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Determination of carbohydrate-deficient transferrin separated by lectin affinity chromatography for detecting chronic alcohol abuse

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Abstract Carbohydrate-deficient transferrin (CDT) has been established as a valuable biological marker for detecting chronic alcohol abuse. To improve the diagnostic efficiency, we studied new CDT determination procedures involving the use of lectin affinity chromatography with *Allomyrina dichotoma* agglutinin (allo A) and *Trichosanthes japonica* agglutinin I (TJA-I) to isolate the CDT isoforms CDT-allo A and CDT-TJA, respectively. These procedures, based on detection of the CDT-allo A and CDT-TJA isoforms in sera, showed high sensitivity (100% and 98%, respectively) and high specificity (93% and 85%, respectively). These results demonstrate that the new procedures involving the use of lectin affinity chromatography are more useful for isolating markers in the CDT test than the conventional charge-based separation method.

Key words: Carbohydrate-deficient transferrin; Chronic alcohol abuse; Carbohydrate-deficient glycoprotein syndrome type 1A; Lectin affinity chromatography

1. Introduction

Many types of plasma proteins are glycosylated with *N*-linked oligosaccharides. Human transferrin has two potential glycosylation sites which are normally occupied by *N*-linked oligosaccharide chains. However, as evidence of its microheterogeneity, carbohydrate-deficient transferrin (CDT) is observed in patients with chronic alcohol abuse (CAA) and the isoforms present are disialo- and asialo-transferrin with isoelectric points of 5.7 and 5.9, respectively [1]. These CDT isoforms were found to lack one or both of the *N*-linked oligosaccharide chains entirely [2,3]. The same transferrin abnormality was found in patients with an autosomal recessive disease with multiple organ manifestations, carbohydrate-deficient glycoprotein syndrome type 1A (CDGS 1A) [4,5].

CDT has been established as a valuable biological marker for detecting individuals with CAA. Several methods based on isocratic anion exchange chromatography followed by immunoassays have been developed for its measurement [1,6]. However, recent studies have shown false-positive CDT test results for individuals with one of several non-alcoholic liver diseases causing hyposialylation by fucosylation [7–11] and some genetic variants causing a shift of isoelectric point [1,12], because the separation of the CDT fraction from non-specific isoforms is insufficient by charge-based separation procedures, owing to the close isoelectric points [6,13]. Unfortunately, because patients with CAA often suffer from liver diseases, their abnormal transferrin isoforms consist of hyposialylated transferrins and deglycosylated transferrins.

To improve the diagnostic efficiency for clinical purposes, we studied CDT determination procedures involving the use of lectin affinity chromatography with *Allomyrina dichotoma* agglutinin (allo A) [14,15] and *Trichosanthes japonica* agglutinin I (TJA-I) [16]. Although both lectins interact fundamentally with sialic acid (Sia)α2→6 galactose (Gal)β1→4 *N*-acetylglucosamine (GlcNAc) residues in *N*-linked oligosaccharides, we recently found that not only non-glycosylated transferrin but also a disialylated transferrin isoform passes through an allo A-Sepharose column using two disialylated transferrins derived from a CDGS 1A patient (Ohkura and Yamashita, unpublished results), and the allo A-Sepharose column is more useful to determine CAA transferrin than the TJA-I-Sepharose column and charge-based separation procedures.

2. Materials and methods

2.1. Patients and controls

The subjects of the present study included three patients with CDGS 1A, 41 patients with alcohol-related disorders, 20 patients with hepatocellular carcinoma (HCC) and 20 healthy controls. These 84 subjects all had the TF C phenotype (TF C1, 59.5%; TF C2-1, 33.3%; TF C2, 7.2%). In addition, a healthy individual with another genetic variant, TF C1-Dsaga [17], was examined. Serum samples were collected from each of the three patients with CDGS 1A; these patients have been described in previous papers [4,5,18,19]. They were diagnosed as having CDGS 1A based on distinctive clinical features [18] and the occurrence of multiple carbohydrate-deficient glycoproteins [19]. Forty-one serum samples were obtained from patients with a well-documented history of continual alcohol consumption. All of them fulfilled the DSM IV criteria (American Psychiatric Association, 1994) for alcohol-related disorders. In each case, the level of ethanol consumption was estimated to be more than 120 g/day during the 2 weeks prior to admission based on personal interviews. Blood was drawn at the time of admission. The 20 patients with HCC had underlying non-alcoholic liver cirrhosis (all of them were HCVAb-positive) as determined by ultrasonography, computed tomography and histo-

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Abbreviations: CDT, carbohydrate-deficient transferrin; allo A, *Allomyrina dichotoma* agglutinin; TJA-I, *Trichosanthes japonica* agglutinin I; CAA, chronic alcohol abuse; Sia, sialic acid; Gal, galactose; GlcNAc, *N*-acetylglucosamine; CDGS 1A, carbohydrate-deficient glycoprotein syndrome type 1A; HCC, hepatocellular carcinoma; IEF, isoelectric focusing

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logical examination. These patients had no history of ethanol consumption for a long time prior to blood sampling. The healthy controls were 20 volunteers, either teetotalers or social drinkers, who consumed 0–30 g ethanol/day on any occasion.

All serum samples were stored and carefully kept at −80°C until analysis. This study was carried out in accordance with the Declaration of Helsinki. Informed consent to participate in the study was obtained from each participant.

2.2. Determination of CDT-allo A and CDT-TJA values

CDT-allo A and CDT-TJA are CDT isoforms isolated by means of an allo A-Sepharose column and a TJA-I-Sepharose column, respectively. The value of each isoform in serum was expressed as a percentage of the total serum transferrin content.

Allo A-Sepharose (5 mg lectin/ml gel) and TJA-I-Sepharose (5 mg lectin/ml gel) were prepared according to the methods described previously [16,20]. Each column (1.0 ml, 7.5 mm inner diameter) was equilibrated with 50 mM Tris-HCl buffer, pH 7.4, containing 0.5 M NaCl. After diluting the serum sample 1:10 with the above-mentioned buffer, a 10 μl aliquot was applied to the column, and it was left to stand for 15 min. Then the column was irrigated with the same buffer. The first 10 ml of eluate was collected as the CDT fraction. The fraction and total transferrin content were measured by the method described below.

2.3. Enzyme immunoassay of transferrin

The assay employed for quantitative determination of transferrin levels was basically a double-antibody sandwich ELISA described previously [21]. Incubations were carried out at room temperature. Rabbit anti-human transferrin (Dako, Glostrup, Denmark) was diluted 5000-fold in 50 mM sodium carbonate, pH 9.6. A 200 μl aliquot was applied to each well of the microtiter plates (Sumitomo Bakelite, Tokyo, Japan) and the plates were incubated for 1.5 h. The plates were then rinsed three times with 10 mM phosphate buffer, pH 7.2, containing 0.15 M NaCl and 0.05% Tween 20 (PBS-Tween), and blocked with 300 μl of 1% bovine serum albumin in 50 mM sodium carbonate, pH 9.6, for 1 h. Each standard or sample was diluted with PBS-Tween, and a 200 μl aliquot was added in duplicate to the coated wells. After incubation for 1 h, the plates were washed three times with PBS-Tween. A 200 μl aliquot of chicken anti-human transferrin (Bethyl, Montgomery, TX, USA) diluted 5000-fold with PBS-Tween was added to the plates, and the plates were incubated for 1 h. After the plates were washed again, 200 μl of rabbit anti-chicken/turkey IgG (heavy+light chain) conjugated with horseradish peroxidase (Zymed, South San Francisco, CA, USA) diluted 5000-fold with PBS-Tween was added to each well, and the plates were incubated for 1 h. Then the plates were washed again, and 200 μl of substrate solution was added to each well. The substrate solution, 13 mg of *o*-phenylenediamine in 50 ml of 0.2 M phosphate buffer, pH 6.0, and 0.01% hydrogen peroxide, was prepared just before use. The plates were incubated in the dark for 30 min. After stopping the reaction with 50 μl of 1 M sulfuric acid, the absorbance of the contents of each well at 490 nm was determined by means of a microtiter plate reader (EIA plate reader, model ELNX 96, TFB, Tokyo, Japan).

2.4. %CDT TIA

The CDT level in all serum samples was also analyzed by anion exchange chromatography followed by a turbidimetric immunoassay performed using a commercially available assay kit %CDT TIA (Axis Biochemicals, Oslo, Norway).

2.5. Isoelectric focusing (IEF) and immunoprinting

IEF was performed according to the methods described previously [18,22], using polyacrylamide gels in the pH range 5–7 (Ampholine, Pharmacia, Uppsala, Sweden). Immunoprinting was carried out using cellulose acetate strips (Separax, Fuji Film, Tokyo, Japan) soaked in anti-human transferrin antibodies (Dako) diluted 1:3 with PBS. Each damp-dried strip was placed on the surface of the gel for 3 min. After washing in PBS for 1 h, the strip was stained with Coomassie brilliant blue R-350 (Pharmacia).

2.6. Statistical analysis

All data are expressed as mean ± S.D. Means of four subgroups were compared by one-way analysis of variance (ANOVA) for global analysis. The significance of differences between two subgroups was analyzed by the Scheffé test. For correlation analysis, the Spearman correlation coefficient (r) was used. Probability values less than 0.05 were considered to be statistically significant. Sensitivity was defined as the proportion of the actively drinking patients and patients with CDGS 1A who had a positive test, whereas specificity was the proportion of healthy controls and patients with HCC who tested negative.

3. Results

3.1. Elution profiles obtained with lectin columns

In analysis of serum samples from the patients with CAA and the healthy controls, after serum samples were applied to both the allo A-Sepharose column and the TJA-I-Sepharose column (Fig. 1), the absorbed and pass-through transferrins were analyzed with IEF (Fig. 2). Pentasialo-, tetrasialo- and trisialo-transferrin became adsorbed to both of these columns and were eluted with lactose. Non-glycosylated (asialo-)transferrin did not bind to either of the columns (Fig. 2, lanes B and D). Disialo-transferrin was found to bind to the TJA-I-Sepharose column (Fig. 2, lane E), in contrast, a portion of the total disialo-transferrin (about 50%) passed through the allo A-Sepharose column (Fig. 2, lane B), the remaining disialo-transferrin was absorbed to the column (Fig. 2, lane C).

3.2. CDT-allo A value

The individual CDT values obtained for patients and healthy controls are shown as dot plots in Fig. 3. For the healthy controls, the mean serum CDT-allo A value was 0.8 ± 0.3%. The cut-off point for a normal serum CDT-allo A value was set at 1.4%, corresponding to 2 S.D. above the mean of the healthy controls. In the patients with CDGS 1A and those with CAA, the serum levels were elevated compared to the control group and the mean CDT-allo A values were 18.1 ± 4.6% and 6.8 ± 4.0%, respectively. In the patients with HCC, the serum levels were not elevated and the mean CDT-

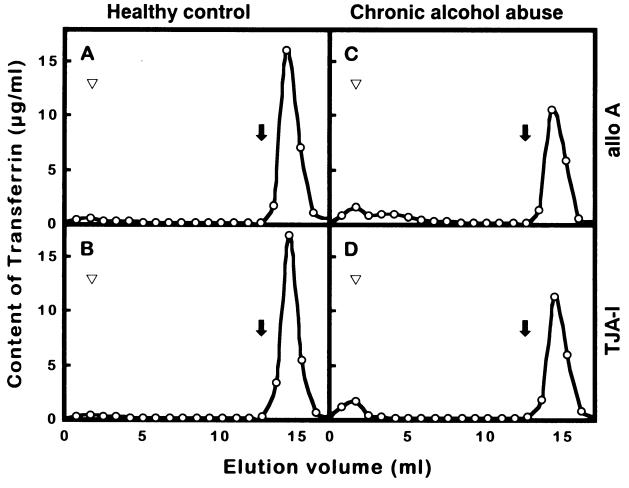

Fig. 1. Elution profiles obtained in allo A-Sepharose and TJA-I-Sepharose column chromatography of transferrin from a healthy control (A, B) and a patient with CAA (C, D). A 10 μl aliquot of each diluted serum sample was applied to each column. Columns were irrigated with a buffer solution consisting of 50 mM Tris-HCl, pH 7.4, and 0.5 M NaCl and then with the same buffer containing 0.1 M lactose from the position indicated by the black arrow. The column temperature was continuously maintained at room temperature. The eluted transferrin in each fraction was assayed by means of an enzyme immunoassay as described in Section 2. ▽ indicates the elution position of bovine serum albumin.

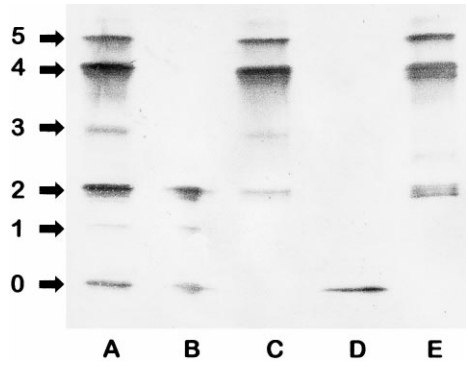

Fig. 2. IEF patterns of serum transferrin from a patient with CAA, as revealed by isoelectric focusing and immunoprinting. Arrows indicate the numbers of sialic acid residues. Before separation by lectin columns (lane A); CDT isoforms separated by allo A-Sepharose (lane B); isoforms adsorbed to allo A-Sepharose (lane C); CDT isoforms separated by TJA-I-Sepharose (lane D); isoforms adsorbed to TJA-I-Sepharose (lane E). The anode is at the top.

allo A value was 0.9 ± 0.4%. Significant differences in CDT-allo A values were detected comparing healthy controls and patients with CDGS 1A ($P < 0.0001$), or comparing healthy controls and patients with CAA ($P < 0.0001$), but no significant difference was detected comparing healthy controls and patients with HCC. The results obtained in the assay of CDT-allo A showed CDT values below the cut-off (1.4%) for 18 of 20 patients with HCC, and 19 of 20 healthy controls, whereas positive results were obtained for all of the three patients with CDGS 1A and for all of the 41 patients with CAA (Fig. 3, top). The sensitivity and specificity within this population were 100% (all of 44) and 93% (37 of 40), respectively.

3.3. CDT-TJA value

For healthy controls, the mean serum CDT-TJA value was 0.7 ± 0.3%. The cut-off point for a normal serum CDT-TJA value was set at 1.3% (mean+2 S.D.). The mean serum CDT-TJA values for patients with CDGS 1A, those with CAA and those with HCC were 9.2 ± 2.9%, 3.3 ± 2.1% and 0.9 ± 0.5%, respectively. Significant differences in CDT-TJA values were detected comparing healthy controls and patients with CDGS 1A, or comparing healthy controls and patients with CAA ($P < 0.0001$), but no significant difference was detected comparing healthy controls and patients with HCC. The results obtained in the assay of CDT-TJA showed CDT values below the cut-off (1.3%) for 15 of 20 patients with HCC, and 19 of 20 healthy controls, whereas CDT-positive values were obtained for all three of the patients with CDGS 1A and 40 of 41 patients with CAA (Fig. 3, middle). The sensitivity and specificity within this population were 98% (43 of 44) and 85% (34 of 40), respectively.

3.4. Axis %CDT TIA value

The results obtained in terms of %CDT TIA for the same subject populations were 21.4 ± 2.9 for the patients with CDGS 1A, 12.2 ± 4.6 for those with CAA, 6.9 ± 1.6 for those with HCC and 2.9 ± 0.8 for the healthy controls. The mean serum %CDT TIA value for the patients with HCC was 2.4-fold greater than that for the healthy controls. Significant differences in %CDT TIA values were detected comparing healthy controls and patients with CDGS 1A ($P < 0.0001$), comparing healthy controls and patients with CAA ($P < 0.0001$) and comparing healthy controls and patients with HCC ($P < 0.005$). %CDT TIA values below the cut-off (6.0%, according to the manual provided by the manufacturer) were obtained for six of the 20 patients with HCC and for all of the 20 healthy controls. False-positive results

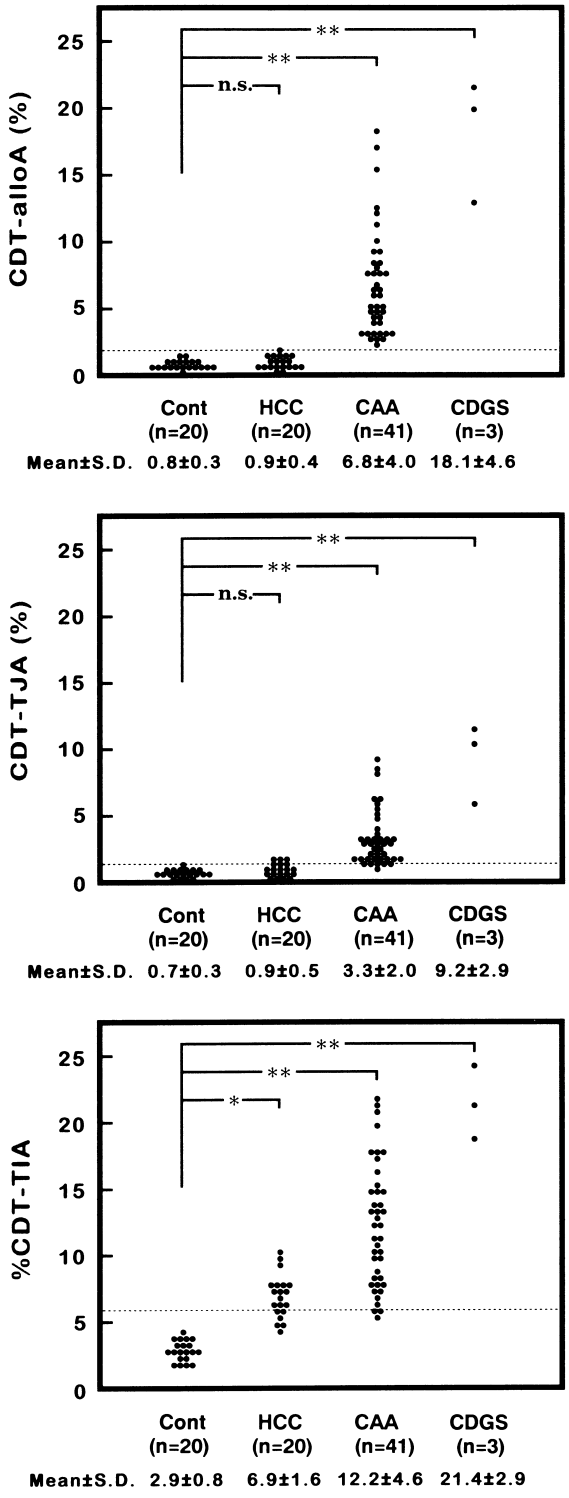

Fig. 3. Distribution of CDT-allo A (top), CDT-TJA (middle) and %CDT TIA (bottom) values. Cont, healthy controls. A significant difference compared with the healthy controls is shown by asterisks: **$P < 0.0001$; *$P < 0.005$; n.s.: not significant.

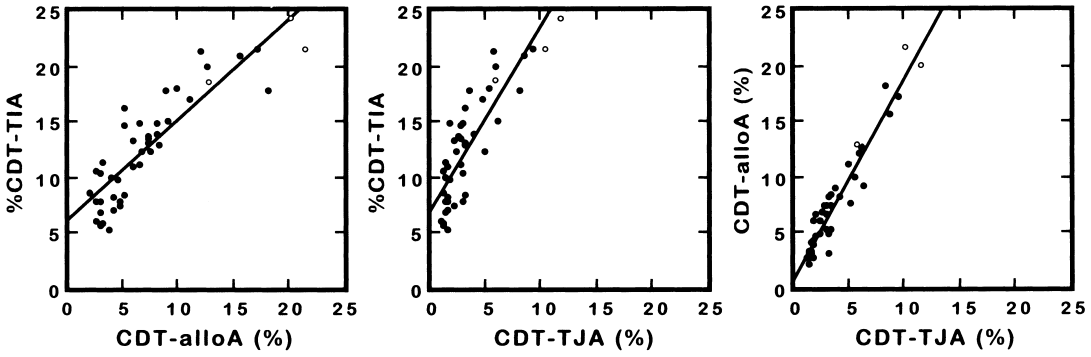

Fig. 4. Correlation between results for CDT tests; between %CDT TIA and CDT-allo A values (left), between %CDT TIA and CDT-TJA values (middle), between CDT-allo A and CDT-TJA values (right), for three patients with CDGS 1A (○) and 41 patients with CAA (●).

were obtained for 14 (70%) of the 20 patients with HCC. CDT-positive values were found for all three of the patients with CDGS 1A and 38 of 41 patients with CAA (Fig. 3, bottom). The sensitivity of the %CDT TIA method was 93% (41 of 44), while its specificity was 65% (26 of 40).

3.5. Correlation between CDT tests

A significant correlation was found between the CDT-allo A and %CDT TIA values ($y = 0.91x + 6.00$, $r = 0.88$, $P < 0.0001$) (Fig. 4, left), between the CDT-TJA and %CDT TIA values ($y = 1.68x + 6.70$, $r = 0.86$, $P < 0.0001$) (Fig. 4, middle), and between the CDT-allo A and CDT-TJA values ($y = 1.81x + 0.87$, $r = 0.96$, $P < 0.0001$) (Fig. 4, right). The similarity of the data obtained by the two lectin column procedures reflects their high correlation coefficient. However, the CDT-allo A values were significantly higher than the CDT-TJA values for the patients with CAA and those with CDGS 1A.

3.6. CDT test results for the subject with TF D

In the case of the healthy individual with the TF C1-Dsaga phenotype, the mean %CDT TIA value was extremely high showing a false-positive result, while the CDT-allo A and CDT-TJA values were below the cut-off (Fig. 5).

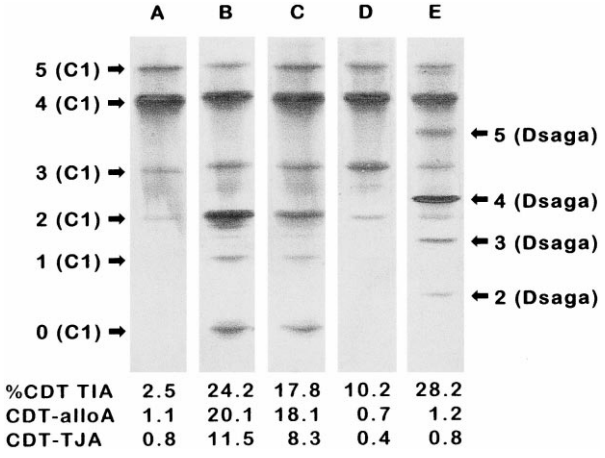

Fig. 5. Comparison of IEF band patterns and CDT values. Arrows indicate the numbers of sialic acid residues in the case of TF C1 and TF Dsaga. A healthy control (lane A), a patient with CDGS 1A (lane B), a patient with CAA (lane C), a patient with HCC (lane D) with TF C1 (lane A-D) and a healthy individual with the genetic variant, TF C1-Dsaga (lane E). The anode is at the top.

4. Discussion

We have clearly demonstrated that underglycosylated transferrin can be detected using allo A-Sepharose and TJA-I-Sepharose, with high sensitivity (100% and 98%, respectively) and high specificity (93% and 85%, respectively). On the other hand, the %CDT TIA method showed fairly high sensitivity (93%), but its specificity was considerably low (65%). Both allo A and TJA-I strongly interact with oligosaccharides containing Siaα2→6 Galβ1→4 GlcNAc residues [15,16]. Because normal transferrin (tetrasialo-transferrin) has two bi-antennary glycans containing Siaα2→6 Galβ1→4 GlcNAc residues [23,24] and the CDT isoforms lack either one or both of the *N*-linked glycans [2,3], it was predicted that non-glycosylated transferrin would pass through either of these two lectin columns, whereas a disialo-transferrin which has a bi-antennary sugar chain possessing Siaα2→ Galβ1→4 GlcNAc residues [2–5] would be absorbed to both columns. As the results demonstrate, both CDT-allo A and CDT-TJA values are more useful than %CDT TIA values for detecting patients with CAA, and those with CDGS 1A. Moreover, false-positive results are not obtained in the case of patients with severe non-alcoholic liver disease such as HCC, because CDT-allo A and CDT-TJA values reflect the levels of partially or completely underglycosylated transferrin in the sera of patients with CAA and those with CDGS 1A.

The correlation coefficient between the CDT-allo A and CDT-TJA values was the highest, and the CDT-allo A values were significantly higher than the CDT-TJA values in spite of the similarity in sugar specificities of the two lectins. In the sera of patients with CAA, not only non-glycosylated (asialo-)transferrin but also monoglycosylated (disialo-)transferrin levels are elevated. The former passed through both columns and the latter became adsorbed to the TJA-I-Sepharose column. However, about 50% of the disialo-transferrin passed through the allo A-Sepharose column. Considering that the non-glycosylated asparagine site in disialo-transferrin has been shown to be random in patients with CDGS 1A [5], and that one of the isolated two disialo-transferrin isoforms was absorbed to the allo A column and the other isoform passed through the column by steric hindrance, the same phenomenon seems to occur in the case of disialo-transferrin isoforms present in the sera of patients with CAA. From these points of view, the CDT-allo A value is a more reliable marker for detecting patients with chronic alcohol abuse than the CDT-TJA value.

The occurrence of false-positive results in the %CDT TIA test in the case of patients with severe non-alcoholic liver dysfunction such as those with HCC has been previously reported [11]. This is due to altered glycosylation of transferrin in patients with HCC resulting in the occurrence of fucosylated, asialyl, or highly branched *N*-linked oligosaccharides of transferrin, responsible for its altered isoelectric point [25], but importantly, underglycosylation does not occur in such cases. Because the abnormal transferrin isoforms observed in cases of HCC should interact with allo A-Sepharose and TJA-I-Sepharose [15,16], the CDT test based on CDT-allo A and CDT-TJA values should not give false-positive results in the case of patients with HCC.

It has been reported that in the case of healthy individuals with the genetic D variant, which occurs more frequently in certain populations [26,27], false-positive results are obtained in the CDT test [1,12]. The %CDT TIA value obtained for a healthy individual with the TF C1-Dsaga phenotype showed a false-positive result, because the D variant isoforms of transferrin have higher isoelectric points. On the other hand, CDT-allo A and CDT-TJA values obtained for this individual with TF Dsaga were below the cut-off, because the variants have two *N*-linked glycans per protein molecule. These results indicate that CDT-allo A and CDT-TJA values are not dependent on the isoelectric points of transferrin isoforms, and that the lectin column procedures employed here are more suitable than charge-based separation methods for detecting CAA.

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