Intellectual disability and bleeding diathesis due to deficient CMP–sialic acid transport
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ABSTRACT

Objective: To identify the underlying genetic defect in a patient with intellectual disability, seizures, ataxia, macrothrombocytopenia, renal and cardiac involvement, and abnormal protein glycosylation.


Results: We performed biochemical analysis and found combined N- and O-glycosylation abnormalities and specific reduction in sialylation in this patient. Homozygosity mapping revealed homozygosity for the CMP–sialic acid transporter SLC35A1. Mutation analysis identified a homozygous c.303G>C (p.Gln101His) missense mutation that was heterozygous in both parents. Functional analysis of mutant SLC35A1 showed normal Golgi localization but 50% reduction in transport activity of CMP–sialic acid in vitro.

Conclusion: We confirm an autosomal recessive, generalized sialylation defect due to mutations in SLC35A1. The primary neurologic presentation consisting of ataxia, intellectual disability, and seizures, in combination with bleeding diathesis and proteinuria, is discriminative from a previous case described with deficient sialic acid transporter. Our study underlines the importance of sialylation for normal CNS development and regular organ function.

GLOSSARY

CDG = congenital disorders of glycosylation; CHO = Chinese hamster ovary; CMP–Sia = cytidine 5′-monophosphosialic acid; GDP–Man = guanosine diphosphate–mannose; HA = hemagglutinin; m/z = mass-to-charge ratio; SLC35A1 = solute carrier family 35 member A1.

Linked to lipids and proteins at cell surfaces, the glycocalyx, comprised of sugars, forms the outermost coating of cells and mediates cellular communication in all living systems. Its complex function is inherent in the unique structural richness of the glycome and explains the essential role that glycoconjugates have in biological processes including embryonic, brain, and nervous system development.

Inborn errors of metabolism causing partial lack of glycosylation are grouped together as congenital disorders of glycosylation (CDG). Clinical manifestations in patients with CDG are highly heterogeneous and multisystemic, but almost all CDGs affect CNS development. Moreover, a number of gene defects in glycosylation pathways are known to cause embryonic lethality.

Sialic acid (Sia) is a negatively charged sugar that caps the nonreducing (outermost) ends of most surface-expressed and secreted glycoconjugates. Golgi-localized sialyltransferases, with differential but partly overlapping acceptor specificities, catalyze the addition of Sia to glycoconjugates. Because of their exposed position, Sias are key elements in cellular communication processes. Genetic alterations that disturb or prevent the formation of sialo-glycoconjugates are embryonically lethal or cause diseases. Herein, we describe a 22-year-old patient who

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METHODS Patient. Beginning at the age of 7 years, a female patient of consanguineous Turkish descent experienced psychomotor developmental delay and generalized tonic-clonic seizures. Behavioral problems (aggression and lack of self-control) manifested at puberty. At 20 years of age, the patient presented with microcephaly, dysmorphic facial features, clinodactyly of the fourth and fifth fingers of both hands, webbed neck, bilateral hallux valgus and joint hyperlaxity, hypotonia of the lower extremities, hyporeflexia, mild ataxia, and a systolic cardiac murmur. Ophthalmologic examination was normal, cerebral MRI showed no structural pathology, and the EEG was consistent with epilepsy. Laboratory analysis showed macrothrombocytopenia (99 \( \times \) 10^9/L), proteinuria (1.75 g/L), aminoaciduria, and decreased free thyroxine and estradiol levels; the patient also had decreased levels of coagulation factors XI (52%), XII (54%), and antithrombin III (77%), which cleared spontaneously.

At 22 years of age, the patient underwent endoscopic retrograde cholangiopancreatography that was complicated by postoperative fever and abdominal pain later diagnosed as hemolytic anemia and renal failure due to tubular necrosis; her condition worsened and she developed respiratory failure and died at the age of 22 years.

Biochemical investigations. Standard protocol approvals, registrations, and patient consents. We collected plasma and leukocyte samples of the patient and her parents with informed parental consent for diagnostic purposes, in agreement with our institutional regulations. Samples from ethylenediaminetetraacetic acid or heparinized blood were centrifuged and stored at −80°C. Unless stated otherwise, we obtained chemical reagents from Sigma-Aldrich (St. Louis, MO). As previously described, we conducted isoelectric focusing of plasma transferrin to evaluate mucin-type O-glycosylation, and found a decrease in ApoC-III1 profile,² and in agreement with reduced sialylation (figure 1B).

To learn more about the N-glycosylation abnormalities, we used mass spectrometry for plasma N-glycan profiling. The spectrum of a healthy person (figure 2A) is characterized by a main ion with mass-to-charge ratio (m/z) 2,794 representing the biantennary, distal-to-trisialo-transferrin and a decrease in tetrasialo-transferrin (figure 1A) similar to a previously described pattern seen in patients with type II CDG.¹⁴ We then performed isoelectric focusing of ApoC-III to evaluate mucin-type O-glycosylation, and found a decrease in ApoC-III2 and concomitant increase in ApoC-III1, consistent with an ApoC-III1 profile,¹⁵ and in agreement with reduced sialylation (figure 1B).

To detect SLC35A1 expression in parallel to the Golgi marker GM130⁶ and the endoplasmic reticulum marker protein disulfide isomerase¹¹ (see appendix e-1 on the Neurology® Web site at www.neurology.org). The complete coding sequences of wild-type and mutant (Gln101His) SLC35A1 were heterologously expressed in Saccharomyces cerevisiae. Expressed constructs contained N-terminal FLAG-tags and C-terminal HA tag to facilitate detection through immunocytochemistry and protein quantification through Western blotting. We isolated Golgi vesicles as described and tested for their ability to incorporate [¹³C]CMP-Sia.¹² The quality of different membrane preparations was controlled by measuring the endogenous transport of guanosine diphosphate-mannose (GDP-Man) and uridine diphosphate-glucose (see appendix e-1).

RESULTS Identification of a combined N- and O-glycosylation disorder with reduced sialylation. We screened the above patient for CDG using isoelectrofocusing of serum transferrin.¹⁴,¹⁵ We identified an abnormal pattern with increased levels of distal- and trisialo-transferrin and a decrease in tetrasialo-transferrin (figure 1A) similar to a previously described pattern seen in patients with type II CDG.¹⁴ We then performed isoelectric focusing of ApoC-III to evaluate mucin-type O-glycosylation, and found a decrease in ApoC-III2 and concomitant increase in ApoC-III1, consistent with an ApoC-III1 profile,¹⁵ and in agreement with reduced sialylation (figure 1B).

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Because elevated sialidase activity in plasma²⁰ causes nonspecific loss of Sia as observed in patients with hemolytic uremic syndrome, we measured sialidase activity. Results were normal for this patient (data not shown).

Genetic studies reveal a homozygous mutation in SLC35A1. To find the genetic defect, we first performed homozygosity mapping and identified 4 large homozygous regions, but no obvious candidate genes except SLC35A1 that encodes the CMP-Sia transporter. SLC35A1 was present in a homozygous region on chromosome 6, spanning 18.7 Mb and 1,913 single nucleotide polymorphisms on 6q15. Via subsequent mutation analysis, we identified a homozygous c.303G>C (p.Gln101His) mutation (figure 3A), which was heterozygous in the parents, but was not found in any of the more than 5,000 publicly available exomes (Exome Variant Server, NHLBI Exome Sequencing Project, Seattle, WA) nor in our in-house

presented with mental retardation, seizures, and bleeding diathesis, as well as renal and cardiac dysfunction. Serum analysis demonstrated significantly reduced Sia levels. The disease-causing mutation was localized to the SLC35A1 gene encoding the Golgi CMP-Sia transporter,¹³ which is essential for supply of metabolically activated Sia (cytidine monophosphate–Sia, CMP-Sia) to sialyltransferases. Herein, we demonstrate involvement of the CMP-Sia transporter in disease development.
The CMP-Sia transporter is a type III protein of the late Golgi complex comprising 10 transmembrane domains. Gln101 is part of the third transmembrane domain and is highly conserved (figure 4B) down to Drosophila melanogaster.\textsuperscript{21} Prediction software, SIFT\textsuperscript{22} and PolyPhen,\textsuperscript{23} predicted that the p.Gln101His exchange identified in our patient was deleterious and probably damaging.

Figure 1  CDG screening for N-glycosylation and mucin-type O-glycosylation

(A) Transferrin isoelectric focusing of a control, a patient with confirmed CDG-II (ATP6V0A2-CDG), and the current patient. Numbers correspond to sialo-transferrin isoforms. The tetrasialo isoform (4) is most abundant in healthy controls, whereas in patients with CDG-II, we observed an increase of lower sialylated isoforms (0–3). (B) Isoelectric focusing of ApoC-III in controls shows similar levels of ApoC-III2 (tetrasaccharide with 2 sialic acids) and ApoC-III1 (trisaccharide with 1 sialic acid). In our patient, we observed decreased sialylation with decreased ApoC-III2 and increased ApoC-III1. CDG = congenital disorders of glycosylation.

Figure 2  Plasma N-glycan profiling

(A) Spectra of a healthy person, characterized by a main ion with \(m/z\) 2,794 representing the biantennary, disialylated complex N-glycan and minor additional peaks (e.g., the triantennary, fully sialylated N-glycan [\(m/z\) 3,606] and the monofucosylated disialylated glycan [\(m/z\) 2,968]). (B) Levels of all undersialylated glycans are increased in our patient, confirming selective reduction in protein N-linked sialic acid levels. \(m/z\) = mass-to-charge ratio.
Functional testing confirmed reduced activity of the CMP-Sia transporter. To study the functional consequences of the p.Gln101His mutation, we cloned the wild-type and mutant gene to allow expression of HA-tagged proteins. After transient expression in CHO cells, we found that both variants of SLC35A1 correctly localized to the Golgi apparatus (figure 4A), with no sign of endoplasmic reticulum retention. Similar levels of SLC35A1 expression for wild-type and mutant constructs in Western blot indicated that the mutant protein was stable (figure e-1A).

To quantify activity, heterologous expression in yeast, providing a suitable background-free model system, was used. Wild-type and p.Gln101His mutant were expressed with N-terminal FLAG-tags; Golgi membranes from transfected yeast cells were isolated and used to determine \[^{14}\text{C}\]CMP-Sia transport. The in vitro assays clearly demonstrated decreased CMP-Sia transport activity (approximately 50%) in Golgi vesicles isolated from p.Gln101His transformed yeast cells (figure 4B). Because expression levels of the analyzed transporters were comparable (figure e-1B) and the measurement of endogenous GDP-Man and uridine diphosphate–glucose transport confirmed equal quality of vesicle preparations, the reduced \[^{14}\text{C}\]CMP-Sia transport can only be explained by a functional defect of the mutant protein.

To test whether an impacted transport of p.Gln101His is also visible in a mammalian cell system, complementation in the SLC35A1-defective 6B2 cells was used. As a readout, we monitored the re-expression of the large sialo-glycoconjugate polysialic acid. Whereas both wild-type and mutant transporter restored polysialic acid expression, cells transfected
with p.Gln101His showed a significantly reduced (approximately 15%) level (figure e-2).

**DISCUSSION** Herein, we describe a patient with CDG-II in whom isoelectric focusing and mass spectrometry revealed a general reduction in the biosynthesis of sialylated proteins. We identified a point mutation in the CMP-Sia transporter (SLC35A1; OMIM *605634). The mutation does not influence protein localization or stability, but causes reduced CMP-Sia transport activity to approximately 50%. Remarkably, the patient’s history indicates that symptoms (developmental delay and generalized tonic-clonic seizures) did not appear until the age of 7 years, but then progressed to multisystem disease including the coagulation system and peripheral organs such as kidney and heart. The case described herein causally links this CMP-Sia transporter defect to disease development.

We identified reports on 2 other patients with macrothrombocytopenia and reduced sialyl-Lewis X structures in leukocytes, but of unknown etiology. The biochemical defect was selective since transferrin isoelectric...
Molecular investigations conducted postmortem in blood samples of one patient identified a compound heterozygous mutation in SLC35A1. However, a recent report showed that one of the identified mutations occurs frequently in the general population (the CACT insertion at the splice donor site of intron 6) and is a common polymorphism (found in 19 of 51 control samples). Whether the second, apparently pathogenic mutation causes a dominant defect or may be additive with a second somatic mutation remains unknown.

In an attempt to elucidate the causative genetic defect in one of the 2 previously mentioned patients, all genes of relevance for biosynthesis of α2,3-sialylated structures, including SLC35A1, were sequenced and to a large extent functional testing was conducted, but no definitive genetic cause of disease was found. According to our in vitro transport studies, the p.Gln101His mutation identified in the current patient specifically reduces transport activity of the CMP-Sia transporter by 50%, suggesting that the mutation causes a shortage of CMP-Sia in the lumen of the Golgi apparatus with a resultant decrease in the formation of sialo-glycoconjugates (N- and O-glycosylated proteins and glycolipids). We hypothesize that the non-neuronal clinical features are foremost a consequence of altered O-glycosylation, because mice deficient in glycoprotein-N-acetylgalactosamine-3-β-galactosyltransferase, the enzyme essential for the synthesis of extended mucin-type O-glycans, recapitulate a number of the observed phenotypic components (i.e., thrombocytopenia and kidney disease). Moreover, our patient developed proteinuria, a symptom also found in a mouse model with a hypomorphic mutation in Cnas, the gene encoding the CMP-Sia synthetase, located immediately upstream of SLC35A1. In line with the above assumption that sialylation defects in the mucin-type O-glycans are key to nephrologic alterations in this patient, the lack of Sia on O-linked glycans of podocalyxin was highly associated with proteinuria in the Cnas mouse model. Other conditions such as cardiac dysfunction, endocrine abnormalities, and dysmorphic features could not be adequately characterized in the Cnas mouse model because the constitutive presence of the defective gene caused early postnatal death due to kidney failure.

In contrast to the earlier described patients, our patient did not show any sign of neutropenia and had no spontaneous bleeding, although thrombocytopenia and reduced coagulation activity was identified and became clinically relevant during surgery. Bleeding diathesis is common in patients with N-linked glycans biosynthesis defects, but in most cases manifests only in stress situations. Generally, the clinical and biochemical features are well explained by the identified CMP-Sia transporter defect, including the observed intellectual impairment. The fact that almost all CDGs affect brain development is not surprising if we consider that glycans are unparalleled in their capacity to store information. Excellent proof is evident in previous identification of a highly specific sialylation defect in patients with intellectual disability. In 2 consanguineous Iranian families, independent mutations inactivating the sialyltransferase ST3Gal-III were shown to cause intellectual retardation without additional clinical features. The shortage of CMP-Sia caused by the SLC35A1 defect in our patient is likely to entail a reduction in the biosynthesis of sialylglycoconjugates and gangliosides, both broadly involved in brain development and memory formation. In analogy to the potential treatment opportunities using dietary fucose in GDP-fucose transporter deficiency, it could be speculated that dietary addition of Sia or its precursor N-acetyl-mannosamine could be used to decrease disease symptoms, especially in case of residual activity.

In conclusion, we identified a CMP-Sia transporter deficiency leading to intellectual disability, epilepsy, bleeding diathesis, and renal as well as heart involvement, in a case of autosomal recessive SLC35A1-CDG leading to a generalized glycosylation defect.

**AUTHOR CONTRIBUTIONS**

M. Mohamed: drafting/revising the manuscript, interpretation of data, and funding. A. Ashikov and M. Guillard: drafting/revising the manuscript and interpretation of data. J.H. Robben and S. Schmidt: analysis and interpretation of data. B. van den Heuvel: drafting/revising the manuscript. A.P.M. de Brouwer: analysis and interpretation of data, acquisition of data, and funding. R. Gerardy-Schahn: drafting/revising the manuscript, study design, and funding. P.M.T. Deen and R.A. Wevers: drafting/revising the manuscript. D.J. Lefebre: drafting/revising the manuscript, study design, and funding. E. Morava: drafting/revising the manuscript and study design.

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