CLINICAL REPORT

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Multi-Systemic Involvement in NGLY1-Related Disorder Caused by Two Novel Mutations

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NGLY1-related disorder is a newly described autosomal recessive condition characterized by neurological, hepatic, ophthalmological findings and associated with dysmorphic features, constipation and scoliosis. It is caused by mutations in *NGLY1*, which encodes an enzyme, N-glycanase 1, involved in deglycosylation of glycoproteins, an essential step in the endoplasmic reticulum–associated degradation (ERAD) pathway. The disorder has been described in eight patients. We investigated the molecular basis and phenotype of NGLY1-related disorder in an additional patient. The proband is a 14-year-old who presented in early infancy with profound hypotonia and elevated transaminases. Liver biopsy showed lipid accumulation with dilated endoplasmic reticulum. He exhibited global developmental delay, acquired microcephaly, seizures, involuntary body movements, muscle atrophy, absent reflexes, and poor growth. He had multiple procedures for lacrimal duct stenosis and strabismus and had intractable blepharitis. He had severe osteopenia and persistent hypocholesterolemia. Whole exome sequencing revealed two novel variants in *NGLY1*: a truncating mutation, c.347C > G (p.S116X), and a splicing mutation, c.881 + 5G (p.IVS5 + 5G>T), predicted to abolish the splice donor site of exon 5. This study, along with previously reported cases, suggests that mutations in *NGLY1* cause a recognizable phenotype and targeted sequencing should be considered in patients with typical presentation. This study expands the molecular spectrum of NGLY1-related condition and suggests that osteopenia and hypocholesterolemia may be part of the phenotype.

Key words: NGLY1; endoplasmic reticulum-associated degradation; liver disease; hypotonia; seizures

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INTRODUCTION

Mutations in *NGLY1*, encoding the enzyme N-glycanase 1, were recently found to cause a multi-systemic recognizable phenotype. This condition is characterized by neurological abnormalities including global developmental delay, severe hypotonia, movement disorder, seizures or abnormal EEG, acquired microcephaly, diminished reflexes and nerve conduction abnormalities. Hepatic findings include neonatal jaundice, elevated liver enzymes, fibrosis, and intrahepatic cytoplasmic inclusions. Ophthalmological aberrations including alacrima/hypolacrima, strabismus, chalazion, and apraxia were documented in most patients. The disorder is associated with dysmorphic features, constipation and scoliosis [Need et al., 2012 Enns et al., 2014].

The enzyme N-glycanase 1 removes the N-glycan species from N-linked glycoproteins and permits degradation of misfolded proteins after translocation from the ER to the cytosol [Park et al., 2001]. This function is an essential step in the endoplasmic reticulum–associated degradation (ERAD) pathway [Park et al., 2001; Kamiya et al., 2012].

NGLY1 deficiency was reported in 2012 in a whole exome sequencing (WES) study for undiagnosed genetic conditions that identified two loss-of-function mutations in a patient who was thought to have a congenital disorder of glycosylation (CDG), but whose transferrin and N-glycan analyses were normal [Need et al., 2012]. Clinical features included developmental delay (DD), multifocal epilepsy, involuntary movements, abnormal liver function with elevated transaminases, and absent tears. Liver biopsy showed "amorphous unidentified substance throughout the cytoplasm, suggestive of stored material in the liver cells," but lysosomal enzymes had been normal [Need et al., 2012]. More recently, Enns et al. [2014] reported seven additional patients with similar neurological, hepatic, and ophthalmologic findings (Table I).

Five unique mutations were observed in the reported patients. Two mutations were frameshifts: c.1891del (p.Q631fs) and c.1370dupG (p.R458fs). The c.1891del mutation was only seen in

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TABLE I. Clinical Findings in Patients With NGLY1-Related Disorder

Clinical finding	Present Case	Previously reported cases [Enns et al., 2014; Caglayan et al., 2014] (n = 10)	(%) of total
Neurological			
Global developmental delay	+	10/10	100
Movement disorder	+	9/10	91
Hypotonia	+	10/10	100
EEG abnormalities	+	8/10	82
Seizures	+	5/10	55
Microcephaly	+	6/10	64
Abnormal brain imaging	−	6/10	55
Abnormal nerve conduction study	+	5/5	100
Reduced tendon reflexes	+	8/10	82
Hepatic			
Neonatal jaundice	+	4/10	45
Elevated liver transaminases	+	7/9	80
Elevated AFP	−	3/7	38
Hepatic intracytoplasmic storage material or vacuolization	+[#]	5/6	86
Liver fibrosis	+	2/8	33
Ophthalmological			
Hypolacrima or alacrima	+	9/10	91
Blephiritis/Chalazions	+	4/8	56
Ocular apraxia	−	4/10	36
Strabismus	+	5/9	60
Skeletal			
Small hands/feet	−	4/10	36
Scoliosis	+	5/10	55
Osteoporosis	+	1/2	[$]
IUGR	−	5/10	45
Dysmorphic features	+*	5/10	55
Hearing abnormalities	−	2/7	25
Constipation	+	7/8	89

(+) indicates presence of the clinical finding and (−) indicates its absence. ([#]) the proband's liver biopsy revealed abundant intracytoplasmic neutral lipid and dilated endoplasmic reticulum. (*) myopathic face, bilateral ptosis, hypertelorism and wide mouth. ([$]) no percentage was assigned because of limited data.

the initial patient who was a compound heterozygote with the mutation c.1205_1207del (p.402del) causing an in-frame deletion of 3 base pairs and introducing a stop codon [Need et al., 2012]. The c.1370dupG mutation was seen in a homozygous state in one patient who had a history of consanguinity. The other two mutations introduced stop codons: c.1201A > T (p.R401X) and c.1570C > T (p.R524X). The mutation p.R401X was the most common,observed in 11 of 16 reported alleles. In this study, we present the clinical and molecular findings of an additional patient with novel *NGLY1* mutations.

MATERIALS AND METHODS

Clinical Report

The patient is a 14-year-old Caucasian male who has been followed by several subspecialties since early infancy. He was born at full term after a pregnancy complicated by a vanishing twin. Birth weight was 2722 g (10th centile). Birth length and OFC are not recorded, but at admission at 6 days of life, his length was 51 cm (75th centile), and OFC was 35.2 cm (70th centile). He exhibited early hypotonia and poor feeding and presented at four days with dehydration, jaundice, and elevated alkaline phosphatase of 293 IU and GGT of 802 IU (70–295). At the time, ALT was 51 (6–50 IU/L) and AST 56 (10–60 IU/L). At age 2 weeks, ALT was 201 and AST 236. At 10 weeks, ALT was 200 and AST 236. Liver biopsy at age 6 months showed cirrhosis with diffuse macrosteatosis. Electron microscopy revealed abundant intracytoplasmic neutral lipid and dilated endoplasmic reticulum (ER), but no inclusions were seen. Repeat liver biopsy at 1 year showed moderate cirrhosis with septal chronic inflammation and mild fatty changes. Muscle biopsy was performed at age 1 year due to concern for mitochondrial conditions and showed minor variation in muscle fiber size, but no evidence of metabolic diseases or mitochondrial changes. Histochemical stains of mitochondrial complexes, phosphorylase, and hydrolytic enzymes were unremarkable. Enzymatic activity of the mitochondrial complexes was not performed.

The patient has had global developmental delay since birth and remains non-ambulatory, non-verbal and communicated using a device. He had absent reflexes as an infant and at 4 years an EMG

showed sensory motor polyneuropathy, predominately axonal. Repeat study at age 8 showed progression of the axonal neuropathy. EEG showed epileptiform changes in the neonatal period, but he did not have overt seizures until age 10, when he had eyelid fluttering, eye deviation, and perioral cyanosis. EEG showed generalized electrodecremental patterns representing subclinical generalized seizures, very frequent multifocal and generalized spike and polyspikes, lack of normal posterior dominant rhythm, and a reverse anterior to posterior gradient during wakefulness. He requires levetiracetam to control his seizures. The proband has had abnormal movements since age 7 years, including chorea and myoclonus in his arms and hands.

He developed marked scoliosis, underwent a spinal fusion at age 11 and required a second repair at 14 years. The proband had a femur fracture at 11 years and was found to have osteopenia. DEXA scan showed a Z score of -3.9 in the left hip and -4.0 in the right hip. He has been treated with pamidronate infusions every 3 months to improve his bone density and reduce risk for fractures.

The initial ophthalmologic finding at 4 months of age was left gaze preference. The patient had normal fundoscopic examinations and normal visual evoked potentials. At 2 years, he had large magnitude infantile esotropia and bilateral epiphora with mattering consistent with congenital dacryostenosis. Shortly thereafter he underwent nasolacrimal duct probing with incision of the punctum and ballooning. This was complicated by chronic dacryocystitis, which required stent placement at age 30 months. At age 3 years, the dacryocystitis recurred and the nasolacrimal ducts were ballooned. The patient developed right upper trichiasis requiring correction. Throughout childhood, he has had recurrent redness and mattering of the eyes. He was diagnosed with chronic severe blepharitis and has been treated with Lacri-lube (artificial tears) and topical antibiotics. At his most recent ophthalmological visit at age 14, he was found to have a chronic inferior exposure keratitis and inferior corneal thickening due to blink apraxia.

Although our patient had normal growth parameters at birth, he later developed short stature and acquired microcephaly. At 14 months, his weight was below the first centile, his length was at the 25th centile, and his OFC was at the 10th centile. By 33 months, his weight, length, and OFC were all below the first centile.

On his physical examination at age 14 years, his height was 137 cm (-3.5 SD), weight was 25.8 kg (-5.1 SD) and OFC was 50.3 cm (-3.9 SD). The proband was alert, smiled, attempted to vocalize with interaction, and followed some commands. He was wheelchair bound. Head was asymmetric and scalp showed seborrheic dermatitis. Face was myopathic with bilateral ptosis. He had hypertelorism, a wide mouth, high arched palate, and gingival hypertrophy (Fig. 1A). Lungs were coarse to auscultation. Abdomen was flat without hepatosplenomegaly. Musculoskeletal exam showed severe muscle atrophy (Fig. 1B), flexion contractures in his hands and 2–3 cutaneous toe syndactyly. There was severe scoliosis with well-healed scars from spinal fusion. Skin showed erythematous perifollicular hyperkeratotic papules on all extremities (Fig. 1B). His neurological exam revealed diffuse severe hypotonia, dystonia and areflexia in the lower extremities, and choreoathetoid movements of the upper extremities. Right bicep and tricep reflexes were 2+ and left bicep and tricep were 1 +. He responded to touch in all four extremities.

The patient had an extensive evaluation including normal results for the following studies: karyotype, Prader-Willi methylation, acylcarnitine profile, transferrin isoelectric focusing, urine organic acids, very long chain fatty acids, lysosomal enzyme panel, fatty acid oxidation enzymes activity in fibroblasts, brain MRI, chromosomal microarray, lactate and pyruvate panels, urine purine and pyrimidines, AFP, and sterol panel. Lab abnormalities included a low cholesterol level of 118 (normal 120–220 mg/dl) at age 7. This was repeated at age 13 and cholesterol was 102 (normal <199 mg/dl). Estradiol (E2) was undetectable at <10 pg/ml (normal: undetectable-16 pg/ml).

Molecular Analysis

Exome sequencing was performed by GeneDx (Gaithersburg, MD) using Agilent SureSelect XT2 All Exon V4 Kit and Illumina HiSeq 2000 100 bp paired-end reads. Sequence was aligned to the UCSC build hg19 reference sequence. Mean depth of coverage was 233× with quality threshold of 99.2%. GeneDx's XomeAnalyzer was used to evaluate sequence changes between the proband, parental samples and reference. Sanger sequencing was used for confirmation of reported mutations.

RESULTS

Sequencing Results

Clinical whole exome sequencing revealed two novel variants in *NGLY1* that are likely pathogenic. The first mutation is a maternally inherited truncating mutation, c.347C > G (p.S116X) in exon 3. The second is a paternally inherited variant, c.881 + 5G (p.IVS5 + 5G > T). This is predicted to abolish the splice donor site of exon 5. Neither change has been previously reported as a disease causing mutation or as a benign polymorphism, and neither was found in approximately 6500 individuals of European and African American ancestry in the NHLBI Exome Sequencing Project. The diagnosis of NGLY1-related disorder was established based on the predicted effect of the mutations on the *NGLY1* protein, their *trans* configuration, and the similarity of the clinical phenotype of our patient with the previously reported cases.

DISCUSSION

Mutations in *NGLY1* have recently been described in 8 patients and cause an inherited disorder of deglycosylation [Need et al., 2012; Freeze, 2013; Enns et al., 2014]. While this manuscript was in review, a paper was published describing two additional siblings with intellectual disability, neuromotor impairment, neuropathy, and corneal opacities caused by a novel homozygous frame-shift mutation, p.Asn511LysfsX51, in *NGLY1* [Caglayan et al., 2014]. This report describes an additional patient with NGLY1-related disorder and compares his findings with reported cases (Table I). Our patient showed similar symptoms and findings as prior patients, suggesting that mutations in *NGLY1* cause a recognizable phenotype and targeted sequencing should be considered in patients with the typical presentation.

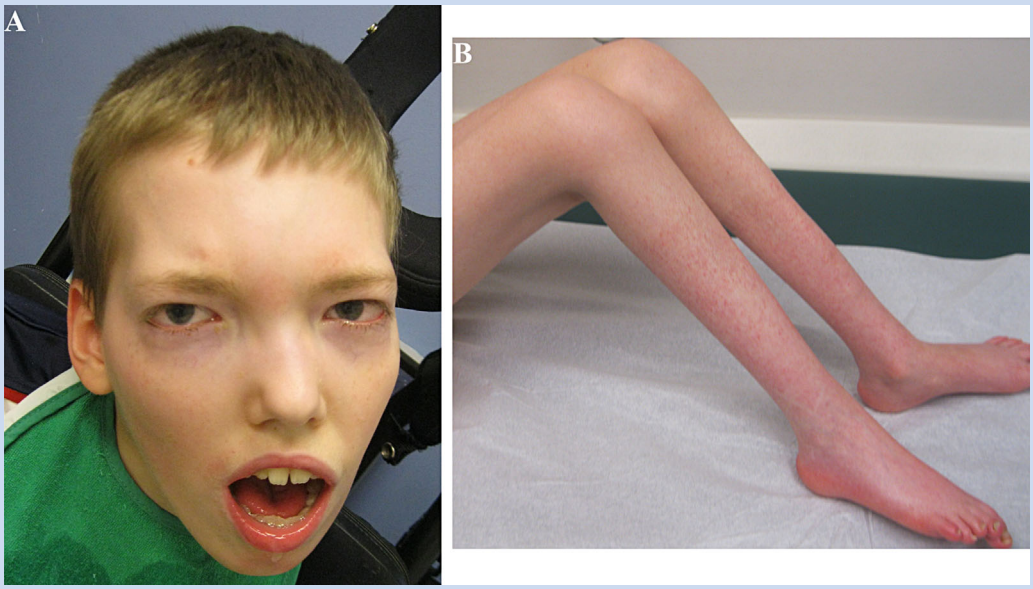

FIG. 1. Physical findings in the proband. (A) The proband had a myopathic face, bilateral ptosis, hypertelorism and a wide mouth. (B) Lower extremities showing muscle atrophy and erythematous perifollicular hyperkeratotic papules.

Similar to the patients described by Enns et al. [2014], our patient was thought to have a congenital disorder of glycosylation, but carbohydrate deficient transferrin patterns were normal. Due to the neurological presentation and liver dysfunction our patient was then thought to have Alpers–Huttenlocher, and was evaluated by Dr. Huttenlocher himself. However, he had symptoms from birth and his other clinical findings were not consistent with POLG-related disorders.

Our patient and another described by Caglayan et al. [2014] had fractures and osteopenia. There are several potential factors for osteopenia including immobility, hypotonia, muscle atrophy, peripheral neuropathy, and antiepileptic medications. However, skeletal manifestations are common findings among patients with the closely related congenital disorders of glycosylation [Coman et al., 2008] and an intrinsic effect of the mutated *NGLY1* protein cannot be excluded. It was hypothesized that the process of glycosylation is very important to proteins implicated in the development of cartilage and bone and in skeletal patterning pathways [Coman et al., 2008]. It is possible that impaired deglycosylation of these proteins plays a role in *NGLY1* deficiency.

The elevation of liver enzymes was first noted at age 14 days in our proband and was mild (ALT $<$ 201 IU and AST $<$ 236 IU). Complete normalization of AST was seen at age 8 and of ALT at age 13 years. The stable hepatic abnormality was not associated with hepatomegaly. Several patients with *NGLY1* mutations had a liver biopsy to evaluate for transaminitis. The almost universal finding of cytoplasmic storage material or vacuolization in hepatocytes seems characteristic for NGLY1-related disorder. It has been suggested that the stored material represents misfolded glycoproteins in the cytoplasm that failed to undergo further processing after their translocation from the ER [Enns et al., 2014]. The findings in our patient were different and consistent with abundant intracytoplasmic neutral lipid and dilated ER. It is possible, however, that these findings reflect different abnormal aspects of the same ERAD pathway.

Ophthalmologic findings in the previously reported patients include alacrima or hypolacrima leading to corneal scarring due to lack of lubrication, chalazion, and strabismus. Our patient had strabismus, lacrimal duct stenosis and chronic dacryocystitis status post multiple procedures to balloon and stent the ducts. Later on he developed blink apraxia and hypolacrima leading to corneal scarring.

Enns et al. [2014] reported low unconjugated estriol level (uE3) in second-trimester maternal screen in three patients negative for trisomy 18 and Smith–Lemli–Opitz syndrome. Interestingly, two of these patients died at ages 9 months and 5 years and had vacuolation of the adrenal cortex. The maternal serum screen in our patient was reported as normal. However, our proband had low cholesterol, which had not been reported in the previous cases. More research is needed to determine if hypocholesterolemia plays a role in this condition and whether it can be a biomarker for this disease.

The novel mutations in our patient expand the molecular spectrum in the NGLY1-related conditions. The p.S116X mutation is expected to cause loss of protein function either through truncation or nonsense-mediated mRNA decay. The IVS5 + 5G $>$ T variant is predicted to abolish the splice donor site for exon 5 and cause abnormal splicing. Approximately 10% of human pathogenic mutations are at the splice site, mostly located at the 5' donor and 3' acceptor sites [Lewandowska, 2013]. The effect of the IVS5 + 5 G $>$ T mutation is difficult to predict without experimental assays but it can cause exon skipping, form new exon/intron boundaries or activate cryptic exons [Lewandowska, 2013], leading to a nonfunctional incomplete protein. Clinically,

the effect of these mutations is similar to the severe phenotype observed in association with the recurrent truncating mutation, p.R401X. Sequencing of RNA from the patient or functional analysis of the *NGLY1* enzyme would support the pathogenicity of the variants, but were not performed.

In summary, we present an additional patient with NGLY1-related disorder with novel compound heterozygous mutations detected through whole exome sequencing. Our data suggest that osteopenia and hypocholesterolemia may be part of the phenotypic spectrum. NGLY1-related disorder should be considered in patients presenting with typical neurological impairment, ophthalmological abnormalities, and hepatic dysfunction. Molecular and clinical studies of additional patients are needed to better define the molecular and phenotypic spectra of the emerging phenotype.

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