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Serum transferrin carrying the xeno-tetrasaccharide NeuAc-Gal-GlcNAc₂ is a biomarker of ALG1-CDG

Per Bengtson, PhD¹, Bobby G. Ng, MS², Jaak Jaeken, MD, PhD³, Gert Matthijs, PhD⁴, Hudson H. Freeze, PhD², and Erik A. Eklund, MD, PhD⁵

¹Division of Clinical Chemistry and Pharmacology, Department of Laboratory Medicine, Lund University, Lund, Sweden

²Human Genetics Program, Sanford-Burnham-Prebys Medical Discovery Institute, La Jolla, CA, USA

³Centre for Metabolic Disease, University Hospital Gasthuisberg, KU Leuven, Leuven, Belgium

⁴Centre for Human Genetics, KU Leuven, Leuven, Belgium

⁵Department of Pediatrics, Clinical Sciences, Lund University, Lund, Sweden

Summary

ALG1-CDG (formerly CDG-Ik) is a subtype of congenital disorders of glycosylation (CDG) where the genetic defect disrupts the synthesis of the lipid-linked oligosaccharide precursor required for N-glycosylation. The initial step in the investigation for these disorders involves the demonstration of hypoglycosylated serum transferrin (TF). There are no specific biomarkers of this CDG subtype known to date. An LC/MS approach was used to analyze sera from patients with ALG1-CDG, PMM2-CDG, suspected CDG and individuals with alcohol abuse. We show mass spectrometric data combined with data from enzymatic digestions that suggest the presence of a tetrasaccharide consisting of two N-acetylglucosamines, one galactose and one sialic acid, appearing on serum TF, is a biomarker of this particular CDG subtype. This is the first time analysis of serum TF can suggest a specific CDG Type I subtype and we suggest this tetrasaccharide be used in the clinic to guide the ALG1-CDG diagnostic process.

Keywords

CDG; ALG1; N-linked glycosylation; LC/MS of transferrin; tetrasaccharide biomarker

Introduction

Deficient protein and lipid glycosylation is the basis of the rapidly growing group of metabolic disorders known as congenital disorders of glycosylation (CDG) (Freeze et al

Corresponding author: Erik A Eklund, MD, PhD, Clinical Sciences, BMC D12, 221 84 Lund, Sweden, erik.eklund@med.lu.se.

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2012; Freeze et al 2014). The most common glycosylation pathway to be disrupted, asparagine (N)-linked glycosylation, today has over 50 known subtypes (Scott et al 2014) the most common being PMM2-CDG (former CDG-Ia) (Barone et al 2014; Monin et al 2014). The phenotypes of CDG patients vary considerably both between and within a given subtype, but often include neurological involvement (developmental delay/intellectual disability, cerebellar hypoplasia, epilepsy), liver disease, coagulopathy, intestinal disease and hormonal dysregulation (Freeze et al 2015). N-linked glycosylation of proteins is preceded by an ER-located synthesis of a 14-sugar lipid-linked oligosaccharide precursor (LLO), consisting of two N-acetylglucosamine (GlcNAc), nine mannose (Man) and three glucose (Glc) residues. The lipid (dolichol (Dol)) bound oligosaccharides are co-translationally transferred from Dol to asparagine acceptor sites of the protein. *ALG1* encodes the first mannosyltransferase in the LLO synthesis (Kranz et al 2004) and its disruption leads to the accumulation of GlcNAc₂-P-P-Dol glycolipids (Grubenmann et al 2004; Schwarz et al 2004). This decreases the amount of full length LLO and, as a consequence, leaves many unoccupied glycosylation sites on proteins. To date, 17 patients with ALG1-CDG (OMIM 608540) have been described in the literature (Grubenmann et al 2004; Kranz et al 2004; Schwarz et al 2004; Dupre et al 2010; Morava et al 2012; Rohlfing et al 2014). Many of the described patients present with a very severe phenotype with early fatality whereas others show psychomotor disability, microcephaly, seizures and coagulopathy, however with a rather stable clinical course (Jaeken et al 2015).

The first step in the diagnostic procedure in a suspected CDG case is usually isoelectric focusing (IEF) of serum transferrin (TF). A vast majority (>95 %) of the TF molecules in the healthy individual carries two N-linked complex type chains, where each chain terminates in two negatively charged sialic acid (NeuAc) residues. If TF is under glycosylated this is reflected by loss of one or more NeuAc (i.e. loss of one or more charges). More recently, the use of mass spectrometric analysis of TF has been introduced in some facilities. This method can distinguish between the absence of entire glycan chains or of individual monosaccharides (Lacey et al 2001). These methods are only useful to diagnose protein N-glycosylation disorders and subgroup them into type I (disruption of LLO synthesis) or type II (deficient trimming and modification of the protein-bound oligosaccharide).

In the analysis of a patient with ALG1-CDG, using the mass spectrometric approach, we unexpectedly found a mass peak suggesting the substitution of a tetrasaccharide (NeuAc-galactose (Gal)-GlcNAc₂) for the mature glycan on a small fraction of the hypoglycosylated serum TF molecules. The structure was partially confirmed using *exo*-glycosidases directed toward NeuAc and Gal. Our initial findings were then expanded to ten more patients with known ALG1-CDG, who all had this tetrasaccharide on TF. In addition, four out of eight PMM2-CDG patients showed a significant but much lower amount of this aberrant substitution. In contrast, no control patients, patients with suspected CDG (i.e. IEF or H.P.L.C TF analysis indicative of CDG), or patients with RFT1-CDG or MPI-CDG showed this tetrasaccharide modification. We therefore argue that mass spectrometry is useful in the direct subtype diagnostics of ALG1-CDG.

Material and Methods

Sera from patients with ALG1-CDG (n=11), PMM2-CDG (n=8), RFT1-CDG (n=4), MPI-CDG (n=2) and from patients with suspected CDG (i.e. patients with an abnormal serum TF IEF) (n=20) were obtained after their legal guardians signed informed consent. The samples were de-identified to the person analyzing the mass spectral data. De-identified CDT-positive sera (i.e. sera with an increased ratio of disialylated/total TF) samples (n=100) were obtained from a clinic, which routinely analyzes hypoglycosylation of TF as an indicator of alcoholism.

POROS-aldehyde self-pack medium was purchased from Applied Biosystems. Polyclonal rabbit anti-human TF used in the immunoaffinity column was purchased from Dako. α 2-3,6,8 neuraminidase (EC 3.2.1.18) 50 U/ μ L and β 1,4 galactosidase (EC 3.2.1.23) 8 U/ μ L (New England BioLabs). All other chemicals and solvents were of the highest analytical LC-MS grade available from commercial sources and were used without further purification.

Immunoaffinity column preparation

Polyclonal anti-TF antibodies were conjugated to POROS-aldehyde self-pack medium according to the manufacturer's instructions. Antibodies (1 mg) were coupled to each 0.1 g of medium. After antibody coupling, the medium was stored in PBS containing 0.01 % (v/v) sodium azide at 4 °C. The medium was packed in empty 20 $\times$ 1 mm pre-columns bought from Upchurch.

LC-MS analysis of TF

Serum was diluted 1:2 with PBS pH 7.4, transferred to sample vials and kept at + 12 °C. Method development and validation were performed using a Thermo Scientific Q Exactive quadrupole-orbitrap mass analyzer. The method (Tham et al 2015) is a refined version of a previously published method (Lacey et al 2001). A 15 μ L aliquot of sample was applied to the immunoaffinity column in PBS (pH 7.4) at a flow rate of 200 μ L/min for 2 min. Non-binding serum components, were diverted to waste. Enriched TF were subsequently eluted from the immunoaffinity column for 2 min with 100 mM glycine containing 2 % formic acid (v/v) flow rate 200 μ L/min, and concentrated on an analytical Thermo Scientific C4 monolith column, Proswift RP-4H 1 $\times$ 50 mm. The analytical C4 column was washed with water/MeCN 95/5 %, containing 1 % (v/v) formic acid at 200 μ L/min for 2 min, before a gradient was applied with increasing ratio of MeCN containing 1 % formic acid (v/v). TF eluted from the C4 column at ~80 % MeCN and was introduced into the ion source. Total instrument acquisition time, 11 min/sample. The mass spectrometer was operated in full scan mode. For MS2, the mass spectrometer was operated in full scan or data-dependent-HCD-MS2 mode. MS2 spectra were acquired by data-dependent acquisition (resolution setting 35000 at 200 m/z) with a 2 m/z isolation window together with a target ion inclusion list for specific charge states.

Enzymatic digestions

For removal of terminal NeuAc, 5 μ L of patient serum was incubated with 30 U α 2-3,6,8 neuraminidase in 0.5 mM CaCl₂, 5 mM sodium acetate, pH 5.5 in a total volume of 34 μ L

for 40 minutes at 37 °C. For removal of terminal NeuAc plus the adjacent Gal, 5 µL of patient serum was incubated with 25 U α 2-3,6,8 neuraminidase and 24 U β 1,4 galactosidase in 0.5 mM CaCl₂, 5 mM sodium acetate, pH 5.5 in a total volume of 37 µL for 96 hours at 37 °C.

Statistics

The non-parametrical Mann-Whitney U-test was used to determine statistical significance between the different subtypes.

Results

Analysis of serum from the index patient showed a typical mass spectrometric pattern for CDG type I with 141 % mono-glycosylated/di-glycosylated TF (ref value < 10 %) and 109 % non-glycosylated/di-glycosylated TF (ref value < 5 %) (Fig. 1C and 1D; for comparison, a spectra from a healthy individual is included in Fig 1A and B). In addition, two peaks (76000 and 78207 Da) were visible and each was ~3 % of the total amount of TF. These peaks correspond to a TF carrying zero (75142 Da) or one complex chain (77348 Da) + 859 Da. In order to test whether other ALG1-CDG patients show these extra peaks, sera from ten more ALG1-CDG patients were analyzed and all showed these additional peaks, with a ratio over TF isoforms without the tetrasaccharide of 3.54 ± 1.99 % (range 0.8-6.7%). Four of the samples from PMM2-CDG patients, contained a detectable amount of these aberrant TF glycoforms (range 0-1.3 %) (Fig. 1E and 1F), whereas all other sera analyzed lacked these glycoforms (see table 1 and Fig. 3 for data summary). This novel mass (859 Da) theoretically corresponds to the combined mass of two N-acetylhexosamines (HexNAc) ($203 \text{ Da} \times 2$), one hexose (Hex) (162 Da) and one NeuAc (291 Da). In order to enzymatically explore the nature of these aberrant peaks, we next incubated serum from the patients with either 2-3,6,8 neuraminidase alone or in combination with β 1,4 galactosidase. Digestion with neuraminidase completely shifted the peaks to 75710 Da (loss one NeuAc) and 77916 Da (loss of three NeuAc) proving that the terminal monosaccharide is a NeuAc (Fig 2B). In addition, when β -galactosidase was present, there was a further partial removal of 162 Da, indicating that the penultimate sugar is a Gal (Fig 2C). Full removal of this monosaccharide was never achieved despite prolonged digestions (up to 96 hours), however there was a steady increase in the de-galactosylated peaks over time. To further analyze the nature of the additional peak, top-down MS2 was employed. Several charge states corresponding to 76000 Da were chosen for fragmentation, due to their strong signal to noise ratios and lack of interfering peaks. This analysis clearly showed peaks corresponding to the fragment ions of the suggested tetrasaccharide, i.e. 292 Da (NeuAc), 366 Da (HexNAc-Hex), and 657 Da (HexNAc-Hex-NeuAc). According to the literature, using the chosen fragmentation technique (collision with nitrogen gas), the innermost GlcNAc remains bound to the amino acid chain and hence no oxonium ion of the full length tetrasaccharide was detected (Segu and Mechref 2010). Data from charge state 37 (2055.09 m/z) is shown as a typical example (Fig S1A and B).

Discussion

Screening for serum TF underglycosylation is often the initial step in the metabolic investigation when deficient N-glycosylation is suspected (Eklund and Freeze 2006). This is often done by IEF, which is a stable and reliable method. With a few exceptions, an abnormal IEF indicates abnormal glycosylation and is diagnostic of CDG. It also indicates whether the defective step is located before or at the transfer of the oligosaccharide to the protein (type I) or after it has become protein-bound (type II). However, it does not indicate the specific gene defect. Analyzing TF glycosylation by mass spectrometry is more accurate as it gives the exact mass of the intact protein and indicates if abnormal glycans are present. This is especially important for type II defects because it can indicate whether sialylation, galactosylation *et cetera* are deficient.

In this paper we show the surprising finding that also a specific type I CDG (ALG1-CDG) disorder can be suspected early on in a routine CDG screening. Sera from eleven ALG1-CDG patients all showed a portion of TF molecules with type I features (loss of one or two complex chains) but with the additional substitution of an 859 Da molecule. Theoretically, this mass adds up to a tetrasaccharide containing two HexNAc, a Hex and a NeuAc. Using enzymatic digestions, we show that the two outer sugars are NeuAc-Gal. Top-down MS2 shows that the Gal is attached to a HexNAc, why the mass of the remaining reducing end corresponds to another HexNAc. It is very tempting to speculate that the two inner HexNAc moieties are GlcNAc, i.e. the tetrasaccharide is NeuAc-Gal-GlcNAc₂. To produce such a structure, we suggest the following biosynthetic pathway (Fig. 4): 1) ALG1-deficient cells produce GlcNAc₂-P-P-Dol, which is the substrate for ALG1-catalyzed mannosylation, as previously shown (Grubenmann et al 2004). 2) This structure flips to the lumen of the ER, possibly by the flippase encoded by RFT1. 3) The GlcNAc₂-P-P-Dol is transferred to newly synthesized proteins using the oligosaccharyltransferase, which normally prefers full length LLO (Glc₃-Man₉-GlcNAc₂-P-P-Dol) but can use other substrates as well (Vleugels et al 2009). 4) Once the proteins move into the Golgi the protein-bound GlcNAc₂ serves as an acceptor for galactosylation by β 1,4-galactosyltransferase. 5) The Gal-GlcNAc₂ is further elongated with NeuAc by a Gal-recognizing sialyltransferase, completing the structure. In theory all CDGs that decrease the available pool of GDP-Man (including PMM2-CDG, PMI-CDG and GMPBB-CDG) could also cause the accumulation of GlcNAc₂-P-P-Dol and hence show aberrant glycosylation with the tetrasaccharide. Indeed we did notice a small but statistically significant peak indicating the presence of a tetrasaccharide on TF from four out of eight PMM2-CDG patients, however at a significantly lower amount than what was found in ALG1-CDG patient sera. Unfortunately, only two sera from MPI-CDG patients not under mannose therapy were available, but in these, no signs of the additional tetrasaccharide were seen. Further, sera from four RFT1-CDG patients were devoid of the modification as were all (n=100) of the CDT-positive sera from patients with excessive alcohol consumption.

The ALG1-CDG patients all had a moderate to severe phenotype with one exception (clinically mild). The milder patient, however, had the second highest level of tetrasaccharide modification, suggesting that also modestly affected ALG1-CDG patients will be detected by this method. However, this notion will have to be addressed further in the future. The sequencing of the *ALG1* gene is known to be difficult as it has several

pseudogenes (Morava et al 2012) and there is a need for a method to suggest direct Sanger sequencing of this gene, instead of relying on high throughput genetic methods. So far we have not found a single confirmed ALG1-CDG patient that tested negative for this marker, implying that it may be used as a discriminator of disease-causing and non-causing mutations in the ALG1 gene. In fact, one of the control patients was suggested to be an ALG1-CDG by a clinical exome-sequencing center (without prior TF analysis), but lacked the tetrasaccharide marker on TF by our method and further enzymatic studies showed normal activity of the *ALG1* encoded mannosyltransferase. As the number of confirmed ALG1-CDG patients has risen quickly, often with a phenotype resembling PMM2-CDG, we suggest the following scheme for the investigation of a potential CDG type I patient: 1) Serum TF analysis by IEF, CDT or mass spectrometry; 2) If possible, confirmation of the result using mass spectrometry; 3) Analysis of PMM2-activity in fibroblasts or white blood cells; 4) If mass spectrometry indicates the presence of the marker tetrasaccharide, Sanger sequencing of ALG1; 5) If steps 3 and 4 yield a normal result, perform a CDG gene panel analysis or untargeted exome sequencing.

In summary we show that a very small, but detectable portion of serum TF from all eleven patients with confirmed ALG1-CDG substitute a tetrasaccharide for the normal large glycan chain, possibly reflecting the nature of the deficient step in this particular subtype. We suggest it may be useful as a biomarker of ALG1-CDG and that mass spectrometric analysis of serum TF directly can inform the physician of an ALG1 deficiency prior to genetic confirmation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Synopsis

Finding the tetrasaccharide NeuAc-Gal-(GlcNAc)₂ on under glycosylated transferrin is indicative of an ALG1-CDG genotype of the patient.

Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all patients for being included in the study.

Representative mass spectra (A, C, E) and mass spectra after deconvolution (B, D, F) for control (A and B), ALG1-CDG patient (C and D) and PMM2-CDG patient sample (E and F). The glycan structures of relevance are depicted next to the peaks. Black squares = GlcNAc; grey circles = Man; white circles = Gal; black diamonds = NeuAc.

Fig 2. LC-MS analysis of neuraminidase or galactosidase treated intact TF

Deconvoluted mass spectra of CDG-ALG1 serum without (A) or with (B) neuraminidase treatment or a combination of neuraminidase and galactosidase (C). Structures of interest are indicated (black squares = GlcNAc; grey circles = Man; white circles = Gal; black diamonds = NeuAc).

Fig 3. Dot-plot presentation of the ratio of tetrasaccharide-modified TF to non-modified TF
 The percentage of TF molecules modified with the aberrant tetrasaccharide in patients with ALG1-CDG (closed diamonds, n=11), PMM2-CDG (closed triangles, n=8), RFT1-CDG (closed circles, n=4), PMI-CDG (closed squares, n=2), suspected CDG according to IEF or CDT (open circles, n=20) and CDT-positivity post excessive alcohol consumption (open diamonds, n=100) are shown. The percentage was calculated as the ratio of all TF molecules carrying the tetrasaccharide/all TF molecules not carrying the tetrasaccharide. An arrow indicates the position of the sample described in figure 1C and D. ** indicates $p < 0.01$.

Fig 4. Suggested biosynthetic pathway of the tetrasaccharide in ALG1-deficiency

Relevant steps of the lipid-linked oligosaccharide (LLO) biosynthesis are indicated. After the deficient step (1.), normal LLO/protein bound oligosaccharides are depicted in black, white and grey whereas the biosynthetic pathway of the tetrasaccharide is in color. 1. Deficiency of the ALG1 catalyzed mannosylation causes the accumulation of GlcNAc₂-P-P-Dol and lack of Man-GlcNAc₂-P-P-Dol. 2. The GlcNAc₂-P-P-Dol is flipped to the inside of the ER, presumably by the RFT1-encoded "flippase". 3. The GlcNAc₂-Dol serves as a donor substrate for the oligosaccharyltransferase (OST) forming N-linked chitobiose. 4. The chitobiosylated protein is recognized by a β 1,4-galactosyltransferase (GalT) in Golgi forming Gal-GlcNAc₂-protein. 5. The structure is finalized by the addition of NeuAc by the action of a sialyltransferase (SiaT).

Table 1

The table summarizes the quantification data on all ALG1-CDG and PMM2-CDG patients included in this study. The first two columns show the amount of disialo (lacking one full chain) and asialo (lacking both chains) TF as a general indicator of underglycosylation. The reference values are indicated. Columns 3-4 show the ratios of TF carrying the tetrasaccharide to its non-tetrasaccharide carrying counterparts and column 5 shows the total ratio (in %) of tetrasaccharide carrying species to non-tetrasaccharide carrying species.

	Asialo	Disialo	Tetrasaccharide (%)		
Structures	1/3	2/3	4/1	5/2	(4+5)/(1+2+3)
Ref. values	<0.05	<0.10			
ALG1	0.57	2.16	3.5	4.1	2.9
	0.28	0.77	5.2	5.9	2.9
	12.26	6.40	3.4	0.3	2.2
	0.58	2.21	2.6	2.9	2.1
	0.45	1.65	4.1	3.4	2.4
	0.23	1.68	1.4	1.3	0.8
	0.09	0.50	2.7	5.8	2.0
	3.94	6.14	11.0	3.1	5.7
	4.40	6.71	8.4	2.3	4.3
	5.03	8.90	11.8	4.5	6.7
PMM2	1.08	2.67	9.4	3.7	4.2
	0.09	0.57	18.1	0.0	0.9
	0.09	0.41	0.0	0.0	0.0
	0.44	1.52	8.5	0.0	1.3
	0.44	1.78	0.0	0.0	0.0
	0.62	2.21	2.1	0.0	0.3
	0.85	1.74	2.3	0.9	1.0
	0.00	0.12	-	0.0	0.0
	0.09	0.32	0.0	0.0	0.0
Schematic structures	1	2	3	4	5

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