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Neurology published online July 19, 2013
DOI 10.1212/WNL.0b013e3182a08f53

Published Ahead of Print on July 19, 2013 as 10.1212/WNL.0b013e3182a08f53

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.

Methods: Genetic studies involved homozygosity mapping by 250K single nucleotide polymorphism array and *SLC35A1* sequencing. Functional studies included biochemical assays for N-glycosylation and mucin-type O-glycosylation and *SLC35A1*-encoded cytidine 5′-monophosphosialic acid (CMP–sialic acid) transport after heterologous expression in yeast.

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. ***Neurology***® **2013;81:1–7**

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.[1] Its complex function is inherent in the unique structural richness of the glycome[2] and explains the essential role that glycoconjugates have in biological processes including embryonic, brain, and nervous system development.[3]

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.[4,5] Moreover, a number of gene defects in glycosylation pathways are known to cause embryonic lethality.[6]

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.[7] 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[6] or cause diseases.[8–12] Herein, we describe a 22-year-old patient who

Supplemental data at www.neurology.org

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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.

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 (99×10^9/L; normal: 150–400 $\times$ 10^9/L), proteinuria (1.75 g/L), aminoaciduria, and decreased free thyroxine and estradiol levels; the patient also had reduced 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 bleeding and followed by emergent laparotomy with cholecystectomy. After surgery, she was readmitted with 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 −20°C. Unless stated otherwise, we obtained chemical reagents from Sigma-Aldrich (St. Louis, MO). As previously described, we conducted isoelectric focusing of plasma transferrin and ApoC-III in 2 independent samples,[14] and used matrix-assisted laser desorption/ionization linear ion trap mass spectrometry for plasma N-glycan profiling,[15] using 10 μL of plasma.

Genetic analyses. We isolated DNA from ethylenediaminetetraacetic acid blood or leukocytes using standard procedures and performed linkage analysis in genomic DNA of the patient using 250K single nucleotide polymorphism array according to the manufacturer's protocols (Affymetrix, Santa Clara, CA). Next, we performed sequence analysis of the *SCL35A1* gene by standard methods using Sanger sequencing for the patient's and parents' samples.

Subcellular localization studies of SLC35A1 and functional testing of wild-type and mutant SLC35A1. We stably expressed human influenza hemagglutinin (HA)-tagged variants of wild-type and mutant SLC35A1 in Chinese hamster ovary (CHO) cells and used immunocytochemistry and Western blotting to detect SLC35A1 expression in parallel to the Golgi marker GM130[16] and the endoplasmic reticulum marker protein disulfide isomerase[17] (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 tags to facilitate detection through immunocytochemistry and protein quantification through Western blotting. We isolated Golgi vesicles as described and tested for their ability to incorporate [^{14}C]CMP-Sia.[18] 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

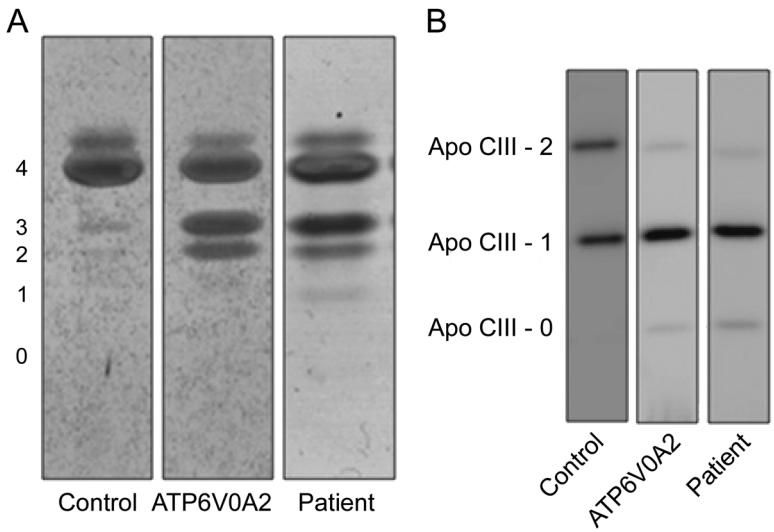
Identification of a combined N- and O-glycosylation disorder with reduced sialylation. We screened the above patient for CDG using isoelectrofocusing of serum transferrin.[14,19] We identified an abnormal pattern with increased levels of disialo- and trisialo-transferrin and a decrease in tetrasialo-transferrin (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,[14] 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, 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]). In our patient, all undersialylated glycans were increased, confirming selective reduction in protein N-linked Sia levels (figure 2B).

Because elevated sialidase activity in plasma[20] 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 our in-house

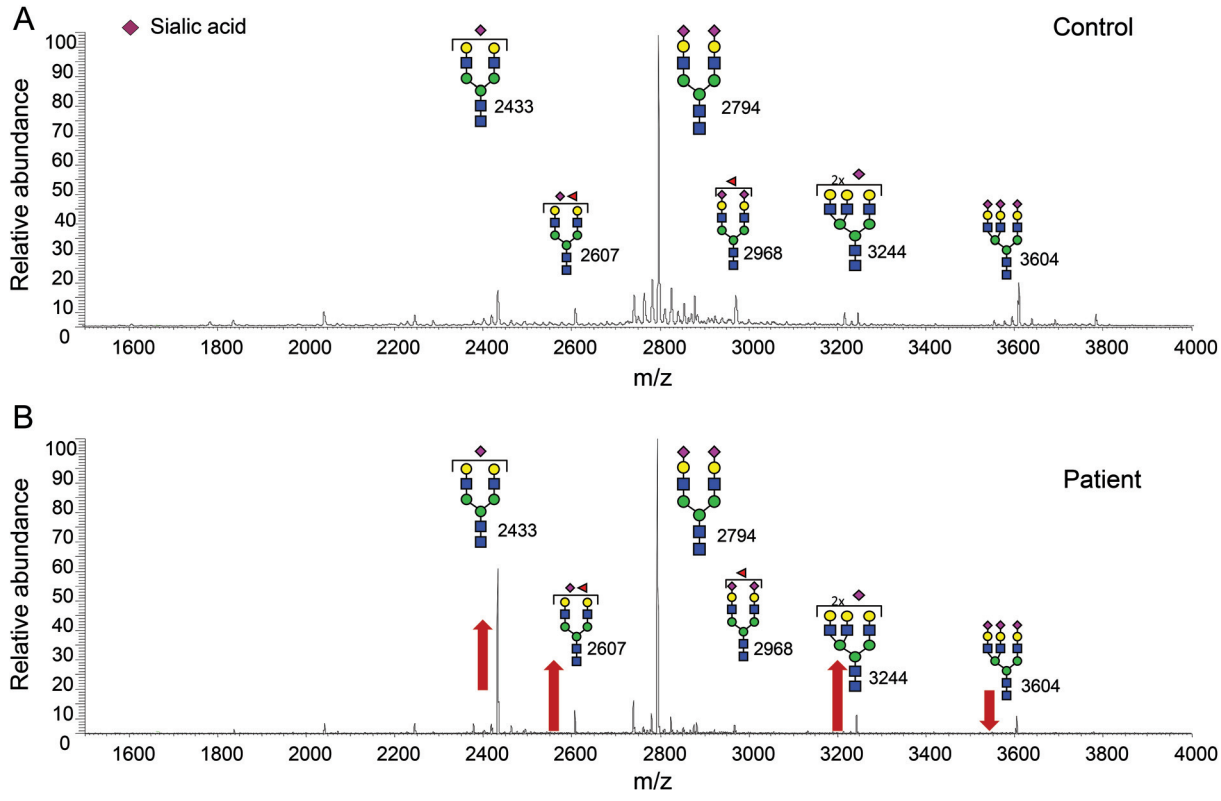
Figure 1 CDG screening for N-glycosylation and mucin-type O-glycosylation



(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.

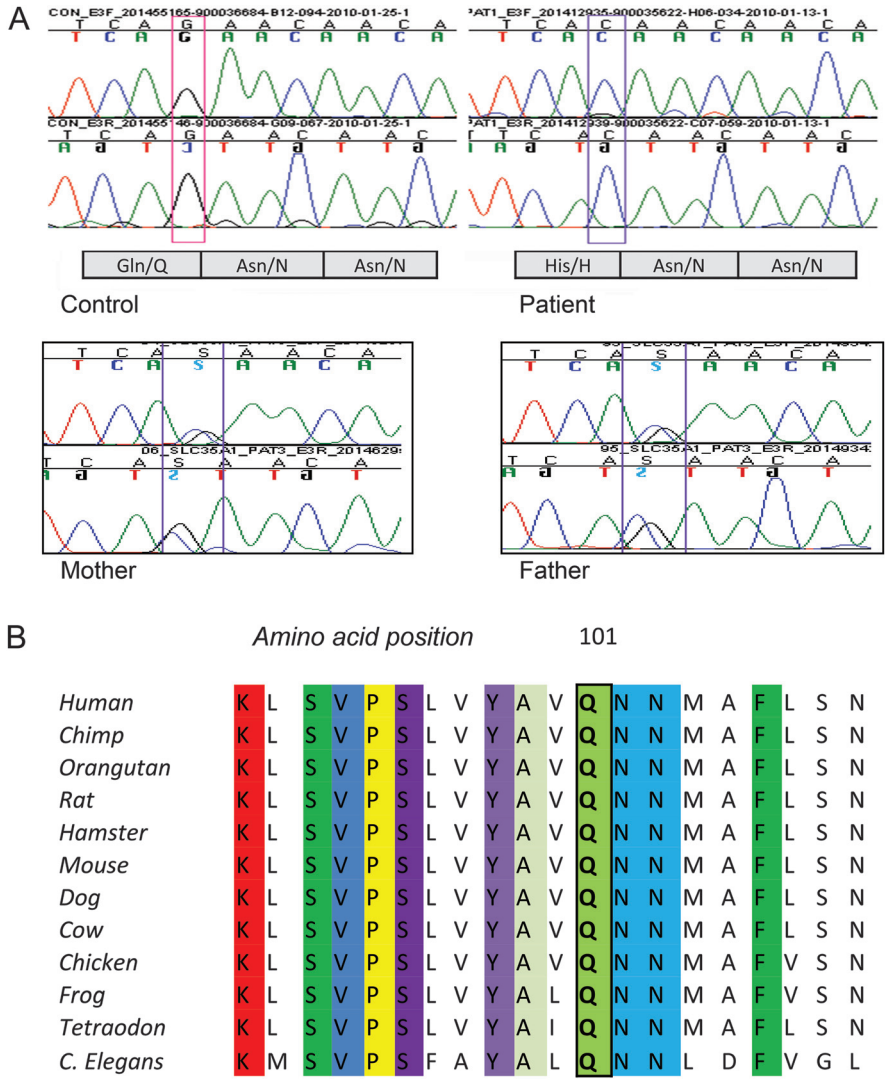
database with more than 1,154 exomes. The CMP-Sia transporter is a type III protein of the late Golgi complex comprising 10 transmembrane domains. Gln101 is part of the third transmembrane domain and is highly conserved (figure 4B) down to *Drosophila melanogaster*.[21] Prediction software, SIFT[22] and PolyPhen,[23] predicted that the p.Gln101His exchange identified in our patient was deleterious and probably damaging.

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.

Figure 3 Localization of the mutation



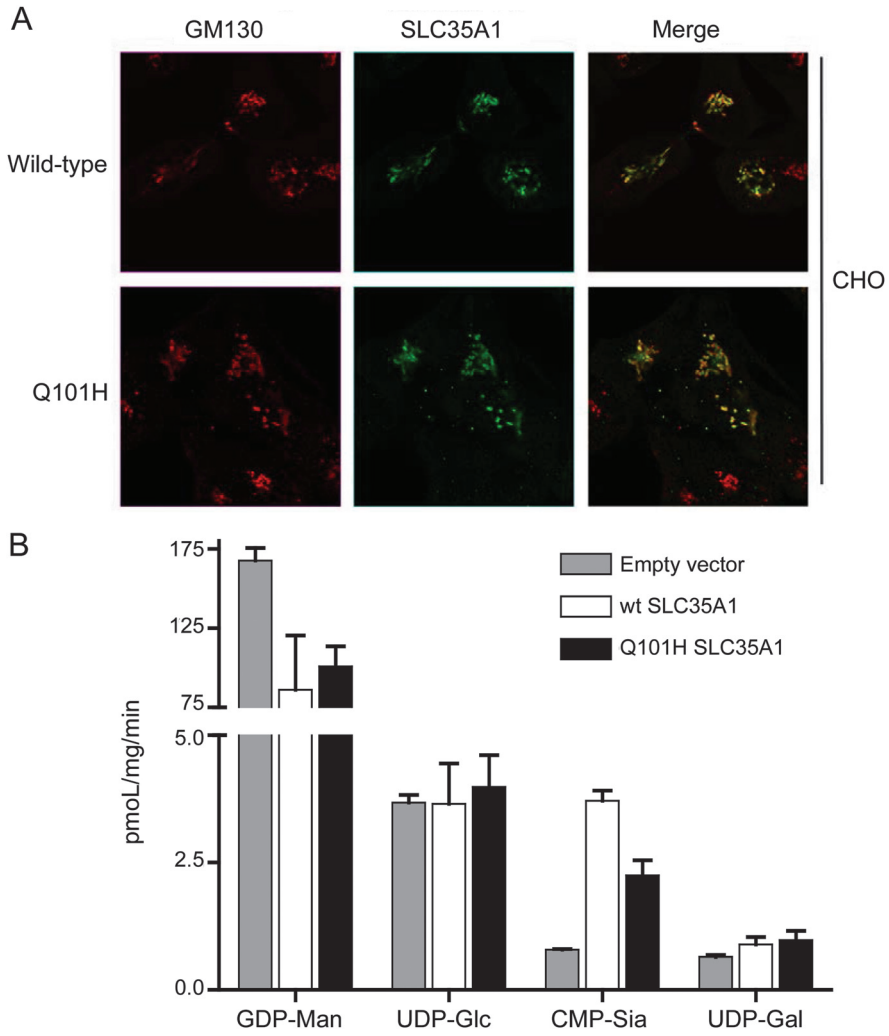
(A) Mutation analysis identified a homozygous c.303G>C (p.Gln101His) mutation. The mutation was heterozygous in the parents, but was not found in a search of both in-house and public databases. (B) Glutamine conservation on position 101 is highly conserved.

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.[24] 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}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}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.[13] As a readout, we monitored the re-expression of the large sialo-glycoconjugate polysialic acid.[25] Whereas both wild-type and mutant transporter restored polysialic acid expression, cells transfected

Figure 4 Localization and functional studies



(A) Golgi localization in Chinese hamster ovary (CHO) cells. CHO cells stably expressing wild-type SLC35A1 or SLC35A1-Gln101His (Q101H) were seeded on glass slides and grown to 80% confluence. Subsequently, cells were fixed with 3% paraformaldehyde in phosphate-buffered saline and subjected to immunocytochemistry using rabbit anti-hemagglutinin antibodies and secondary Alexa 488–conjugated goat anti-rabbit antibodies to stain SLC35A1 (shown in green). Cellular markers for the Golgi compartment GM130 were stained with mouse antibodies and secondary Alexa 647–conjugated goat anti-mouse antibodies (shown in red). Using an Olympus confocal laser scanning microscope with 63× objective and 3× digital zoom, we observed colocalization of SLC35A1 with GM130 (shown in merge). (B) Functional analysis of Golgi vesicles isolated from yeast cells transformed with either wild-type (white bars) or SLC35A1-Gln101His (Q101H) mutant (black bars). Wild-type and mutant SLC35A1-Gln101His were compared for cytidine monophosphate–sialic acid (CMP-Sia) transport ability using radiolabeled nucleotide sugar. Yeast endogenous transport of guanosine diphosphate–mannose (GDP-Man) and uridine diphosphate–glucose (UDP-Glc) was used as a control for membrane preparation quality, as UDP-galactose (UDP-Gal) transport is not present in yeast and therefore is appropriate as a negative control. Error bars indicate SD of the mean from 3 experiments using independently prepared membrane fractions from single cell clones. Each assay was performed twice.

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,[10,12] but of unknown etiology. The biochemical defect was selective since transferrin isoelectric

focusing patterns were normal in both patients, but sialyl-Lewis X was missing in neutrophils. Furthermore, the lack of Sia in those 2 patients resulted in early disease onset with massive spontaneous hemorrhages.[10,12] Molecular investigations conducted postmortem in blood samples of one patient[10] identified a compound heterozygous mutation in *SLC35A1*.[11] 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).[12] 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,[12] 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).[26] Moreover, our patient developed proteinuria, a symptom also found in a mouse model with a hypomorphic mutation in *Cmas*, the gene encoding the CMP-Sia synthetase, located immediately upstream of *SLC35A1*.[9] 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 *Cmas* mouse model.[9] Other conditions such as cardiac dysfunction, endocrine abnormalities, and dysmorphic features could not be adequately characterized in the *Cmas* mouse model because the constitutive presence of the defective gene caused early postnatal death due to kidney failure.[9]

In contrast to the earlier described patients,[10,12] 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 glycan 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[4,5] is not surprising if we consider that glycans are unparalleled in their capacity to store information.[3] Excellent proof is evident in previous identification of a highly specific sialylation defect in patients with intellectual disability.[8] In 2 consanguineous Iranian families, independent mutations inactivating the sialyltransferase ST3Gal-III were shown to cause intellectual retardation without additional clinical features.[8] The shortage of CMP-Sia caused by the *SLC35A1* defect in our patient is likely to entail a reduction in the biosynthesis of sialoglycoproteins and gangliosides, both broadly involved in brain development and memory formation.[27] In analogy to the potential treatment opportunities using dietary fucose in GDP-fucose transporter deficiency,[28] 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. Lefeber: drafting/revising the manuscript, study design, and funding. E. Morava: drafting/revising the manuscript and study design.

STUDY FUNDING

Supported by The Netherlands Organisation for Scientific Research (NWO), Metakids Foundation, Hersenstichting Nederland, Dutch Kidney Foundation, and the German Research Foundation.

DISCLOSURE

M. Mohamed received funding from de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO, The Netherlands Organisation for Scientific Research, 017.008.052) and from de Stichting Metakids (Metakids Foundation, grant 2012). A. Ashikov and M. Guillard report no disclosures. J.H. Robben is supported by a Kolff grant from the Dutch Kidney Foundation (KJPB 09.012). S. Schmidt and B. van den Heuvel report no disclosures. A.P.M. de Brouwer received funding from Hersenstichting Nederland (2010[1]-30). R. Gerardy-Schahn received funding from the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) in the frame of the Cluster of Excellence REBIRTH (From Regenerative Biology to Reconstructive Therapy). P.M.T. Deen received funding from NWO (The Netherlands Organisation for Scientific Research, 865.07.002). R.A. Wevers reports no disclosures. D.J. Lefeber received an NWO Medium Investment Grant (40-00506-98-9001) from de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO, The Netherlands Organisation for Scientific Research). E. Morava reports no disclosures. Go to Neurology.org for full disclosures.

Received January 22, 2013. Accepted in final form April 26, 2013.

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Appendix e-1

Patient and Methods

Patient

The female patient had two siblings were both healthy, but a cousin reportedly showed the same phenotype and still lived in Turkey. The patient was 20 years old upon arrival in the Netherlands. Patient history included delivery after an uneventful and term pregnancy. At that age, multiple dysmorphic features were noted including microcephaly (head circumference 54 cm [> -2 SD]), flat occiput, hypotelorism, deep placed eyes and short philtrum. Body Mass Index was 29. Further examination showed a grade II-III systolic cardiac murmur (previously noted in Turkey). There were no signs of strabismus, lipodystrophy and bone deformities. Echocardiography of the heart showed a thin membrane subaortal without a significant stenosis and a grade I aortic insufficiency. Laboratory analysis showed TSH, LH and FSH were all in the low-normal range. Glycine in urine was slightly elevated (201 umol/mmol creatinine) and cystine was more evidently elevated (43 umol/mmol creatinine). Transaminases, AF and gamma-GT were all in the normal range.

Genetic analysis

We extracted genomic DNA from peripheral blood lymphocytes using standard salting out procedures[e1], and performed genotyping using the Affymetrix NspI 250K SNP array. We performed and analysed SNP array experiments according to manufacturer's protocols (Affymetrix, Santa Clara, CA, USA). For homozygosity mapping, we used PLINK v1.06, using a homozygous window of 50 SNPs tolerating two heterozygous SNPs and ten missing SNPs per window on the patient to restrict the number of candidate genes for mutation analysis[e2]. We then genotyped SNPs for homozygous stretches using an in-house algorithms and performed genomic DNA analysis.

We designed oligonucleotide primers from the human gene sequence, and checked the size of the amplification products obtained after PCR reactions on 1.5% agarose gel and subjected amplimers to DNA sequence analysis with BigDye terminator cycle sequencing chemistry on an Applied Biosystems ABI PRISM 3130*x*l Genetic Analyzer, using DNA from 100 healthy control individuals for comparison. We developed primers using the primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi)[e3.]

Immunocytochemistry and Western blot in CHO cells

We maintained Chinese Hamster Ovary (CHO) cells in minimum essential culture medium-α medium (Sigma) supplemented with 5% fetal calf serum, ciproxin and 4mM glutamine, and transfected cells with 2.5 µg of expression constructs encoding the FLAG and HA-tagged wild-type mouse SLC35A1, or its mutant -Gln101His using lipofectamine 2000 (Invitrogen). To select stable transfectants, we grew cells for 10 days in culture medium supplemented with 800 µg/ml G418, and pooled resulting colonies for use in subsequent experiments. For western blotting, pooled colonies were seeded in 6 well plates and grown to 80% confluence. Cells were lysed in 1X Laemmli buffer with 0.1 M DTT, followed by sonication. Samples were run on a 12% PAAG and subjected to immunoblotting using a rabbit-anti-HA antibody or mouse anti-β-actin antibody. Signals were visualized using a horseradish-peroxidase-conjugated goat-anti-rabbit or goat-anti-mouse antibody and enhanced chemiluminescence using a Chemidoc XRS Imager (Biorad).

Sialic acid transport analysis in yeast

To clone the human SCL35A1, we prepared total RNA with Trizol® reagen (Invitrogen) from human white blood cells according the manufacture protocol. Then we synthesized cDNA using a Transcriptor High Fidelity cDNA Synthesis Kit (Roche). To amplify the wild type

SLC35A1 primers, we used AA-SLC35A1-F 5´gataggatccgctgccccgagagacaat 3´) and AA-SLC35A1-R (5´atcactcgagtcacacaccaataactct 3´). We inserted the polymerase chain reaction (PCR) product was in the pYEScupFLAG K plasmid[4] utilizing BamHI / XhoI restriction sites, controlling the plasmid by restriction digest mapping and sequencing (GATC biotech, Germany). To generate the Gln101His mutant, we used a joint PCR technique with AA-Q101H-F(5´tagtgtatgctgttcacaacaacatggct3´) and AA-Q101H-R (5´agccatgttgttgtgaacagcatacacta3´) primers and confirmed 303G>C substitution by sequencing.

Expression of mutant and wildtype SLC35A1 in *Saccharamyces cerevisiae:* S. cerevisiae cells (YPH500 (MATα ura3-52 lys2-801 ade2-101 trp1-Δ 63 his3-Δ200 leu2-Δ1)) were transformed using the lithium acetate method provided by Invitrogen. Transformed cells were selected on plates without uracil. Protein expression of FLAG tagged transporter was proved by SDS-PAGE and western blot.

We extracted Golgi vesicles and performed transport assays as previously described[4]. Briefly, we induced one liter of yeast culture with an optical density (OD_{600}) of 0.8 with 0.5 mM $CuSO_4$ for 3 h. at 30° C; then we harvested cells by centrifugation (5 min at 1,500 × g) and washed twice with ice-cold 10 mM NaN_3. For partial digestion of the cell walls, we weighted and partially suspended wet cells in zymolyase buffer (3 ml/g of cells; 50 mM potassium phosphate, pH 7.5; 1.4 m sorbitol; 10 mM NaN3 and 0.3% β-mercaptoethanol) containing 0.6 mg/ml of zymolyase-100T. We collected spheroplasts after centrifugation (5 min at 1,000 × g) and lysed them via homogenization (10 strokes in a Dounce homogenizer in lysis buffer (4 ml/g of cells; 10 mM Hepes-Tris, pH 7.4; 0.8 M sorbitol; 1 mM EDTA and complete EDTA-free protease inhibitor mixture (Roche Applied Science)). We repeated the homogenization

procedure twice and removed non-lysed cells and debris by centrifugation (two times 5 min at 1,500 × g). Then, we collected endoplasmic reticulum- and Golgi-rich fractions centrifugation at 10,000 × g for 10 and 100,000 × g for 1 h respectively and resuspended pellets in lysis buffer (0.8 ml/g of wet cells); finally, we froze aliquots and stored them at at -80 °C for further analysis. We estimated protein expression by SDS-PAGE and western blot. 5µl of the isolated ER or Golgi enriched fractions were analysed by SDS-PAGE, western blotting and immunostaining with FLAG® M5 monoclonal antibody (Sigma-Aldrich). For details see[e4].

To determine protein concentration, we used a BCA™ kit (Pierce). For the transport assay, we added 50 ul of 2 mM radioactive nucleotide sugar (in assay buffer (10 mM Tris-HCl, pH 7.0; 0.8 m sorbitol; 2 mM $MgCl_2$) to pre-warmed 50 ul vesicles and incubated at 30°C for 30 sec. To stop the reaction, we added 1 ml of ice cold assay buffer containing 1 µM of corresponding cold nucleotide sugar. The separation of vesicles and nucleotides that were not internalized was achieved by filtration through nitrocellulose filter (MF™ membrane filters Millipore, Bedford, MA), and we measured radioactivity associated with the filter by liquid scintillation in LS 6500counter (Beckman Coulter). We calculated transport as pmol nucleotide-sugars transported per 1 mg of protein during 1 min incubation time (pmol/mg/min), using yeast endogenous transports (GDP-Man and UDP-Glc) as a quality control. All assays were performed in duplicate with 3 independent single cell clones.

Complementation experiments in CHO 6B2 cells

CHO 6B2 cells exhibit an asialo-phenotype due to a defect in the CMP-Sia transporter[e5]. Cells were grown on DMEM-Ham's F-12 (1:1) medium (Biochrom) supplied with 10% FCS. For complementation analyses, cells were seeded into 6-well plates and transfected with 0.5 µg of plasmid DNA and 6 µl Metafectene (Biontex) according to the manufacturer's instructions.

24 h posttransfection, cells were washed twice with PBS, lysed on ice for 10 min in lysis buffer (50 mM Tris buffer pH 8.0, 150 mM NaCl, 1 mM, 1% NP-40, 10% glycerol, and cOmplete, EDTA-free Protease Inhibitor Cocktail (Roche)) and lysates collected with a cell scraper into 0.5 ml Eppendorf tubes. Debris was spun down at 1 500xg for 5 min and supernatants mixed with 5xLaemmli buffer containing 5% beta-ME. Samples were incubated at 60ºC for 5 min and run on 7% or 10% PAGE. After western blotting, polysialic acid was detected with the mouse IgG2a antibody 735[e6]. For loading control, actin was visualized with a rabbit anti-actin antibody (Sigma-Aldrich). After incubation with IRDye conjugated secondary antibodies, signals were detected by use of a Li-Cor Odyssey Infrared Imaging System and quantified using the Odyssey V3.0 software. In this step the house-keeping protein beta actin was used for normalisation. Results are expressed as relative band intensities calculated from 5 independent transfection experiments.

e-References

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Supplemental Figure Legends

Figure e-1: Western blot analysis of SLC35A1 and its mutant Gln101His in CHO and yeast cells.

(A) shows protein lysates of CHO cells, stably expressing SLC35A1 or its mutant Gln101His, after immunoblotting. Protein weight is indicated on the left in kDa. As a control for equal protein loading, blots were stripped and subsequently probed for β-actin. (B) shows Golgi-enriched fractions isolated from yeast transformed with an empty vector (mock), or vectors encoding the FLAG-tagged wild-type (wt) and p.Gln101His SLC35A1. No changes were observed in intensity and electrophoretic mobility pattern of the wild type and mutant SLC35A1.

Western blot analysis of Golgi-enriched fractions isolated from yeast cells transformed with empty vector (mock). With the mouse monoclonal antibody FLAG® M5 (Sigma-Aldrich) protein bands of similar intensity and electrophoretic mobility were detected.

Figure e-2: PolySia analysis in Lec2 cells

CHO 6B2 (Lec2) cells do not express a functional CMP-Sia transporter and therefore do not synthesize sialo-glycoconjugates. After transfection with wild-type (wt) SLC35A1 or p.Gln101His, the cells re-gain sialylation capacity shown by the polysialic acid signal in the western blot (A). However, quantification and normalization of the obtained polysialic acid signal with the help of the loading control beta actin (B) revealed a 15% reduction of the signal in cells transfected with Gln101His (C). Results shown in C summarize 5 independent transfection experiments.