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Encephalopathy caused by Novel Mutations in the CMP-Sialic Acid Transporter, *SLC35A1*

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Abstract

Transport of activated nucleotide-sugars into the Golgi is critical for proper glycosylation and mutations in these transporters cause a group of rare genetic disorders termed congenital disorders of glycosylation. We performed exome sequencing on an individual with a profound neurological presentation and identified rare compound heterozygous mutations, p.Thr156Arg and p.Glu196Lys, in the CMP-sialic acid transporter, *SLC35A1*. Patient primary fibroblasts and serum showed a considerable decrease in the amount of N- and O-glycans terminating in sialic acid. Direct measurement of CMP-sialic acid transport into the Golgi showed a substantial decrease in overall rate of transport. Here we report the identification of the third patient with CMP-sialic acid transporter deficiency, who presented with severe neurological phenotype, but without hematological abnormalities.

Keywords

Congenital disorders of glycosylation; *SLC35A1*; Golgi; sialic acid; nucleotide-sugar transporter; seizures

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INTRODUCTION

It is estimated that approximately 1–2% of all human genes encode for proteins involved in various aspects of glycosylation and more than a hundred-glycosylation related genetic disorders have been described [Freeze et al., 2015; Hennet and Cabalzar, 2015]. These disorders are collectively termed, congenital disorders of glycosylation (CDG) and categorized by the gene name with a CDG suffix [Freeze et al., 2014; Jaeken et al., 2009].

Mutations affecting the N-linked glycosylation pathway are the most common type with seventy distinct genes identified [Freeze et al., 2014; Scott et al., 2014]. The identification of affected individuals with CDG is often achieved by analyzing the glycosylation status of the abundant serum glycoprotein, transferrin (Tf) [Wada, 2016] using a variety of methods including isoelectric focusing (IEF), high performance liquid chromatography (HPLC) and electrospray ionization mass spectrometry (ESI-MS) to name a few [Lacey et al., 2001; Carchon et al., 2004; Parente et al., 2010]. We collectively refer to the testing of Tf by any of these or other methods as carbohydrate deficient transferrin (CDT) testing.

CDG defects involving the N-linked pathway are divided into two types, type I and type II. CDG-I involves defects in synthesis or transfer a mature lipid linked oligosaccharide (LLO) from its dolichol carrier onto the recipient proteins [Freeze et al., 2014; Buczkowska et al., 2015]. CDG-II involves processing of the protein bound oligosaccharide, which mostly occurs in the Golgi. Dysfunctional Golgi-associated multifunctional protein complexes, alterations in Golgi trafficking, dynamics, homeostasis, pH or ion balance can all lead to CDG-II defects [Freeze et al., 2014]. Another way to directly affect Golgi-dependent glycosylation is by limiting the availability of high energy donor substrates, which are synthesized in the cytoplasm and must be transported into the Golgi to access glycosyltransferases. Some individuals have pathogenic mutations in these nucleotide-sugar transporters that limit the amount of donor substrate co-located with the glycosyltransferases [Lübke et al., 2001; Lühn et al., 2001; Martinez-Duncker et al., 2005; Hiraoka et al., 2007; Ng et al, 2013; Edvardson et al., 2013; Mohamed et al., 2013].

Due to the extensive utilization of each nucleotide-sugar by multiple glycosylation pathways, individuals with mutations in these transporters often have biochemical defects involving multiple glycosylation pathways [Xia et al., 2013]. Genetic disorders involving nucleotide-sugar transporters include SLC35A1-CDG (CMP-sialic acid) [MIM 603585], SLC35A2-CDG (UDP-galactose) [MIM 300896], SLC35A3-CDG (UDP-N-Acetylglucosamine) [MIM 615553], SLC35C1-CDG (GDP-fucose) [MIM 266265] and SLC35D1 (UDP-glucuronic acid / UDP- N-Acetylgalactosamine) that causes Schneckenbecken dysplasia [MIM 269250]. Here we report the identification of the third patient with CMP-sialic acid transporter deficiency. We present data to expand the molecular, clinical and biochemical knowledge on SLC35A1-CDG and present a novel cell-based, quantitative assay.

METHODS AND MATERIALS

Patient samples

Consent was provided for receiving and using biological sample for family 374 under an approved Sanford Burnham Prebys Medical Discovery institute IRB protocol. Whole blood was drawn from both parents, the affected female and genomic DNA purified using a Qiagen Puregene blood core kit B (Qiagen, Valencia, CA, USA). Fibroblast cultures were established from a skin biopsy using a standard protocol.

Exome and Sanger sequencing

Exome sequence was performed as previously described [Simon et al., 2017]. Polymerase chain reaction (PCR) using genomic DNA from family-374 was used to amplify exons 4 and 6 of *SLC35A1* (NM_006416.4). PCR conditions and primers available upon request.

SLC35A1 Transport assay

Primary fibroblast or Chinese Hamster Ovary (CHO) cells were grown to 80% confluence and permeabilized using hypotonic conditions [Kim et al., 2001]. Permeablization efficiency was determined by trypan blue staining. CMP-sialic acid transport was carried out as described with the exception that [H^3] CMP-sialic acid (American Radiolabeled Chemicals, Inc Saint Louis, MO) was used as the nucleotide-sugar substrate [Kim et al., 2001].

Flow cytometry

Primary fibroblast or Chinese Hamster Ovary (CHO) cells were grown to 80% confluence and detached using phosphate buffered saline (PBS) supplemented with 5mM EDTA. Cells were collected and thoroughly washed to remove the residual EDTA then fixed using PBS containing 2% PFA with 2% sucrose for 15 minutes at room temperature. Cells were again washed with PBS followed by incubation for one hour in FACS blocking buffer, 3% IgG free bovine serum albumin (BSA) in PBS. Cells were then incubated for thirty minutes in blocking buffer with 0.1ug/mL of FITC labeled PNA lectin (EY Labs, Foster City, CA) followed by three washes with blocking buffer to remove unbound lectin.

RESULTS

Clinical Summary

CDG-374 is a twelve-year-old female of German ancestry born to healthy non-consanguineous parents. She presented early in life with generalized muscular hypotonia and developed seizures at four months of age with orofacial tics in clusters of 5–30 seizures (Table I). Her seizure type at present involves versive seizures (unnatural, sustained turning of the eyes or head to one side) to the right. She initially responded to a combination of valproate and topiramate and currently is treated with topiramate and lamotrigine. She responded partly and continues to show clusters of three versive seizures to the right every 3–6 months.

Brain magnetic resonance imaging (MRI) showed no gross abnormalities, although there was a small left parietal arachnoidal cyst noted at the age of eleven months.

Electroencephalogram (EEG) was normal at onset, but showed slowing over the posterior areas and generalized as well as frontal spikes and polyspikes in the following years.

She has a severe cognitive deficit and the last neurocognitive testing demonstrated an age equivalent to three to four years, with an IQ level below 55. She communicates with sounds, or single words (~50–100), articulation is difficult to understand due to dysarthria. She has mild ataxic - dyskinetic movements. In addition, she displays autistic features with behavioral problems. Nystagmus is also noted. She has never had coagulation abnormalities nor issues with infections, but has developed thoracolumbar scoliosis (Table I). Currently, she receives physical and speech therapies.

All testing for inborn errors in metabolism were normal, until, at one year of age she showed a serum Tf type 2 isoelectric focusing pattern. Follow up analysis of serum TF by electrospray ionization mass spectrometry (ESI-MS) confirmed a type 2 pattern with loss of both one and two sialic acids.

Molecular and biochemical analysis

Exome sequencing of the proband identified two rare compound heterozygous variants, c.467C>G (p.Thr156Arg) and c.586G>A (p.Glu196Lys), in the CMP-sialic acid transporter *SLC35A1* (Table I). We searched the Genome Aggregation Database (gnomAD) database (http://gnomad.broadinstitute.org/) [v2 accessed 26.4.2017] of 126,136 exomes and 15,496 genomes and found a single heterozygous carrier c.467C>G (p.Thr156Arg) (123,066 individuals) while the c.586G>A (p.Glu196Lys) was not present. Segregation analysis confirmed each mutant allele was inherited from a separate parent.

We utilized three separate *in silico* analytical programs to predict potential pathogenicity for each variant. Polyphen2 predicted both variants to be damaging, while SIFT scored the p.Thr156Arg as deleterious and the p.Glu196Lys as tolerated. Our experience is that Combined Annotation Dependent Depletion (CADD) scoring is a better tool for predicting the deleteriousness of a given variant. The CADD score for p.Thr156Arg was 25.8 and for p.Glu196Lys it was 27.7. In comparison, the previously reported pathogenic missense mutation p.Gln101His scored a 27.4. This would place all three variants within the top 0.5% of predicted deleterious mutations in the human genome [Kircher et al., 2014].

SLC35A1 is predicted to have ten transmembrane domains (TMD) with the p.Thr156Arg localized to TMD-5 and p.Glu196Lys to TMD-6. The p.Gln101His reported by Mohamed et al occurs within TMD-3. We speculate that these mutations likely disrupt how the transporter is oriented within the membrane. However, it is known that sugar nucleotide transporters can form hetero or homo-dimers with other transporters and we cannot exclude the possibility that either mutation disrupts these interactions.

We prioritized *SLC35A1* as a causal candidate for further biochemical characterization based on CDG-374 having a type 2 pattern showing loss of both one and two sialic acids from serum Tf and the presence of these rare variants.

We used two biochemical approaches to assess the potential defect in CMP-sialic acid transport. The first involved flow cytometry to determine if primary fibroblasts had reduced

levels of cell-surfaced glycoproteins terminated with sialic acid (Sia). If an N- or O- glycan lacks a terminal sialic acid, its glycan terminates in a β-galactose (Gal) [Novogrodsky et al., 1975]. Since a defect in SLC35A1 will affect both N- and O- linked glycans, we choose to use the O-glycan specific peanut lectin (PNA) which recognizes a terminal Gal-β(1–3)-GalNAc [Lotan et al., 1978]. We determined that CDG-374 had approximately 2.5-fold more PNA reactivity than a control fibroblast line, suggesting less terminal sialic acid on O-linked glycans (Fig. 1). As a positive control, we used the established SLC35A1-deficient Chinese hamster ovary (CHO) line, Lec2, to show that SLC35A1 deficiency affects PNA lectin reactivity [Eckhardt et al., 1998; Aoki et al., 2001] (Data not shown).

A second biochemical technique is based on the method described by Kim et al, which uses an artificial glycoside as an acceptor for the transported nucleotide-sugar. In the present case, the glycoside, 4-methylumbelliferone β-galactose (4MU-β-Gal), functions as an acceptor for transported CMP-[H^3] sialic acid. The glycoside freely diffuses into the intact Golgi [Etchison et al., 1996; Kim et al., 2001] providing an abundant endogenous acceptor, far in excess of the endogenous glycoprotein acceptors. Under these conditions, the artificial substrate can be sialylated within the Golgi only by the CMP-[H^3] sialic acid delivered by transporter. Here permeabilized cells with sealed Golgi [Etchison et al., 1996] are pre-incubated with 4MU-β-Gal which equilibrates within the Golgi. Addition of CMP-[H^3] sialic acid then allows for its transport into the Golgi using SLC35A1. The endogenous sialyltransferase adds sialic acid to the 4MU-β-Gal substrate and the product 4MU-β-Gal[H^3] Sia is purified and counted. Using this method, the patient fibroblast line showed up to a nine-fold lower transport compared to the control. (Fig. 2). Similar results were initially seen when measuring direct transport without using the artificial acceptor (data not shown). CHO-Lec2 was used as a positive control to validate the transport assay (Fig. 2). The results of both the direct CMP-[H^3]sialic acid transport and glycoside acceptor assays both agree and show a severe loss of SLC35A1 function in the patient's fibroblasts. These are the first SLC35A1 functional assays performed in patient cells.

Finally, we wanted to test if supplementing the fibroblast culture medium with either sialic acid, N-acetylmannosamine (ManNAc) or fetuin could improve the sialylation deficiency in a similar manner as supplementing GDP-fucose deficiency with fucose. However, there was no improvement in lectin binding using several concentrations or treatment for several days (data not shown).

DISCUSSION

SLC35A1 is thought to encode the sole transporter of CMP-sialic acid in humans and mutations result in a congenital disorder of glycosylation [Eckhart et al., 1999; Song, 2013]. In 2001, Willig et al identified a patient with major immunological complications including macrothrombocytopenia and neutropenia with polymorphonuclear cells (PMNs) showing a complete lack of the sialyl- Le^x antigen, or CD15s [Willig et al., 2001]. The individual would require a bone marrow transplantation and ultimately experienced complications that led to death at thirty-seven months of age [Martinez-Duncker et al., 2005]. Total serum N- and O-glycans and transferrin glycosylation were normal, however, ApoCIII did show a mild under sialylation [Martinez-Duncker et al., 2005; Wopereis et al., 2007]. Initial functional

work using CHO-Lec2 cells suggested that the two variants identified in the patient were non-functional [Martinez-Duncker et al., 2005]. However, their subsequent studies showed that one of the variants, an intronic CACT insertion thought to be responsible for the abnormal splicing, in fact was not responsible since non-carriers of the insertion also produced the same splice products [Salinas-Marín et al., 2016]. Furthermore, later work from another laboratory determined one of the two variants identified by Martinez et al is very likely benign, since it was reported to be in the homozygous state in at least six unrelated healthy individuals [Jones et al., 2011]. It remains possible, but less likely, that the second variant identified by Martinez et al is truly pathogenic.

Only one other patient has been reported with proven SLC35A1-CDG. This patient showed macrothrombocytopenia and severe neurological problems including microcephaly, severe developmental disability, hypotonia and seizures [Mohamed et al., 2013]. She also had dysmorphic features in addition to cardiac abnormalities. As in our patient, this individual had clear sialylation deficiencies in both N- and O-linked glycans on serum glycoproteins, as well as increased levels of transferrin missing either one and two sialic acids [Mohamed et al., 2013] (Table I).

The roles of sialic acid in pathogen-host interactions have been well documented both evolutionarily and via animal models [Varki and Schauer, 2009]. More recently, genetic screens have shed light on the critical role SLC35A1 plays in how pathogens such as Enterovirus D68 and Lassa virus utilize sialic acid to highjack cellular machinery [Jae et al., 2013; Baggen et al., 2016]. Knockout cells completely lacking SLC35A1 or other key components of the sialic acid biosynthetic pathway were completely resistant to the effects of either Enterovirus D68 or Lassa virus [Jae et al., 2013; Baggen et al., 2016].

Perhaps more extensively studied is the role sialylation plays in neurological development, specifically as it relates to polysialic acid (PSA) modification of neural cell adhesion molecule (NCAM). It is well documented that PSA modification of NCAM is critical for neurological development and synaptic plasticity [Hildebrandt et al., 2007; Schnaar et al., 2014; Hildebrandt and Dityatev, 2015]. Thus, loss of SLC35A1 should result in deficient PSA modification of NCAM. Unfortunately, neuronal tissue deficient for SLC35A1 has not been isolated or studied. CHO mutant cell lines deficient for SLC35A1 have been used to study these effects on PSA, and as expected, global PSA is dramatically altered [Eckhardt et al., 1996; Eckhardt et al., 1999]. This suggests an explanation for the severe neurological phenotype seen in our patient as well as the one reported by Mohamed et al.

More recently, CRISPR based screens have shown that SLC35A1 plays an important role in regulating the O-mannosylation of alpha-dystroglycan [Jae et al., 2013]. This O-mannosylation is critical for alpha-DG function and its disruption causes various forms of muscular dystrophy-dystroglycanopathy (MDDG) syndromes. Interestingly, sialic acid itself seems to be dispensable for alpha-dystroglycan function suggesting that there may be other roles for SLC35A1 [Riemersma et al., 2015]. It is possible that SLC35A1 can transport other nucleotide sugars besides CMP-sialic acid.

In summary, here we describe a patient with compound heterozygous mutations in *SLC35A1* and use lectin staining along with a newly developed biochemical assay to confirm the loss of transport activity in patient cells.

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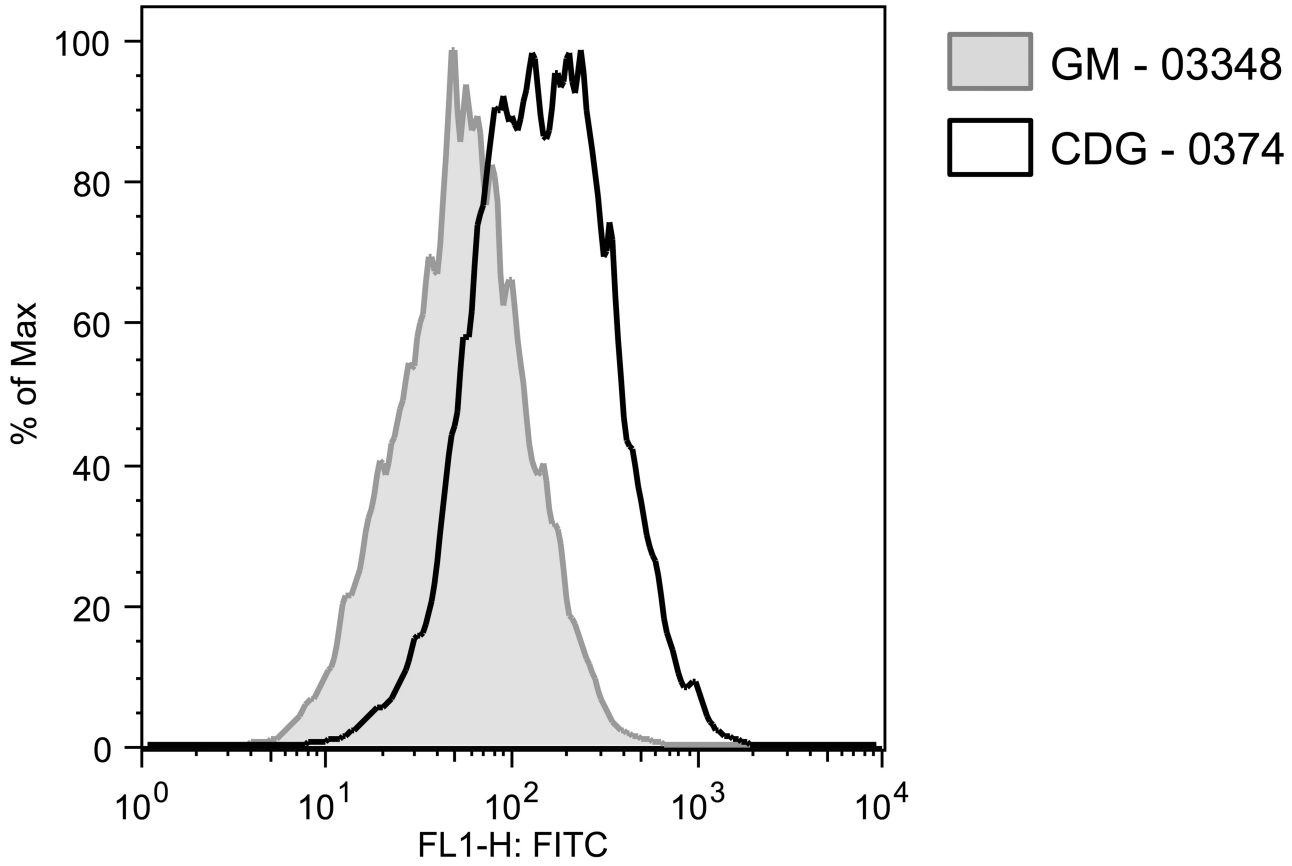

Figure 1.

Cell surface analysis of terminal Gal-β(1–3) using PNA Lectin

Flow cytometry analysis of FITC-conjugated PNA (Arachis hypogaea) lectin in control and patient fibroblasts. The presented control (GM03348) is representative of three controls.

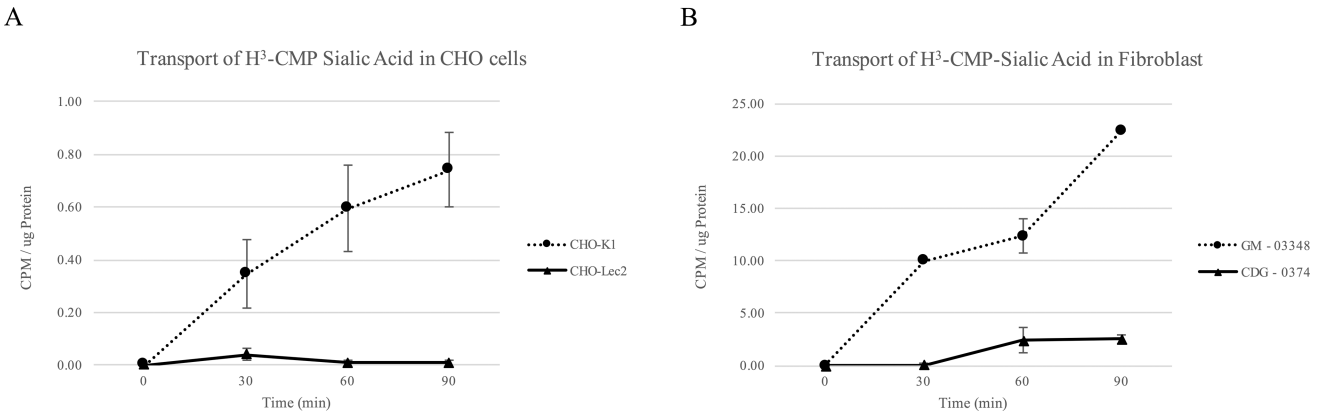

Figure 2.

CMP-sialic acid transport in permeabilized cells with intact Golgi.

Transport of CMP-sialic acid into the Golgi of hypotonic permeabilized cells for A.) Chinese hamster ovary (CHO) wild-type (K1) or SLC35A1 deficient (Lec2) and B.) GM03348 and CDG-0374 primary fibroblast.

TABLE I

Clinical comparison of two known SLC35A1-CDG patients.

	Patient 1	**Patient 2**	**Patient 3**
	Martinez-Duncker et al., 2005	Mohamed et al., 2013	Ng et al., 2017
Ancestor	NR	Turkish	German
cDNA Mutations (NM_006416.4)	c.277_280delGTGCinsTG, c.752-158_752-157insCACT (rs144543370)	c.330G>C, c.330G>C	c.467C>G, c.586G>A
Protein Mutations (NP_006407.1)	p.Val93Cysfs*17 p.Val208Phefs*20	p.Gln101His, p.Gln101His	p.Thr156Arg, p.Glu196Lys
Developmental or Intellectual disability	NR	Yes	Yes
Behavioral problems	NR	Yes	Yes
Hypotonia	NR	Yes	Yes
Ataxia	NR	Yes	Yes
Epilepsy / Seizures	NR	Yes	Yes
Microcephaly	NR	Yes	No
Dysmorphic features	NR	Yes	No
Macrothrombocytopenia	Yes	Yes	No
Coagulopathy	Yes	Yes	No
Status	Deceased (3yrs)	Deceased (22yrs)	Living (12yrs)
CDT Result	Normal	Increased Di, Tri sialo	Increased Di, Tri sialo
ApoCIII Results	Decreased ApoC-III2 Increased ApoC-III1	Decreased ApoC-III2 Increased ApoC-III1	N/A
Serum/Plasma glycans	Normal	Decrease N and O	Decrease N and O

N/A (not available)