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False low holotranscobalamin levels in a patient with a novel *TCN2* mutation

Abstract

Background: Measurement of holotranscobalamin (holoTC) is increasingly used as a screening test for cobalamin (Cbl) deficiency. A level well below the reference interval strongly supports a deficient state. We examined a 21-year-old woman diagnosed as Cbl deficient because of an extremely low holoTC level as measured by the Abbott Architect Assay.
Methods: The patient was evaluated for Cbl deficiency employing an in-house holoTC method as well as other routine markers of Cbl status. Further analyses included exploration of the Cbl binding proteins employing gel filtration of a serum sample saturated with 57 Co-labeled Cbl and Sanger sequencing of exons 1–9 and the intron-exon boundaries of the *TCN2* gene, the gene coding for transcobalamin (TC).
Results: The patient had normal hematological variables throughout. Despite initial treatment with Cbl, holoTC as measured by the Abbott assay remained low, while holoTC measured with the in-house assay was normal, and behaved as TC upon gel-filtration. By Sanger sequencing, we detected a homozygous single point mutation c.855T>A in exon 6 of *TCN2*, corresponding to a asparagine (Asn) to lysine (Lys) substitution in position 267 of the mature protein.
Conclusions: We describe a novel point mutation of the *TCN2* gene. The mutation does not seem to interfere with the function of TC, but the mutation may well explain the low level of holoTC detected by the Abbott assay. Our results underscores that mutations of *TCN2* have to be considered when implausible holoTC results are obtained.

Keywords: active B12; gene mutation; holoTC; immunoassay; *TCN2*; transcobalamin; vitamin B12.

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Introduction

Measurement of circulating holotranscobalamin (holoTC) (also named active B12) is increasingly employed as a first line screening of patients suspected to suffer from vitamin B12 (cobalamin, Cbl) deficiency, and a low value of this biomarker supports the presence of a deficient state [1–8]. HoloTC denotes the part of circulating Cbl bound to transcobalamin (TC), a protein that is essential for the transport of Cbl into the cells of the body [8–10]. The remaining part of circulating Cbl is bound to haptocorrin, a protein of unknown function [9].

Lack of TC is rare. The condition leads to severe Cbl deficiency most often diagnosed within the first year of life [11, 12], and is caused by mutations in the *TCN2* gene. So far, small insertion/deletion variants (indels), large deletions and mutations leading to exon skipping have been identified in TC deficient patients [13]. Healthy individuals also harbor variations in the *TCN2* gene. Specific SNPs are associated with minor differences in the plasma concentration of holoTC, but have not been associated with an impaired Cbl metabolism [5].

The most widely used test of holoTC, "active B12" is established on the high throughput Abbott architect platform. It employs a sandwich immunoassay based on a specific monoclonal antibody [14] that recognizes an epitope in the N-terminal part of TC, formed in the course of Cbl binding, but not directly involving Cbl as part of the epitope itself [15].

Here we describe a patient with undetectable or extremely low holoTC levels as measured by the Abbott assay, but with no other biomarker values suggesting Cbl

deficiency. We show the patient to harbor a mutation of the *TCN2* gene, likely to alter the epitope of the holoTC assay. A possible model of the conformational epitope is presented.

Materials and methods

Case report

A 21-year-old woman visited her GP complaining of several non-specific and not very severe symptoms including fatigue, asthenia, headache, sleeplessness, flatulence, acid regurgitation, moderate diarrhea, and paresthesia of the fingers. In addition, she presented mild psychiatric and family problems, both judged to aggravate the somatic symptoms. The final work up gave no somatic explanation for the experienced symptoms. Initial laboratory examinations revealed no major abnormalities except for an undetectable holoTC (<5 pmol/L, reference interval >40 pmol/L). Cbl, methylmalonic acid (MMA) or homocysteine were not requested. The patient was started on i.m. hydroxo-Cbl (VITARUBIN, Streuli Pharma AG), 1 mg, and was referred to the Hematology out-patient clinic, Bern University Hospital, Inselspital, Switzerland, for further evaluation of Cbl deficiency. Cbl injections had no impact on the clinical symptoms, and 1 month after the second i.m. injection of hydroxo-Cbl, holoTC remained low (6 pmol/L). At this point, the patient underwent extensive examination in order to clarify whether the low holoTC indeed mirrors Cbl deficiency.

Biochemical analysis

Blood samples were drawn for routine testing adhering to the routine of the laboratory. Additional blood samples were collected 7 weeks after the third and last injection of 1 mg hydroxo-Cbl for analysis of Cbl related variables and the serum was stored at –20 °C. Whole EDTA blood for isolation of DNA was stored at –80 °C. Shipment to the Department of Clinical Biochemistry, Aarhus University Hospital, Denmark, was performed on dry ice.

We used routine laboratory methods for evaluation of hematological variables (Advia 2120i Cellcounter, Siemens Healthcare, Zürich, Switzerland), Cbl and creatinine (Cobas 8000 system, Roche Diagnostics, Rotkreuz, Switzerland), homocysteine (HPLC, Agilent 1100 series, Agilent technologies, Basel, Switzerland), MMA (mass spectrometry, Agilent GC-MS 6890N/5973N, Agilent technologies, Basel, Switzerland), holoTC (AxSYM method on an Architect 2000 analyzer, Abbott AG, Baar, Switzerland), autoantibodies against intrinsic factor (ELISA, Alphadia, B1300, Wavre, Belgium) and autoantibodies against parietal cells (ELISA, nova Diagnostics, San Diego, CA, USA).

Total TC was measured by a sandwich ELISA assay employing two polyclonal antibodies specific for TC as described in [16]. HoloTC was in addition to the routine method measured by an in-house ELISA based on removal of apoTC by absorption to B12 coated beads followed by analysis of the remaining TC in the supernatant (expected to be equal to holoTC) [17]. Haptocorrin was measured by an in-house ELISA as previously described [18].

Gel filtration was employed to explore the molecular behavior of TC as described in [19]. In brief, serum labeled with 57 Co-Cbl (KemEnTec A/S, Taastrup, Denmark) was run on a Superdex® 200 HR 10/30 column (GE Healthcare Europe, Broendby, Denmark) on a Dionex® ICS-3000 chromatograph (Dionex Corporation, Sunnyvale, CA, USA) and was eluted with a flow rate of 400 μL/min for approximately 70 min employing a Tris buffer (0.1 mol/L) (Sigma Aldrich, Broendby, Denmark), 1 mol/L NaCl (Merck A/S Denmark, Hellerup, Denmark), 0.5 g/L bovine albumin (Sigma Aldrich), 0.2 g/L sodium-azide, pH 8.0 (Merck A/S Denmark). The eluted fractions were counted in a Wizard Automatic Gamma Counter to monitor the elution of 57 Co-Cbl. Molecular mass markers Blue Dextran (Sigma Aldrich) and 22 Na (GE Healthcare Europe) were used for determination of void volume (V_o) and total volume (V_t). Reference serum was used for determination of the elution volume of haptocorrin (V_{HC}), albumin (V_{alb}) and transcobalamin (V_{TC}).

Genomic DNA was isolated from EDTA blood using the Maxwell 16 Blood DNA Purification Kit (Promega Biotech AB, Stockholm, Sweden) following the manufacturer's instructions. The coding regions of the *TCN2* gene, encoded by exon 1–9, and the intron-exon boundaries, were amplified by PCR (primer sequences available on request). The quality of the PCR products were assessed by gel-electrophoresis using a 1.5% ethidium-bromide stained agarose gel (Ultra Pure™ Agaraose, Life technologies, Nærum, Denmark). PCR products were purified using the Exo-SAP procedure (Affymetrix, High Wycombe, UK). Amplicons were sequenced bidirectional using Big-Dye terminator version 1.1 (Applied Biosystems, Naerum, Denmark). The ethanol-precipitated fragments were separated on an Applied Biosystems 3500 Genetic Analyzer. The obtained sequences were aligned to the reference sequence NM_000355.2 using SeqScape (version 2.7, Applied Biosystems, Naerum, Denmark). Structural analysis was done using software PyMol (Schrödinger.com), Swiss-PdbViewer (Swiss Institute of Bioinformatics) and the available 3D structure of human holoTC [20].

The study was performed in accordance with the Declaration of Helsinki and the regulations of the local Ethical Commitee. The patient gave written informed consent.

Results

We report data on a patient initially treated with Cbl injections because of clinical symptoms suggesting Cbl deficiency combined with an unmeasurable level of holoTC. During Cbl treatment (total dose of 3 times 1 mg over a period of 4.5 months) the patient did not improve. She continuously showed low levels of holoTC, but normal levels for the hematological variables, total Cbl, MMA and homocysteine (Table 1). No autoantibodies against intrinsic factor or parietal cells were present.

In order to find an explanation for the low level of holoTC as measured by the routine Abbott Architect method, we explored the level of total TC and also measured holoTC employing the in-house ELISA methods [16, 17]. Total TC was 590 pmol/L and holoTC 50 pmol/L (Table 1). We examined the elution pattern of TC upon

Table 1: Laboratory data from a patient misdiagnosed as cobalamin deficient due to an initial low level of holoTC. Months after the first visit are indicated.

Component Reference interval in brackets	First visit[a]	3 months[b]	4 months[c]	4.5 months	6 months
B-Hemoglobin (121–156), g/L	151	163	157	158	158
S-HoloTC, Abbott (>40), pmol/L	<5		7.0	6.5	5.3
S-Cobalamin (141–489), pmol/L		565		563	592
S-MMA (76–271), nmol/L			148	105	
Homocysteine (<15), μmol/L				9	
S-Folate (10.4–42.4), pmol/L	18.5	10.6			
S-Creatinine (45–84), μmol/L	59.4			60	
S-Total TC in-house (610–1400), pmol/L					590
S-HoloTC, in-house (>40), pmol/L					50

[a]After first visit treated with 1 mg hydroxo-Cbl i.m.; [b]1 month after Cbl injection; [c]at the time of Cbl injection.

gelfiltration of a serum sample saturated with labeled Cbl, (Figure 1). The patient's serum showed distinct peaks eluting as TC and haptocorrin both judged by the elution profile for 57Co-Cbl and by ELISA assays of the eluted fractions.

Sequencing of TCN2, the gene encoding TC, revealed a homozygous single point mutation c.855T>A in exon 6, leading to an amino acid substitution p.Asn285Lys, corresponding to a asparagine (ASN) to lysine (LYS) substitution in position 267 of the mature protein. The variant is not present in the dbSNP (http://www.ncbi.nlm.nih.gov/snp/)

Figure 1: Gelfiltration of serum from a patient harboring a TCN2 mutation.
A serum sample was labeled with 57Co-Cbl to saturate unsaturated Cbl binding capacity and separated on a Superdex® 200 HR 10/30 column (see Materials and method section for details). Indicated at the top of the curve is the void volume (V_0), the elution volumes for haptocorrin (V_{HC}), albumin (V_{alb}), transcobalamin (V_{TC}) ant the total volume (V_t). Haptocorrin (HC) and transcobalamin [13] were measured in the fractions by ELISA and are indicated in pmol/L. 57Co-Cbl was counted in the fractions and is indicated in counts per minute (CPM).

database and is therefore unlikely to be a normal polymorphism. Bioinformatic analysis using PolyPhen2 (http://genetics.bwh.harvard.edu/pph2/) resulted in a HumVar score of 1.000, indicating that the amino acid exchange might be damaging. Furthermore, we notice complete homozygosity in the entire TCN2 gene sequence (several exonic and intronic polymorphic SNPs assayed) (data not shown).

Discussion

We report a novel TCN2 mutation detected in a 21-year-old patient misdiagnosed to suffer from Cbl deficiency because of clinical symptoms and an unmeasurable level of holoTC by the Abbott assay. The symptoms remained after treatment with Cbl injections. No increase in holoTC was observed, while all other indicators of Cbl deficiency were within the reference interval. In addition, our in-house assay revealed normal levels of holoTC.

From a clinical point of view, the results warrant against diagnosing Cbl deficiency if solely based on the measures of holoTC. Currently, the Abbott method for measurement of holoTC is used in certain laboratories in Switzerland as the initial screening assay. However, until further experience using the commercially available holoTC assays has accumulated, it is recommended to follow the strategy to use at least two markers in order to diagnose Cbl deficiency [21].

The Abbott assay and the in-house assay for holoTC are based on different principles. While the Abbott method depends on an antibody specifically recognizing holoTC [14], the in-house assay measures holoTC by ELISA after removal of all TC not saturated with Cbl [17]. Previous studies have shown the two methods to reveal
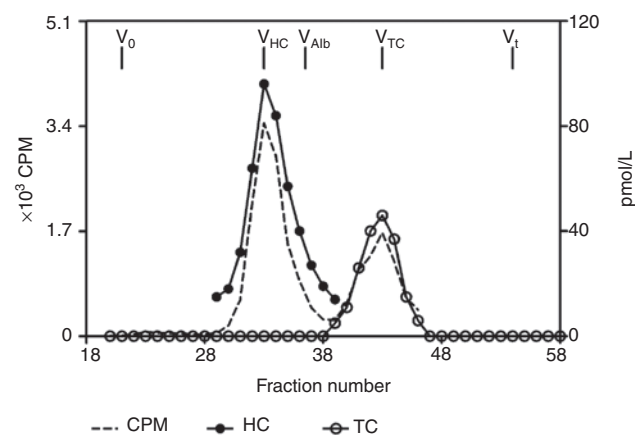
comparable results [22]. This was not the case in our patient.

Our patient harbors a mutation predicting an amino acid change from Asn to Lys at position 267 in the mature protein sequence of TC. The Asn to Lys mutation appears to be of importance for the structure of TC as mirrored in lack of detection by the Abbott holoTC assay. The assay employs a monoclonal antibody that recognizes the complex between Cbl and transcobalamin (holoTC), but not the Cbl-unsaturated TC. Cbl itself is not part of the epitope [15]. Based on the available 3D structure of the transcobalamin-Cbl complex [20], we can compare the wild type and the mutated TC (Figure 2). The TC molecule consists of two large domains (α and β), connected by a flexible link. Cbl assembles the two domains together, whereupon the "hairpin" loop of the β-domain (between folds β3 and β4) moves under the plane of Cbl and contacts both Cbl and the α-domain. The mutated Asn-267 from the α-domain is positioned at the beginning of α12 helix and under the b-amide of Cbl (pyrrol ring A). Asn-267 interacts with Ser-359 and Asp-393 (both from the β-domain), as shown in Figure 2A. The adjacent residues Met-270 and L-268 (α12 helix) have hydrophobic bonds to the methyl group of pyrrol A (Cbl moiety) and the CH_2-group of Ser 359, respectively. These bonds provide additional stabilization of the contact between the two domains. The whole structural element is hidden inside the protein and cannot be involved into any direct contacts with the antibody. At the same time, Asn-267 lies at the bottom of a cavity constructed by the two loops, connecting the α11–α12 and β7–β8 blocks. This cavity slightly expands toward the protein surface and ends with the surface residues Asp-262 (α-domain) and Lys-397 (β-domain). Both loops are rather flexible and are stabilized in the proximity to each other exclusively by the inner protein-Cbl and domain-domain interactions. This region is suggested as the conformational epitope recognized by a gag-like site on the antibody. Mutation of Asn-267 to Lys-267 significantly affects the basis of this cavity. The introduced Lys has a longer and positively charged residue, which would turn toward the negatively charged Asp-393 and clash against it, thereby hindering the walls of the cavity from close approach to each other (Figure 2B and C). This rearrangement should increase the gap of the hole and preclude a tight fitting of the gag-like antibody. In addition, the binding characteristics of Cbl to TC are likely to be affected. Movement of the mutated Lys-267 toward Asp-393 and adjustments of the bulk Lys-residue are expected to disrupt connections of α12 helix to Cbl and the hairpin loop β3–β4. Loss of at least one protein-ligand contact plus partially disturbed
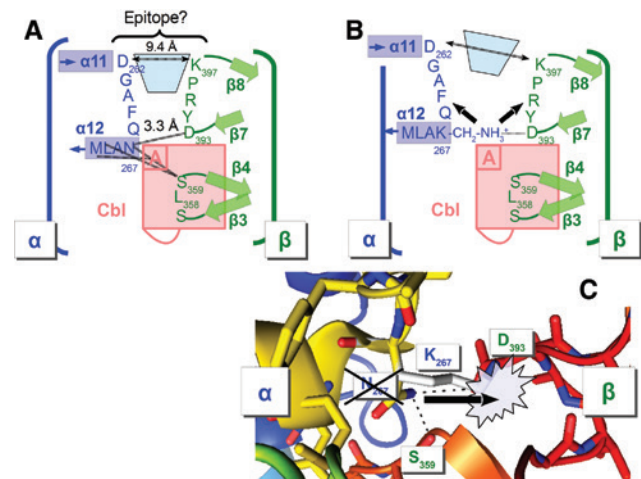

Figure 2: Schematic drawing of transcobalamin showing the possible effect of the observed Asn-267 Lys mutation on the structure of holoTC.

(A) Wild type holoTC. Domains α and β are schematically depicted by left and right braces. The central square indicates the corrin ring with pyrrol A placed at its upper left corner. The notated α-helices and β-folds are situated under the plane of corrin ring. The relevant contacts of N_{267} and the neighboring residues are shown as dotted lines. The suggested scheme of interaction between the antibody and the conformational epitope is indicated by trapezium and upper brace. (B) Mutated TC. Substituted residue K_{267} swings toward D_{393} and widens the cavity. The gag-like antibody does not fit the binding site. (C) Close view at the mutated site. The mutated K_{267} pushes D_{393} aside (arrow) and does not allow closer approach of the cavity walls. Standard one letter abbreviations are used for the amino acids. Notably, N denotes asparagine, K denotes lysine and D denotes aspartic acid.

domain-domain fixation would somewhat decrease TC affinity for Cbl. The possible alteration of binding of Cbl to TC does not critically affect the function of TC, since the patient showed values for the metabolic markers of Cbl deficiency (MMA and homocysteine) within the normal range.

In conclusion, we present data on a patient harboring a *TCN2* mutation that apparently does not prevent the function of the protein, but causes falsely low levels of holoTC if measured by the commercially availableAbbott assay. Our results warrant a caution when relying solely on a low level of holoTC for the diagnosis of Cbl deficiency.

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