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Diagnostic value of Western blotting in carbohydrate-deficient glycoprotein syndrome

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Abstract

Carbohydrate-deficient glycoprotein syndrome (CDGS) is a newly recognized family of diseases characterized by the absence from the transferrin molecule of at least one glycan chain (type I) or an antenna of the glycan chain (type II). CDGS is currently diagnosed by studies of serum transferrin sialylation. We have developed an alternative Western blot-based method to detect serum transferrin species with reduced molecular masses due to altered glycosylation. Two additional bands are observed in type I CDGS, while a single lower band is observed in type II CDGS, relative to healthy subjects. *N*-glycanase treatment of serum from type I CDGS patients and normal subjects yields a single band of the same mass in the two cases, confirming that the glycan is the only moiety involved in the differential Western blot pattern. Similar results were found with serum α1-acid glycoprotein, haptoglobin and α1-antitrypsin. Western-blot analysis of one or more serum glycoproteins permits the differential diagnosis of CDGS.

Keywords: Haptoglobin; α1-Acid glycoprotein; α1-Antitrypsin; Transferrin; SDS-PAGE; CDG

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1. Introduction

In recent years a new group of inborn errors affecting glycoprotein synthesis has been identified as a metabolic disorder in which infants develop severe neurologic disorders and variable multisystem abnormalities [1,2]. Associated with unique carbohydrate defects in circulating glycoproteins, they have been termed carbohydrate-deficient glycoprotein syndromes (CDGSs), of which three clinical and biochemical types have been identified.

Type I CDGS was the first variant to be described and is probably the most common. Patients develop multisystem disease shortly after birth, as well as severe nervous system involvement [3] and ocular [4], cardiac [5], endocrine [6,7], hepatic [8], coagulation [9] and renal [10] abnormalities. It is inherited as an autosomal recessive trait [11]. CDGS types II [12,13] and III [14] differ considerably from type I in clinical terms, with dysmyelination, variable extra-neurologic manifestations, more pronounced developmental retardation and dysmorphic features.

Structural analysis of transferrin purified from serum of type I CDGS patients has revealed the presence of heterogeneous glycoforms bearing 1, 2 or no glycan chains [15,16], whereas normally glycosylated transferrin has 2 chains (Fig. 1). The corresponding primary enzyme defect has been recently

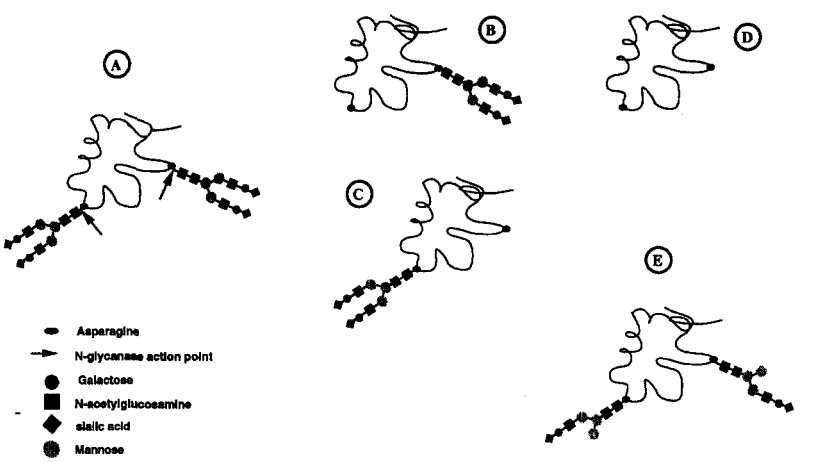

Fig. 1. Schematic representation of the serum transferrin molecule carrying two N-linked glycans (A) in normal subjects; two (A), one (B,C) or no (D) glycans in type I CDGS patients; two truncated glycans (E) in type II CDGS patients. Point of action of *N*-glycanase is also noted.

determined as a defective cytosolic phosphomannomutase [17]. Type II CDGS is characterized by the loss from serum transferrin of one antenna of each glycan chain (Fig. 1) [18], due to defective *N*-acetylglucosamine transferase II (EC 2.4.1.143), an enzyme located in the trans-Golgi apparatus [18]. Type III CDGS is also associated with abnormally glycosylated transferrin [14], but glycan structure has not yet been elucidated and the primary defect thus remains unknown.

The characteristic biochemical abnormalities of CDGS were discovered during isoelectrofocusing of serum transferrin, a method originally devised to screen for alcohol abuse in otherwise healthy adults. Isoelectric focusing separates normal transferrin, with high resolution, into different isoforms depending on iron saturation, sialic acid content or amino acid substitution. After complete iron saturation, transferrin is normally separated into 4 isoforms distinguished on the basis of their approximate isoelectric point (p*I*). The p*I* 5.7 fraction normally accounts for $< 0.8\%$ of total transferrin, but may be increased more than 10-fold after heavy alcohol consumption [19]. Consistent cathodal migration of serum transferrin from CDGS patients was attributed to a reduction in the number of sialic acid residues from 4 or 5 to 4, 2 or 0 in type I [3] and to usually 2 in type II [12]. Isoelectrofocusing of serum transferrin is thus used to diagnose CDGS. Carbohydrate-deficient serum transferrin (CDT) can also be disclosed by quantitative analysis by means of rapid microanion-exchange chromatography [20], which is also based on variations in sialic acid content.

We have developed a different strategy to detect the absence of one or more glycan chains on glycoproteins, or the absence of one antenna of the glycan chains. Instead of detecting electric charge variations, we identify changes in the apparent molecular mass of serum transferrin by submitting serum to Western blot analysis. We have also tested other serum glycoproteins such as haptoglobin, α1-acid glycoprotein and α1-antitrypsin.

2. Materials and methods

2.1. Sera

Serum was collected from a patient hospitalised in the gastroenterology unit (Prof. Navarro) of Robert Debré Children's Hospital (Paris, France) and diagnosed as having type I CDGS on clinical grounds. Serum from 2 patients diagnosed clinically and biochemically as having type I and type II CDGS, were from Professor J. Jaeken (Leuven, Belgium). Serum was also obtained from 9 healthy subjects (5 adults with alcohol consumption between 0 and 15 g/day and free of liver disease, and 4 newborns free of

CDGS) and from 12 hospital patients with a well-documented history of chronic alcoholism.

All samples were stored at −20°C until use.

2.2. Reagents

Peptide-N^4-(N-acetyl-β-glucosaminyl) asparagine amidase^F (EC 3.5.1.52) (N-glycanase) from *Flavobacterium meningosepticum* was from Boehringer (Meylan, France). Goat anti-human transferrin, haptoglobin, (α1-acid glycoprotein and α1-antitrypsin antisera, prestained SDS-PAGE standards and nitrocellulose membrane (0.45 μm) were from Bio-Rad (Ivry S/Seine, France). Donkey anti-rabbit IgG antibody conjugated to horseradish peroxidase, enhanced chemiluminescent luminescent (ECL) substrate and Hyperfilm-ECL were from Amersham (Les Ulis, France). Rabbit anti-human transferrin, haptoglobin, α1-acid glycoprotein and α1-antitrypsin antisera were from Behring (Rueil-Malmaison, France). The CDTect EIA kit was kindly provided by Pharmacia (St Quentin-en-Yvelines, France).

2.3. Apparatus

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransfer were performed on a Mini Protean II cell (Bio-Rad).

2.4. Protein assays

Serum total transferrin, haptoglobin, α1-acid glycoprotein and α1-antitrypsin were determined by means of automated immunoturbidimetric assay on a centrifugal analyser (Monarch, I.L., Paris, France) with corresponding goat anti-human specific protein antisera.

2.5. Carbohydrate-deficient transferrin assay

Serum carbohydrate-deficient transferrin (CDT) was determined using the CDTect EIA kit, according to the manufacturer's instructions. Briefly, transferrin in the sample (50 μl) saturated with ferric citrate is passed though a minicolumn retaining transferrin with a high sialic acid content. Enzyme-labelled transferrin is added to the eluate and competes with CDT in the serum sample for binding to a mouse monoclonal anti-transferrin antibody. These antibodies bind to anti-mouse antibodies coated on the surface of a microplate well. Unbound material is washed away and bound transferrin conjugate is measured by addition of enzyme substrate to the wells. The absorbance of the product is inversely proportional to the concentration of CDT in the sample.

2.6. Enzymatic N-deglycosylation [20]

Before enzyme treatment, serum proteins were denatured by boiling samples for 4 min in the presence of 0.1% SDS and 1% 2-mercaptoethanol. The potential inhibitory effect of SDS on *N*-glycanase activity was neutralized by Triton X-100 (10-fold excess). Samples were then treated with *N*-glycanase (20 U/ml) in 0.2 mol/l sodium phosphate buffer (pH 8.6) plus 13 mmol/l EDTA at 37°C for 24 h [21].

2.7. Western blot analysis

Diluted samples (serum or *N*-glycanase-treated serum) corresponding to 30 ng of transferrin, haptoglobin, α1-acid glycoprotein or α1-antitrypsin, were subjected to SDS-PAGE in reducing conditions, using 7% (for transferrin) and 9% (for the other glycoproteins) homogeneous separating gel and 4% homogeneous stacking gel, according to Laemmli et al. [22], in parallel with molecular mass standards. Proteins in the gel were electrotransferred to a nitrocellulose membrane in 25 mmol/l Tris, 132 mmol/l glycine, 20% methanol (v/v) according to Towbin et al. [23] at 100 V for 1 h. The incubation time of the following steps was 1 h at room temperature, and was followed by a washing step (4 washes lasting 10 min each with 20 mmol/l Tris, 0.5 mol/l Na, 0.1% Tween 20, pH 7.5). Non-specific binding sites on the nitrocellulose membrane were blocked by immersion in 10% (w/v) non-fat dried milk. Specific protein bands were detected with a 1:2000 dilution of corresponding rabbit anti-human specific protein antibodies and by a 1:5000 dilution of donkey anti-rabbit IgG antibodies conjugated to horseradish peroxidase. Western blots were developed by means of ECL according to the manufacturer's instructions.

3. Results

As shown in Fig. 2a, Western blotting of serum transferrin from healthy adults (lane 3) yielded a single band of 80 kDa, and the same results were obtained with serum transferrin from alcoholic patients (lane 4). Serum from both patients with type I CDGS yielded 3 well-separated bands of immunoreactive transferrin (80, 77 and 74 kDa) with variable relative intensities (lanes 1, 2). Western blotting of serum transferrin from the type II CDGS patient (lane 5) revealed a single band with a lower molecular mass (78 kDa) than normal transferrin.

To investigate the differences in the apparent molecular masses of transferrin observed on Western blots, serum from healthy subjects and a patient

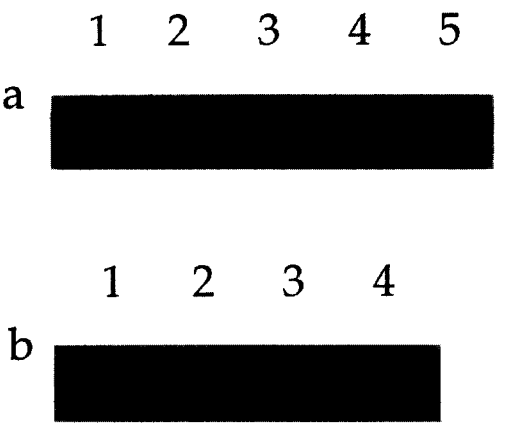

Fig. 2. (a) Western blot of serum transferrin from a healthy subject (lane 3), an alcoholic patient (lane 4), type I CDGS patients (lanes 1, 2) and a type II CDGS patient (lane 5); (b) Western blot of serum transferrin from a healthy subject before (lane 1) and after (lane 2) *N*-glycanase treatment and from type I CDGS patients before (lane 3) and after (lane 4) *N*-glycanase treatment.

with type I CDG syndrome were submitted to *N*-glycanase treatment followed by Western blotting. As shown in Fig. 2b, deglycosylation of transferrin gave rise to bands of the same molecular mass level whatever the origin of the serum, indicating that the glycan moiety is the only structure involved in the modified Western blot pattern.

In addition, Western blots of other serum glycoproteins (α1-acid glycoprotein, α1-antitrypsin and haptoglobin) from CDGS patients were compared to those of healthy subjects. As shown in Fig. 3, a single band was observed for the 3 proteins in healthy subjects, whereas 1–3 additional bands were found in the 2 patients with type I CDGS. A single band with a lower molecular mass than that of the native glycoprotein band was observed in type II CDGS serum. Finally, as with transferrin, deglycosylation of the other serum glycoproteins led to the disappearance of the former differences between type I CDGS patients and healthy subjects.

CDT serum levels in the healthy subjects were within the normal range (mean $\pm$ S.D. = 15 $\pm$ 5 U/l), whereas those in 7 of the 12 alcoholic patients were elevated (mean $\pm$ S.D. = 53 $\pm$ 47 U/l). Results for the 2 type I CDGS patients were far above those of the alcoholic patients (186 and 200 U/l).

4. Discussion

CDGS is a disorder characterized biochemically by a complex carbohydrate deficiency in glycoproteins, which has mainly been studied with

respect to transferrin. The absence of one or more glycan chains or antennae on transferrin finds expression in the loss of sialic acid. The diagnosis of this syndrome has so far been based on the study of serum transferrin sialylation, by means of isoelectrofocusing or anion-exchange chromatography. However, the presence of CDT is not exclusive to CDGS [24], as allelic D variants of transferrin [25] and other pathological conditions such as primary biliary cirrhosis, alcoholic cirrhosis [19], hypertension [26] and idiopathic hemochromatosis [27] may generate false-positive results.

Another way of diagnosing CDGS is to test for different molecular mass forms of a glycoprotein generated by the lack of glycan chains. A glycan chain represents about 2.4–4.0 kDa, depending on whether the chain is bi-, tri- or tetra-antennary. Methods based on variations of protein molecular masses have been successfully applied to serum transferrin and include electrospray ionization-mass spectrometry [28] and matrix-assisted laser desorption time-of-flight mass spectrometry [29]. However, these methods require purification of transferrin and equipment unavailable in standard clinical laboratories. A two-dimensional electrophoresis followed by silver staining has also been applied to total serum proteins from type I CDGS patients [30] but did not allow easy localization of specific glycoproteins.

The loss of at least one chain is clearly manifest on SDS-PAGE, and creates another approach to measuring glycoprotein molecular masses. In Western blot with immunospecific detection, serum transferrin of type I

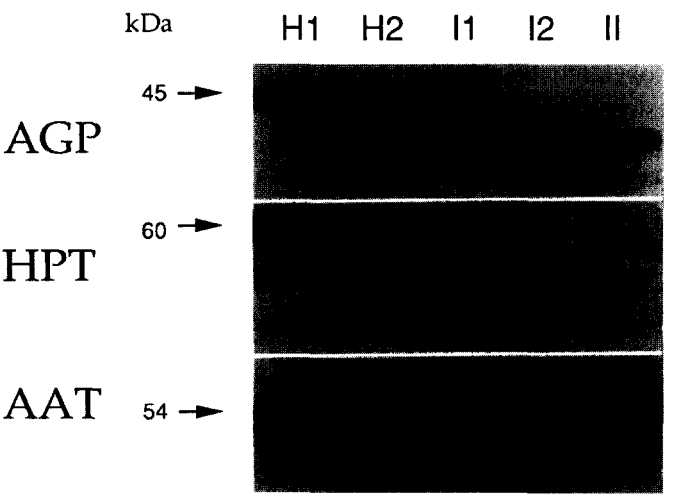

Fig. 3. Western blot of serum α1-acid glycoprotein (AGP), haptoglobin (HPT) and α1-antitrypsin (AAT) from a healthy subject (H) and a type I CDGS patient (I) before (1) and after (2) *N*-glycanase treatment, and from a type II CDGS patient (II).

CDGS patients yielded 3 bands, the molecular masses of which were comparable to those of native, partially and totally deglycosylated transferrin (*N*-glycanase treatment). In addition, Western blotting of the other serum glycoproteins gave similar patterns to that of transferrin, i.e. several bands in type I CDGS and a single and lower band in type II CDGS relative to normal subjects. *N*-glycanase treatment of serum from type I CDGS patients and normal subjects gives rise to a single band of the same molecular mass level in the two cases, whatever the glycoprotein, confirming that the glycan is the only moiety involved in this differential pattern.

Western blots of α1-acid glycoprotein and of haptoglobin are more sensitive than those of transferrin and α1-antitrypsin for distinguishing CDGS patients from normals, especially the type II patient. Haptoglobin bands are better separated than those of α1-acid glycoprotein but haptoglobin has very low serum levels during early post-natal life time and thus is hardly detectable in neonates and newborns.

Paralleling our work, another study was performed by Yuasa et al. [31] in which the electrophoretic mobilities of several glycoproteins of type I CDG patients were compared to those of healthy patients. SDS-PAGE of native α1-acid glycoprotein and transferrin gave similar results in both studies.

However, the α1-acid glycoprotein Western blot pattern contained only 3 bands, rather than the expected 6 bands corresponding to partial and total absence of the 5 chains of the native glycoprotein; similarly, α1-antitrypsin yielded 2 or 3 bands instead of the 4 bands corresponding to the 3 glycan chains it contains, and haptoglobin yielded 3 or 4 bands instead of the 5 corresponding to the 4 glycan chains it contains. These latter glycoproteins have more complex glycan moieties than transferrin, and the absence of the most deglycosylated forms of these glycoproteins on SDS-PAGE calls for further investigation.

The Western blot assay we propose as a diagnostic tool for CDGS is adapted to routine laboratory conditions. It is as easy to perform as isoelectrofocusing and more specific. All the reagents are commercially available. As the assay is highly sensitive, very small volumes of diluted samples are used, making the method suitable for testing children and even neonates. Whole blood eluted from filter paperspots has also been used with success (data not shown).

In conclusion, our results indicate that Western blot analysis of one or more serum glycoproteins can be used to diagnose CDGS. We obtained no false-positive results with samples from alcoholics, alcoholism being the main interfering pathology in diagnostic analysis of serum transferrin sialylation. Different Western blot patterns were found between types I and II CDGS, permitting differential diagnosis. Our results also provide additional evidence that transferrin is not the only abnormally glycosylated

serum glycoprotein in CDGS, which thus involves glycosylation abnormalities of multiple liver-synthesized glycoproteins.

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References

[1] Hagberg BA, Blennow G, Kristiansson B, Stibler H. Carbohydrate deficient glycoprotein syndromes: peculiar group of new disorders. Pediatr Neurol 1993;9:255–262.
[2] Jaeken J, Carchon H. The carbohydrate-deficient glycoprotein syndromes: an overview. J Inherit Metab Dis 1993;16:813–820.
[3] Stibler H, Jaeken J. Carbohydrate deficient serum transferrin in a new systemic hereditary syndrome. Arch Dis Child 1990;65:107–111.
[4] Andreasson S, Blennow G, Ehinger B, Strömland K. Full-field electroretinograms in patients with the carbohydrate-deficient glycoprotein syndrome. Am J Ophthalmol 1991;112:83–86.
[5] Clayton PT, Winchester BG, Keir G. Hypertrophic obstructive cardiomyopathy in a neonate with the carbohydrate-deficient glycoprotein syndrome. J Inherit Metab Dis 1992;15:857–861.
[6] Heyne K, Marg W, Walther F, Stephani U, Hermanussen M, Weidinger S. Hypothyroidism phenocopy in glycanosis CDG (carbohydrate-deficient glycoprotein syndrome). Eur J Pediatr 1994;153:866.
[7] Ohzeki T, Motozumi H, Hanaki K, Ohtahara H, Urashima H, Tsukuda T, Kobayashi S, Shiraki K, Ohno K. Carbohydrate-deficient glycoprotein syndrome in a girl with hypogonadism due to inactive follicle stimulating hormone. Horm Metab Res 1993;25:646–648.
[8] Conradi N, De Vos R, Jaeken J, Lundin P, Kristiansson B, Van Hoof F. Liver pathology in the carbohydrate-deficient glycoprotein syndrome. Acta Paediatr Scand 1991;375:50–54.
[9] Van Geet C, Jaeken J. A unique pattern of coagulation abnormalities in carbohydrate-deficient glycoprotein syndrome. Pediatr Res 1993;33:540–541.
[10] Strom EH, Stromme P, Westvik J, Pedersen J. Renal cysts in the carbohydrate-deficient glycoprotein syndrome. Pediatr Nephrol 1993;7:253–255.
[11] Eeg-Olofsson KE, Wahlström J. Genetic and epidemiological aspects of the carbohydrate-deficient glycoprotein syndrome. Acta Paediatr Scand 1991;375:63–65.
[12] Ramaekers VT, Stibler H, Kint J, Jaeken J. A new variant of the carbohydrate-deficient glycoproteins syndrome. J Inherit Metab Dis 1991;14:385–388.
[13] Jaeken J, De Cock P, Stibler H, Van Geet C, Kint J, Ramaekers V, Carchon H. Carbohydrate-deficient glycoprotein syndrome type II. J Inherit Metab Dis 1993;16:1041.
[14] Stibler H, Westerberg B, Hanefeld F, Hagberg B. Carbohydrate-deficient glycoprotein (CDG) syndrome — a new variant, type III. Neuropediatrics 1993;24:51–52.

[15] Yamashita K, Ideo K, Ohkura T, Fukushima K, Yuasa I, Ohno K, Takeshita K. Sugar chains of serum transferrin from patients with carbohydrate deficient glycoprotein syndrome. J Biol Chem 1993;268:5783–5789.
[16] Powell LD, Paneerselvam K, Vij R, Diaz S, Manzi A, Buist N, Freeze H, Varki A. Carbohydrate-deficient glycoprotein syndrome: not an N-linked oligosaccharide processing defect, but an abnormality in lipid-linked oligosaccharide biosynthesis? J Clin Invest 1994;94:1901–1909.
[17] Van Schaftingen E, Jaeken J. Phosphomannomutase deficiency is a cause of carbohydrate-deficient glycoprotein syndrome type I. FEBS Lett 1995;377:318–320.
[18] Jaeken J, Schachter H, Carchon H, De Cock P, Coddeville B, Spik G. Carbohydrate-deficient glycoprotein syndrome type II: a deficiency in Golgi localised *N*-acetyl-glucosaminyltransferase II. Arch Dis Child 1994;71:123–127.
[19] Stibler H. Carbohydrate-deficient transferrin in serum: a new marker of potentially harmful alcohol consumption reviewed. Clin Chem 1991;37:2029–2037.
[20] Stibler H, Borg S, Joustra M. Micro anion exchange chromatography of carbohydrate-deficient transferrin in serum in relation to alcohol consumption (Swedish patent 8400587-5). Alcohol Clin Exp Res 1986;10:535–544.
[21] Hirani S, Bernasconi RJ, Rasmussen JR. Use of *N*-glycanase to release asparagine-linked oligosaccharides for structural analysis. Anal Biochem 1987;162:485–492.
[22] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond) 1970;60:680–685.
[23] Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 1979;76:4350–4354.
[24] De Jong G, Feelders R, Van Noort WL, Van Eijk HG. Transferrin microheterogeneity as a probe in normal and disease states. Glycoconj J 1995;12:219–226.
[25] Bean P, Peter JB. Allelic D variants of transferrin in evaluation of alcohol abuse: differential diagnosis by isoelectric focusing-immunoblotting-laser densitometry. Clin Chem 1994;40:2078–2083.
[26] Fagerberg B, Agewall S, Berglund A, Wysocki M, Lundberg PA, Lindstedt G. Is carbohydrate-deficient transferrin in serum useful for detecting excessive alcohol consumption in hypertensive patients? Clin Chem 1994;40:2057–2063.
[27] Jensen PD, Peterslund NA, Poulsen JH, Jensen FT, Christensen T, Ellegaard J. The effect of iron overload and iron reductive treatment on the serum concentration-deficient transferrin. Br J Haematol 1994;88:56–63.
[28] Yamashita K, Ohkura T, Ideo H, Ohno K, Kanai M. Electrospray ionization-mass spectrometric analysis of serum transferrin isoforms in patients with carbohydrate-deficient glycoprotein syndrome. J Biochem 1993;114:766–769.
[29] Wada Y, Gu J, Okamoto N. Diagnosis of carbohydrate-deficient glycoprotein syndrome by matrix assisted laser desorption time of flight mass spectrometry. Biol Mass Spectr 1994;23:108–109.
[30] Harrison H, Miller K. Multiple serum protein abnormalities in carbohydrate-deficient glycoprotein syndrome: pathognomonic finding of two-dimensional electrophoresis? Clin Chem 1992;38:1390–1392.
[31] Yuasa I, Ohno K, Hashimoto K, Iijima K, Yamashimata K, Takeshita K. Carbohydrate-deficient glycoprotein syndrome: electrophoretic study of multiple serum glycoproteins. Brain Dev 1995;17:13–19.