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Transferrin D Protein Variants in the Diagnosis of Congenital Disorders of Glycosylation (CDG)

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Congenital disorders of glycosylation are a rapidly growing group of inherited (neuro)-metabolic disorders characterized by defects in glycosylation of proteins and lipids. This study discusses an analytical problem in the differentiation between hypoglycosylation and transferrin (Tf) protein variants. Analysis of serum Tf by isoelectric focusing is used as a common method suitable for screening 19 out of a total of 22 types of glycosylation defects identified so far. In three members of a family, several indicators showed evidence of a Tf protein variant, however, routine neuraminidase-based demonstration failed to confirm this result. On the assumption that we should be able to exclude Tf protein variants at the screening-level of the diagnostic algorithm, our concern is a possible cause of our failure to confirm some of the Tf D variants (in contrast to the other C, B and D allelic combinations that are commonly well identified). Several explanations are discussed. J. Clin. Lab. Anal. 23 : 77–81, 2009. © 2009 Wiley-Liss, Inc.

Key words: inherited metabolic disorders; isoelectric focusing; hypoglycosylation; protein variant

INTRODUCTION

Transferrin (Tf) is the major iron-binding glycoprotein in human plasma. It consists of a single polypeptide chain with two homologous N-terminal and C-terminal iron-binding domains and two *N*-(asparagine)-linked glycans.

Analysis of serum Tf by isoelectric focusing (IEF) is used as a common screening method for a rapidly growing group of (neuro)metabolic, autosomal recessive congenital disorders of glycosylation (CDG, *N*-glycosylation defect). Considering the great number of CDG types (two main types and 22 subtypes: CDG Ia-In and CDG IIa-IIh) including those as yet unrecognized (CDG-x), an aberrant Tf result detected on IEF may indicate a potential glycosylation defect even in a subject clinically nearly asymptomatic (1).

Tf shows large microheterogeneity, attributable to various Tf Fe^{3+} loads, different *N*-glycan chains and protein (genetic) Tf variants with a modified core polypeptide.

Incompletely iron-saturated serum Tf forms (Fe0-Tf and Fe1-Tf, normally accounting for about 60% of total Tf) may result in additional bands (co-focusing effect) and thus present a cause of interpretation difficulties. This effect can be prevented by pre-analytical saturation of the sample with iron converting all forms to Fe2-Tf (2).

Tf also separates into several isoforms based on the differences in carbohydrate site units (with a different content of negatively charged terminal sialic acid residues) attached to the polypeptide chain. Apart from the normal Tf–IEF pattern, where the isoform with four sialic acid residues prevails, several CDG-pathognomic patterns, typically combining an increase in at least two of the less sialylated isoforms (asialo-, monosialo-, disialo-, and trisialo-Tf), can be recognized (1) (Fig. 1A).

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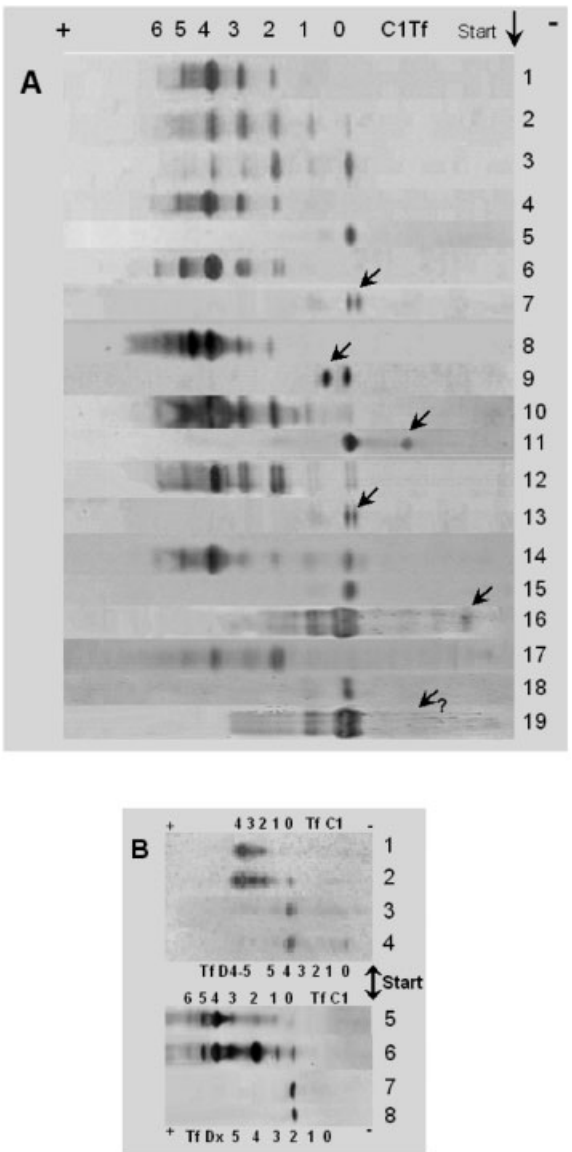

Fig. 1. IEF with direct immunofixation and Coomassie blue staining; 1.5 μL of iron-saturated (diluted five times with water) serum sample was applied; N digested with neuraminidase, diluted 1 in 39; N 40, digested with neuraminidase, not diluted (4 μL applied when neuraminidase-treated). Horizontally: isoforms of Tf C1 and Tf D2 and Tf D-x (unknown variant) are indicated; 0–6, a-, mono-, di-, tri-, tetra-, penta-, and hexa-sialo-Tf. (**A**) Immobiline DryPlate gel, pH ranges 4–7. Vertically: **1, 4, 5**—controls, TfC1C1; **2**—CDG type II, TfC1C1; **3**—CDG type I, TfC1C1; **5, 7, 9, 11, 13, 15, 18**—neuraminidase treated samples, diluted 40 times; **16, 19**—neuraminidase treated samples, not diluted; **6, 7**—healthy control, TfC1C2; **8, 9**—healthy control, TfC1B1-2 ; **10, 11**—healthy control, TfC1D-2; **12, 13**—our CDG II-x patient, TfC1C2; **14, 15, 16**—Case 1 (and his father, the same pattern), TfC1D4-5; **17, 18, 19**—Case 2 (his mother and his grandmother, the same pattern), C1D? Arrows indicate a band of the second Tf allele; ?—estimated position. (**B**) CleanGel with ampholyte pH ranges 3–10. Vertically: **1, 5**—controls; **3, 7**—controls, neuraminidase treated; **2**—Case 1; **4**—Case 1, neuraminidase treated; **6**—Case 2; **8**—Case 2, neuraminidase treated.

In CDG diagnostics, however, one should also be aware of differences in the Tf protein structure (more than 38 Tf protein variants are known to exist that are products of codominant alleles at a single autosomal locus). The most frequent variant is Tf C (Tfs C1 and C2 account for the majority of forms in all races), whereas the more anodic Tf B and more cathodic Tf D variants are less common (3). The homozygous allelic C, B, and D set that can be differentiated on the basis of a shifted electrophoretic position (4,5) usually do not pose any serious diagnostic problem. On the other hand, the heterozygous combinations, namely the Tf CD variants, may lead to misinterpretation caused by co-focusing of various shifted isoforms (sometimes of only slight pI differences).

Several experimental techniques can be used for differentiation between Tf protein variants and CDG already at the screening level of the investigation: These include digestion with neuraminidase or changing the pH range of gel, sample volume, application point for IEF and the staining method. An important indicator is also correlation with family history: in contrast to the mostly normal IEF pattern in CDG-heterozygotes, in the case of a rare Tf protein (genetic) variant detected in a child, the same IEF pattern will be found in one of the subjects' parents.

Under certain circumstances, however, none of these approaches is discriminative enough.

MATERIALS AND METHODS

More than 1,500 subjects including unrelated healthy controls (blood-donors or patients scheduled for surgery) and patients (investigated for various reasons, mostly for a suspected inherited metabolic disorder), children and adults aged 6 weeks–55 years were screened for the evaluation of Tf hypoglycosylation as well as Tf protein variants. An informed consent/patient agreement was obtained in each case and the institutional ethical board approved the study. Sera (or dry blood/serum spots) were obtained by a standard venipuncture and supernatant separation (or dry-blood spotting (6)) techniques. All samples were stored at −20°C before analysis.

A common IEF method used for Tf (and possibly also for α_1-antitrypsin, aAT) analysis with direct immunofixation was described earlier (7). In short, a Pharmacia Multiphor apparatus cooled to 12°C, Immobiline DryPlate gels pH 4–7 and 5–6 or CleanGel IEF with Ampholyte pH 3–10 (GE Healthcare, Uppsala, Sweden), PhastGel Blue R (Coomassie brilliant blue R-350, Amersham Biosciences, Uppsala, Sweden) staining and densitometric evaluation were used for the CDG screening. Tf and aAT antibodies were supplied by

DAKO Cytomation (Glostrup, Denmark, No A0061 and No A0012, respectively).

For Tf analysis, all samples were iron-saturated with Fe-citrate and diluted 1 in 5, as described in (7). The IEF procedure was further amended by pre-treating the sample with neuraminidase (from Cl. perfringens; enzyme No N-2876 supplied by Sigma-Aldrich, Germany) for Tf and aAT protein variant identification, when necessary (after the sialic acid removal by enzymatic cleavage, all isoforms are reduced to single asialo- bands at the position corresponding to the relevant protein variant of a glycoprotein) (7,8) (Fig. 1).

Tf and aAT standards (Sigma-Aldrich, No T0665 and A6150, respectively) were used for isoform identification. Tf protein variants were checked by comparing the band positions with the literature data acquired under the same IEF conditions (4), and by ion-exchange HPLC on an Agilent series 1100 HPLC system and a ResourceQ column (Amersham/Bioscience), using a Bis-Tris/NaCl gradient and detection of the iron-Tf complex at 460 nm (9).

All the details of the IEF method have been published before, only slight modifications are mentioned here: usually a 1.5 μL of saturated (and then diluted 1 in 5) serum or 5–10 μL of an eluate from a dry serum/blood spot (saturated, not diluted) and 4 μL of neuraminidase-treated (diluted 40 times) serum samples were applied. For better visualization of some rare variant faint bands, larger volumes and undiluted enzyme-treated samples were also used. Gels with pH ranges the most suitable for the analysis of the respective Tf protein variants (pH 4–7, 5–6 or 3–10 for Tf C, Tf B, and Tf D, respectively) were used in some doubtful cases. Apart from Coomassie blue, more sensitive silver staining (10) was also applied in an effort to visualize some minor isoforms.

In all subjects showing an abnormal Tf IEF pattern, investigation was also extended to include other family members, namely the parents.

RESULTS

In our set of surveyed individuals, two common (ClC1 in 79% and C1C2 in 19%) and five rare (C2C2, C1C3, C1B1-2, C1D2, and C1D4-5, 2%) Tf protein variants were identified.

Mild (secondary) glycosylation abnormalities associated with specific diagnoses, e.g., Hashimoto thyroiditis, Budd–Chiari syndrome, systemic lupus erythematosus, liver diseases, or treatment with antiepileptics were detected in about 6.2% of the total group of patients (7). Serum from several CDG-symptomatic children was analyzed, showing typical Tf IEF patterns, indicative of hypoglycosylation (samples from CDG Ia patients kindly provided by another laboratory and from our patient with CDG type II) (Fig. 1A).

Besides these, a markedly abnormal IEF pattern was found in two males without any CDG-typical clinical attributes. Even though several indicators led to the diagnosis of a rare Tf protein variant instead of CDG, reliable confirmation (based on the detection of a double-band pattern on the IEF gel after digestion with neuraminidase) could not be achieved or was not convincing enough.

Case 1. A distinct isolated increase in the asialo-Tf C1 band was found in a 15-year-old male with rheumatoid arthritis, tested for a possible long-term therapy effect on protein glycosylation, as noted previously (7,11). This laboratory finding as well as the clinical picture, none of them attributable to any of the known CDG types, implied a Tf protein variant (we supposed that tetrasialo-Tf D, owing to a shift, focused to the position of asialo-Tf C1). The same Tf IEF pattern was also found in the patient's father, whereas aAT, another serum glycoprotein, showed a normal IEF pattern in all family members. Total serum levels of both Tf and aAT were within the reference ranges. However, in contrast with other Tf protein variants, giving two bands after digestion with neuraminidase, in this case, just one band, corresponding to the asialo-Tf C1 appeared after Tf desialylation. Finally, a very faint cathodic band of asialo-D variant was detected close to the position of sample application, however, only when more concentrated (neuraminidase treated) sample was applied (Fig. 1A), and larger pH ranges (3–10) of gel were used (Fig. 1B).

Case 2. An 8-year-old male was tested for CDG because of five attacks of cyclic vomiting between 4 and 6 years of age, requiring hospitalization and IV therapy. He is otherwise healthy (without typical CDG symptoms such as dysmorphy, mental retardation, coagulation defects, hepathopathy, or diarrhea combined with protein loosing enteropathy, etc.). Symptoms of CDG (including the frequently present female hypogonadism) are also absent in the patient's nonconsanguineous parents, younger twin sisters, and grandparents. As an identical Tf–IEF pattern (intensive disialo-isoform of Tf C1) was found in the boy, his mother, and his maternal grandmother, serum aAT was properly glycosylated and also serum levels of Tf, aAT and clotting factors IX and X were repeatedly normal, a rare Tf protein variant was suspected. However, aside from the asialo-Tf C1 band, no asialo-Tf D could be detected in the neuraminidase-digested serum of the patient or his mother (carrier of the same allele), regardless of sample concentration or the position of sample application. Neither a larger pH-range of the gel (e. g. pH 3–10, more suitable for Tf D variants as compared to pH 4–7, optimal for the

common Tf C) (Fig. 1B), nor a sensitive silver staining could reveal the shifted cathodal D-band. Enzyme tests for the most common CDG type Ia (phosphomannomutase) and clinically the most likely type Ib (mannose phosphate isomerase) (7) were normal. In addition to that, no pathologic mutation in the genes for CDG Ib and CDG Ia was found in the patient's mother, which showed identical IEF abnormality. In the last 15 months, the boy is without any health problems.

As the patient's parents refused further investigation, more detailed analyses of Tf cannot be performed.

DISCUSSION

In the Tf-based CDG screening, Tf B, and Tf D may lead to diagnostic misinterpretation, either as a result of a false negative owing to "missing" hypoglycosylated bands, shifted anodally in a CDG subject carrying a Tf B variant, or a false positive because of the normal tetra- and pentasialo-Tf bands that shift cathodally to the "hypoglycosylated zone of Tf C" in a healthy carrier of Tf D variants (12).

This effect of variation in the protein portion of Tf on the IEF pattern can usually be discerned by various techniques. The described abnormality may have resulted from the following:

(1) Unknown pre-analytical factors could have been involved, although sample storage and iron-saturation were carefully checked, avoiding use of urea or mercaptoethanol, reportedly influencing proteins during IEF.
(2) Instability of some rarer Tf D variants (13,14), which includes increased susceptibility to proteolysis during IEF.
(3) Altered immunoreactivity of Tf D variant to commonly used antibodies as seen with some other glycoproteins (15).
(4) Another protein, migrating to the position of Tf and cross-reacting with the commonly used antibodies, which would be similar to a case reported in (16).
(5) A hypoglycosylation defect on a rare Tf D variant, when replacement of amino acid involves one of the branching positions resulting in a loss of the entire carbohydrate chain; such an abnormal hypoglycosylated Tf variant was found among the Japanese and New Zealand populations (17).
(6) Variation in the level of expression of some Tf variants thus falling under the detection limit; similarly, an unequal expression of some combined Tf alleles was reported in the horse (18).

Other potential secondary causes of hypoglycosylation in our patient, such as galactosemia, fructose intolerance, viral hepatitis C, alcohol abuse, or sepsis with increased bacterial production of neuraminidase, were excluded.

CONCLUSION

In rare cases, some Tf variants are not easily identified. We discuss the causes of failure to demonstrate Tf D variant in the context of the presented cases, where the diagnosis of CDG is highly improbable.

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