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Case report

High CDT without clinical context: Beware of the variant

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ABSTRACT

Carbohydrate-deficient transferrin (CDT) is a performant biomarker used for the diagnosis of chronic alcohol abuse. Here, we describe the case of a 39-year-old male of Tamil ethnicity who had extremely elevated (20%) CDT using capillary electrophoresis (but without glycoforms profile analysis), putting his driving license regranting at risk. However, the patient had no symptoms of chronic alcohol abuse, normal mean corpuscular volume and gamma-glutamyl transferase, and did not admit to any alcohol consumption. Re-analysis by *N*-Latex CDT immunoassay revealed a CDT at 1.7%. Further investigation by whole-exome sequencing revealed a c.1295A>G missense variant at the heterozygous state on the *TF* gene. This variant is characterized by an amino-acid change at a consensus sequence for *N*-glycosylation. Therefore, half of the patient transferrin proteins were lacking a complete *N*-glycan chain out of two, despite no alcohol consumption. This also explains the discrepancies between the techniques, as the NLatex antibodies did not recognize the mutated sequence. In conclusion, this case highlights the importance of comparing laboratory results between themselves and the clinical description, the absolute requirement for glycoforms profile analysis before delivering results, and the necessity to confirm intriguing results by another technique in a specialized laboratory.

Case description

Carbohydrate-deficient transferrin (CDT) is a biomarker commonly used in forensic medicine to detect chronic alcohol consumption [1]. In healthy individuals, transferrin (Tf), typically exhibits two *N*-linked glycan chains with two terminal sialic acid (SA) residues (i.e., tetrasialoTf). In the context of chronic alcohol consumption, a partial or total loss of *N*-glycan chains induces a shift in Tf glycoforms, with a decrease in tetrasialoTf and the increases of di- and asialoTf fractions [2], the sum of both being designated as CDT [3]. The sensitivity and specificity of CDT as a marker of alcohol intake are superior to historical biomarkers such as gamma-glutamyl transferase (GGT) and mean corpuscular volume (MCV), which lead to an important growth in its use [4].

Here, we describe the case of a 39-year-old male of Tamil ethnicity living on the French island of La Réunion who was tested for CDT in the context of a driving license regranting. A CDT value of 20%, which is unusually high (IFCC reference <2%) [3], was first reported by a local private laboratory using capillary electrophoresis (CE) of Tf. In CE of Tf, glycoforms are separated with a high-voltage electrical current following saturation with iron, to ensure that the only cause of charge variation should be the difference in the number of terminal SA residues. Separated glycoforms are then detected with a UV–visible spectrophotometer at 200 nm [5].

In light of this strongly abnormal result [3], a control was performed using the same CE method in a private metropolitan France laboratory, reporting equivalent results. We were then contacted by the case's gastroenterologist, who asked us for advice as a laboratory of expertise regarding CDT analysis. The discussion revealed the denial of alcohol intake by the case, the absence of any symptom evocative of alcohol abuse, and normal hepatic exploration. Withdrawal of the driving license was originally due to speeding. However, due to the elevated CDT result, the regranting was delayed for months. Therefore, we asked the physician to send us a new sample for investigation.

First, we performed immunonephelometric measurement of CDT

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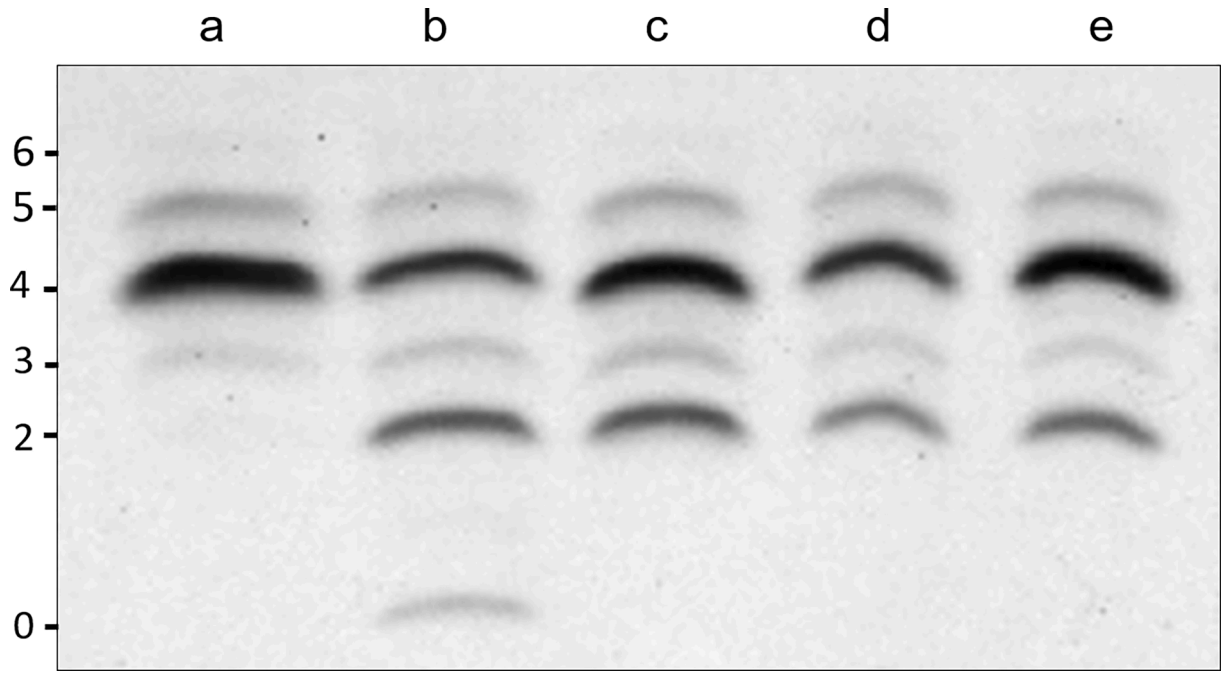

Fig. 1. Distribution of Tf glycoforms using Tf isoelectric focusing of the patients compared to control and a common Tf variant (a) Normal serum, (b) CDG-I (MPI-CDG), (c) Index case, (d) Brother of the index case, (e) Cousin of the index case. Left of the figure are the numbers of sialic acid residues present on the Tf.

with the N Latex CDT assay (Siemens® Healthineers, Marburg, Germany). In this assay, monoclonal antibodies specifically recognize Tf glycoforms lacking one or two complete glycan chains, by targeting empty *N*-glycosylation sites. Here, the patient's CDT value, expressed as the percentage of total Tf (% CDT), was measured at 1.7%. The manufacturer's reference is <2.5 % for CDT N latex (Siemens® Healthineers, Marburg, Germany) [6], while the IFCC reference is <2 % for HPLC [3]. This result was surprising, and led us to suspect the possibility of a Tf variant since numerous cases of teetotalers with elevated CDT due to Tf protein polymorphisms have previously been reported [7–13]. In this situation, an amino acid change will modify the charge of the protein backbone, altering profiles obtained with charge-based separation techniques. However, N Latex is insensitive to variants affecting the isoelectric point of the protein backbone, which would explain the discrepancy observed here [6]. Tf variants are not rare (they are believed to be found in about 1% of individuals in Europe) and are characterized by anodal (B type variant) or cathodal (D type variant) migration compared to the wild-type (C type variant) allele [14].

We then performed isoelectric focusing (IEF) of the patient Tf and also two other members of the family (Fig. 1) [15]. As with CE, IEF relies on the charge-based separation of Tf glycoforms and common Tf variants can be readily identified with this technique. [16]. Using IEF, the normal % values for Tf glycoforms were respectively 2 ± 2, 17 ± 7, 68 ± 13, 12 ± 7, and 2 ± 2% for hexasialo-, pentasialo-, tetrasialo-, trisialo-, and disialoTf. For the patient, an elevation of disialoTf (25.4%) **(**Fig. 1**, lane c**) was observed as compared to a healthy individual **(**Fig. 1**, lane a)**. Due to the absence of asialoTf, the pattern was different from the typical profile of a patient with a congenital disorder of glycosylation type 1 (CDG-I), a hereditary disease characterized by abnormal protein glycosylation **(**Fig. 1**, lane b)** (5). A similar profile was observed for the brother **(**Fig. 1**, lane d)** and the cousin **(**Fig. 1**, lane e)** of the index case, with an increase of disialoTf (respectively 20.5% and 21.8%) without asialoTf. Thus, this suggested that the elevated CDT value reported using CE could be only due to elevated disialoTf, without asialoTf. This was strongly evocative of a variant as in the situation of chronic alcohol consumption with such a high CDT value, asialoTf should have been detectable. Furthermore, similar IEF Tf profiles in family members were consistent with a hereditary trait.

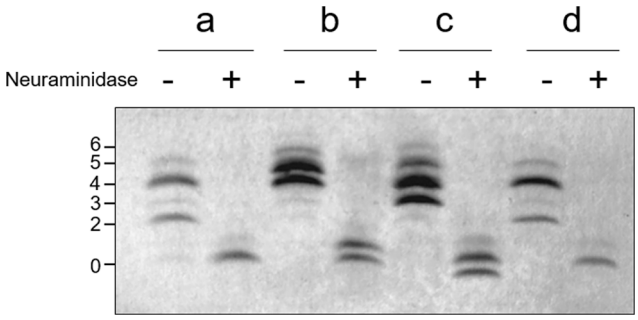

Fig. 2. Tf isoelectric focusing profiles before and after neuraminidase incubation (a) CDG-I profile, (b) Tf heterozygous variant BC, (c) Tf heterozygous variant CD, (d) Index case. Patients lanes a and are homozygous for Tf CC. Left of the figure are the numbers of sialic acid residues present on the Tf.

The second step in Tf variant characterization by IEF is to repeat the technique following neuraminidase treatment. This treatment removes the terminal SA residues of Tf-bound *N*-glycans. Therefore, resulting IEF profiles only reflect the protein backbone's charge, allowing discrimination of variants with modified charge: in an individual with a differently charged variant at the heterozygous state, two bands may be observed (the wild-type protein and the variant). Here, the index case presented only one band after neuraminidase treatment **(**Fig. 2**, lane d)**, comparable to a CDG-I **(**Fig. 2**, lane a)** but different from typical profiles of patients with heterozygous Tf variants (BC or CD) **(**Fig. 2**, lane b–c)** (9). The unique band for the index case thus suggested that the possible variant did not modify the electric charge of the protein backbone.

Therefore, considering all results, we raised the possibility of a variant affecting one of the two *N*-glycan binding sites of Tf but sharing the same charge as the wild-type amino acid. Such occurrences have previously been reported in the literature, particularly in the context of CDG diagnosis [10]. This would explain that charge-based separation techniques only show increased disialotTf, as half of the Tf proteins would be missing one of their two *N*-glycan chains. However, N Latex antibodies would not be able to recognize the free *N*-glycosylation site,

because of the sequence change.

Whole-exome sequencing was performed using next-generation sequencing MiSeq Illumina© technologies, which confirmed the presence of a heterozygous missense variant NM_001063.4(*TF*):c.1295A>G in the *TF* gene. This variant, previously reported [11], leads to a change of Asn^{432} located in the *N*-glycosylation site into a serine residue (p.Asn432Ser), resulting in Tf molecules carrying a single *N*-glycan instead of two. As suspected, this modification does not lead to a charge change, as both asparagine and serine are uncharged polar amino acids.

As the N Latex antibodies recognize three of the four *N*-glycosylation sites (two on the wild-type protein, one on the mutated protein), we can hypothesize that CDT measurement with this technique amounts to about 75% of the "real" one being about 2.1%. This would only be a valid possibility if the antibody has similar affinity for both *N*-glycosylation sites on one Tf protein, which is likely as the method was shown to be comparable with the reference HPLC technique [3,6].

Case discussion

This uncommon *TF* variant fully explains the results obtained using CE, immunonephelometry (the presence of a Ser^{432} residue prevents the antibody from recognizing the *N*-glycosylation site), and IEF with and without neuraminidase. A genetic report was sent to the physician mentioning a contra-indication of charged-based separative techniques (CE and HPLC) for CDT assay for the entire family. Though monoclonal antibodies used in the immunonephelometry method are unable to recognize one free *N*-glycosylation site out of four, the estimated % CDT value is nevertheless not suggestive of chronic alcohol abuse. This is the first report of the use of N Latex to both raise the question of a Tf variant affecting one of the *N*-glycosylation sites, and to provide a rough estimate of CDT in the case of the loss of a *N*-glycosylation site. Interestingly, the *TF*:c.1295A>G,p.Asn432Ser was previously reported by another team in an unrelated patient, indicating that this variant is not a unique occurrence. Here, both unspecialized laboratories who investigated the case only reported the % CDT value without interpretation of the electrophoretic profiles, which is however mandatory for CE or HPLC profiles as per ISO guidelines, and could have had major forensic impacts.

Therefore, in case of an unusually elevated CDT value, this report brings to our attention the absolute necessity, i) to interpret results in light of the clinical interpretation, medical history, and other laboratory data, and ii), to rapidly orientate the sample to a specialized laboratory to avoid potentially dramatic forensic consequences.

Nonstandard abbreviations

CDT (Carbohydrate-Deficient Transferrin); IEF (Isoelectric Focusing); CDG (Congenital Disorders of Glycosylation); GGT (Gamma-Glutamyl Transferase); Mean Corpuscular Volume (MCV); High-Performance Liquid Chromatography (HPLC).

Author contributions

All authors confirmed they have contributed to the intellectual content of this paper and have met the following 4 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; (c) final approval of the published article; and (d) agreement to be accountable for all aspects of the article thus ensuring that questions related to the accuracy or integrity of any part of the article are appropriately investigated and resolved.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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