 2015) as well as benign and transient conditions.

The diagnostic evaluation of CH includes obtaining the family history, laboratory studies, physical examination and detailed neurologic examination for determining the precise site of neurological abnormality. Thus, revealing the underlying cause of CH is a "diagnostic odyssey" requiring multiple clinical evaluations by multidisciplinary specialists, combined with imaging, biochemical, metabolic and genetic studies (Thevenon et al., 2016).

Of late, the use of whole exome/genome sequencing (WES/WGS) has become of paramount importance in defining the underlying molecular basis of rare genetic disorders when comprehensive clinical studies could not lead to definitive diagnosis (Cordoba et al., 2018).

We describe five patients originating from a highly consanguineous Druze kindred. All presented with fatal CH and underwent a "diagnostic odyssey". WES uncovered two rare genetically distinct disorders: fatal central core myopathy (MMD) (OMIM 11700) and NGLY1 deficiency, a congenital disorder of deglycosylation (OMIM 615273).

2 | FINDINGS

We present five patients originating from a large consanguineous kindred (Figure 1a). All patients presented with severe CH. We hypothesized that the patients had the same autosomal recessive (AR) disorder.

The main clinical and laboratory tests are depicted in Table 1, Figure 1 and Figure S1. Full case stories are detailed in the supplementary materials. In short, four females and one male were included in the study. Age at last follow-up ranged between 0.5 and 12 years. All patients were born full term with Apgar score ranging between 7/8 and 8/9 at first and fifth minute postpartum, normal weight and head circumference. All presented from birth with hypotonia and weakness, myopathic face and hypo- or a-reflexia. Due to severe hypotonia and reduced neonatal reflexes, all were fed by nasogastric tube followed by gastrostomy during the first year of life. None acquired sitting position. All patients were intubated during the first months of life and placed on mechanical ventilation due to recurrent pneumonia and nonreversible respiratory failure. Four of the five died between ages 0.5 and 4 year. The cause of death was respiratory failure due to aspiration pneumonia.

Patients VIII-1 and VIII-2 (Subfamily-I) had social communication expressed by social eye contact and social smile. Patients of subfamily II (VI-11, VI-13, VI-14) never acquired social smile or social communication. Additionally, in subfamily-II, symptoms indicated progressive CNS involvement developed with time including irritability, seizures and involuntary movements. VI-11 and VI-14 had hypo-alacrima, a very rare clinical symptom.

2.1 | Pathology and molecular genetic studies

The histopathological findings of a muscle biopsy (MB) in patients VIII-1 and VIII-2, subfamily-I (Figure 1b) suggested the rare AR myopathy, MMD, known at that time to be associated with variations in the genes Selenoprotein N1 (*SEPN1*, MIM#606210) and Ryanodine receptor 1 (*RYR1*, MIM#180901). The MB of patient VI-13 from subfamily-II revealed neurogenic atrophy. Linkage to these genes was ruled out in subfamily-II. Homozygosity mapping including four patients from both subfamilies did not reveal a shared homozygous region between the two subfamilies. In fact, two homozygous regions were found in siblings of subfamily-II, VI-13 and VI-14 (chr3:19837243–40110750, chr8:4571335–18954398), not shared by subfamily-I. However, at that time, no gene included in this interval matched the peculiar phenotype of subfamily II that included congenital hypotonia followed by severe intellectual disability and regression, seizures, peripheral neuropathy, alacrima and hypertransaminsemia. Thus, when WES became available, a trio WES was performed in each subfamily. WES in subfamily-I (patient VIII-2 and her parents) did not reveal any homozygous variants. Three unreported variations in *RYR1* gene (NM_001042723.1) were identified: a missense variant c.9623C>T (p. Pro3208Leu) at exon 65,
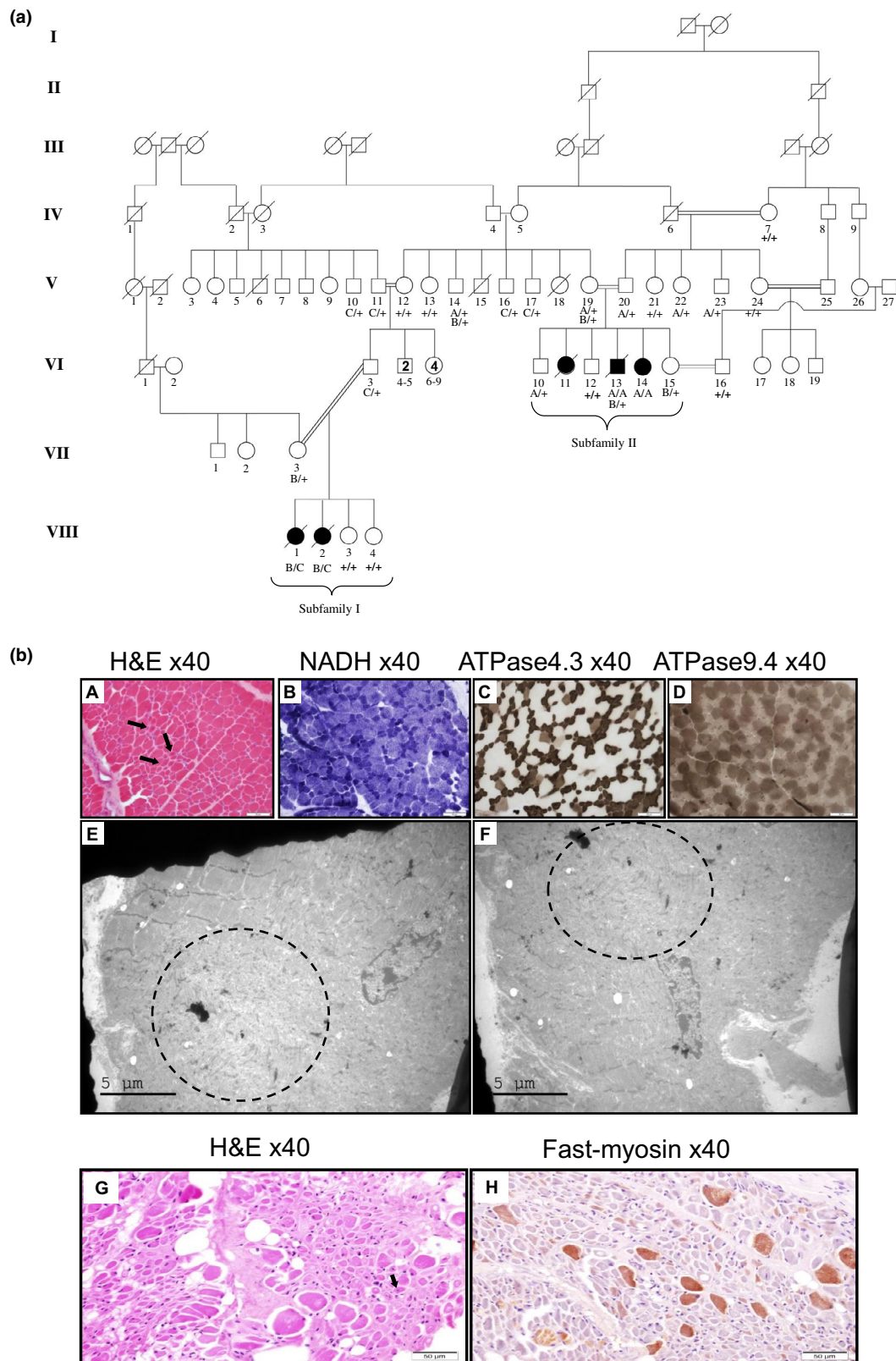
FIGURE 1 (a) Pedigree of the extended family. Filled symbols indicate affected members. Arrows indicate the probands. Circles-females; squares-males; Slant-deceased. Genotype designation: A: Ngly1 c.1294G>T, B: RYR1 c.9623C>T, C: RYR1 c.12373-12395del and c.1366G>A, + WT allele to A, B, C. (b) MB findings. VIII-1 first biopsy (a–d) display fibre-type size disproportion, whilst second biopsy EM (e, f) reveal unstructured large minicores (encircled). VIII-2 (g, h): paraffin sections display a predominance of small type 1 myofibres and scattered hypertrophic type 2 myofibres. Few internal nuclei are evident in both cases (black arrows in a and g)

TABLE 1 Main clinical features and laboratory studies in two patients with *RYR1* and three patients with *NGLY1* genetic variants

No. in pedigree	VIII−1	VIII−2	VI−11	VI−13	VI−14
	Subfamily – I (*RYR*)		Subfamily – II (*NGLY1*)		
SEX (F/M)	F	F	F	M	F
Parents Consanguinity	+	+	+	+	+
Pregnancy (reduced fetal movement)	−	−	−	−	+
Delivery: spontaneous (S)/cesarean section (CS)	S	S	S	S	CS
APGAR score	7/8	7/8	NA	8/9	8/9
Birth weight (grams)	3058	3000	NA	3500	2930
Head circumference at birth	34	34	NA	36	34
Progressive microcephaly	−	−	NA	+	+
Congenital hypotonia	+	+	+	+	+
myopathic facies	+	+	+	+	+
Hyperreflexia/Hyporeflexia	−/+	−/+	−/+	+/+	+/+
Social eye contact, smile and alertness	+	+	−	+ Lost later	−
Irritability	−	−	NA	+	+
ABR abnormality	−	−	−	−	+
Feeding difficulty and aspiration	+	+	+	+	+
Hypolacrima/alacrima	−	NA	+	NA	+
Respiratory failure (age months)	+	+	+	+	+
Scoliosis (progressive)	−	−	−	−	+
Sitting unassisted	−	−	−	−	−
Seizures	−	−	−	+	+
Global developmental delay	+	+	+	+	+
Movement disorder	−	−	NA	+	+
Neurodegenerative progression	−	−	−	+	+
EEG abnormal	−	−	NA	+	+
EMG – myopathic/periph. neuropathy	+/−	NA	NA	−/+	−/+
Brain MRI	NA	NA	NA	atrophy	NA
Muscle histopathology	myopathy	myopathy	NA	Neurogenic atrophy	Neurogenic atrophy
Elevated plasma lactate	+	−	NA	+	+
Urinary 3-methylgutaconic aciduria	+	−	−	−	−
Elevated CPK	−	−	NA	+	+
Elevated transaminase and GGT	−	−	NA	+	+
Deceased (age in years)	2	4.5	0.5	3	Alive 12

inherited from the mother (Supplementary Figure 1A), and two variants including a deletion of 22 nucleotides c.12374_12395del (p.E4125_Q4132delfsX15) (Figure S1b) in exon 90 resulting in a premature stop codon, and c.1366G>A (p. Glu456Lys) (Figure S1c) at exon 13, inherited from the father. Sanger sequencing confirmed these findings. Population screening in 100 healthy individuals from this village revealed a prevalence of 1:33 for c.9623C>T, 1:100 for c.1366G>A and 1:100 for c.12374_12395del. Bioinformatic tools predicted the three variations to be disease causing. *RYR1* encodes a 565 KDa protein. This protein is found primarily in skeletal muscle and functions as a calcium release channel in the SR. Western blot (WB) analysis of skeletal muscle tissue from the patient VIII-2 showed 40% reduction of RYR1 expression compared to the control sample. Desmin immunoreactivity was used as a muscle-specific internal control. (Figure S1d,e).

WES was also performed in subfamily II (patient VI-14 and her parents). A novel pathogenic variant in *NGLY1*, (MIM# 610661), was determined (p. Glu432*, c.1294G>T) (NM_018297) (rs992161646). Sanger sequencing confirmed homozygosity of this variant in all affected patients (Figure S2a). Segregation was compatible with AR inheritance. A survey of 100 healthy individuals from the same village revealed a carrier frequency of 1:25. To further verify the pathogenicity of the genetic variant (GV), *NGLY1* mRNA expression level was studied compared to healthy control. A reduction of 90% and 75% of mRNA extracted from fibroblasts and leukocytes respectively, derived from patient VI-14 were found (Figure S2b). WB analysis determined the entire abolishment of NGLY1 protein in patient's cells compared to healthy controls (Figure S2c).

3 | DISCUSSION

We describe five patients from one consanguineous kindred, who presented with severe CH. Despite extensive laboratory and metabolic studies including invasive procedures, referred to as a "diagnostic odyssey," diagnosis was not reached. However, as WES became available, we could "crack" the enigma (Cordoba et al., 2018; Thevenon et al., 2016) WES revealed in subfamily-I three genetic variants in *RYR1* causing progressive fatal myopathy, and a homozygous variant in *NGLY1* in subfamily-II, causing congenital disorder of deglycosylation (NGLY1-CDDG). Arriving at the molecular diagnoses of the two unrelated genetic disorders in this kindred allowed us to perform carrier screening among the village population, identify couples at risk, provide genetic counselling, and prenatal testing for interested couples and administer optimal management to patients and their families.

In retrospect, the possibility of more than one recessive disorder existing in the present consanguineous kindred was not initially expected. However, in consanguineous families, multiple genetic variants may originate from common founders between spouses (Hamamy et al., 2011; Lal et al., 2016; Shalev et al., 2017) increasing the possibility of co-occurrence of two or more rare disorders even if similar phenotypes exist within a single kindred.

The implementation of WES/WGS in the paediatric clinical practice for diagnosis of rare and heterogeneous genetic disorders shows a diagnostic yield between 25 and 40%, making it the most powerful individual diagnostic test for such condition, especially in a context of no clear clinical diagnosis (Cordoba et al., 2018) The clinical use of WES highlights the necessity of close collaborations between physicians, molecular geneticists, bioinformaticians and researchers for accurate data interpretation (Cordoba et al., 2018; Thevenon et al., 2016) and for re-evaluations every one to two years when the diagnosis is not determined since new genes and novel genotype/phenotype correlations are continuously reported.

In the studied kindred, WES established the finding of two unrelated rare genetic disorders, NGLY1 deficiency and the RYR1 myopathy.

NGLY1 is a cytosolic peptide: N-glycanase, which is the first reported de-N-glycosylating enzyme acts on N-glycoproteins in mammals, generating free, unconjugated N-glycans and deglycosylated peptides. This enzyme is involved in the quality control system for the newly synthesized glycoproteins in the endoplasmic reticulum (ER). In this system, misfolded (glyco) proteins are retro-translocated to the cytosol, where the 26S proteasomes play a central role in degrading the proteins: a process referred to as ER-associated degradation or ERAD in short (Bhattacharya and Qi, 2019)

To-date, about 50 patients with NGLY1-CDDG were reported (Enns et al., 2014; Lam et al., 2017), however, fewer than 100 patients were diagnosed worldwide (Lam et al.), mostly using WES. NGLY1-deficiency patients typically display a clinical tetrad of intellectual disability in the mild-to-profound range, a complex of involuntary movements, hypo- or alacrima, abnormal liver function and markedly elevated α-fetoprotein, the last two resolved in most cases with time. Seizures develop in about half of the patients. Other clinical abnormalities may include feeding difficulties, peripheral and auditory neuropathy and progressive scoliosis (Enns et al., 2014; Lam et al., 2017).

Congenital myopathies are clinically, and genetically heterogeneous conditions characterized by muscle weakness and multiple structural abnormalities in muscle biopsy samples (Jungbluth et al., 2018; Ravenscroft et al., 2017).

Patient VIII-1 presented with severe congenital hypotonia and was seemingly intellectually intact. Two MBs were performed. In the second, the ultrastructural findings were compatible with MMD, which is a recessive congenital myopathy characterized by the presence of small cores or areas lacking oxidative enzymes in skeletal muscle fibres. Using WES, we uncovered three variants in the skeletal muscle ryanodine receptor *RYR1*. This gene encodes the principal sarcoplasmic reticulum calcium release channel with a crucial role in excitation-contraction coupling (Bertini et al., 2011; Jungbluth, 2007; Jungbluth et al., 2018).

To date, congenital myopathies have been attributed to pathogenic variants in over 20 genes, which encode proteins implicated in skeletal muscle Ca^{2+} homeostasis, excitation–contraction coupling, thin–thick filament assembly and interactions, and other mechanisms (Jungbluth et al., 2018) *RYR1* variants are the most frequent

genetic cause. WES has improved variant detection and enabled the identification of novel genetic backgrounds (Ravenscroft et al., 2017; Waldrop et al., 2019) Specific genetic panels for rapid genetic evaluation of CH are available for paediatricians, paediatric neurologists, PICU and NICU physicians and should be utilized to avoid multiple unnecessary testing, to provide a clear and rapid diagnosis for the treating staff and parents, and to reduce costs and hospitalization.

CONFLICT OF INTEREST
The authors have stated that they had no interests which might be perceived as posing a conflict or bias.

DATA AVAILABILITY STATEMENT
The data are available from the corresponding author, upon reasonable request.

ETHICAL STATEMENT
The Israeli Supreme Helsinki committee approved the study. All study participants and parents of minors signed informed consents.

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