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ORIGINAL

Evaluation of serum transferrin microheterogeneity for the diagnosis of congenital N-glycosylation defects

Evaluación de la microheterogeneidad de la transferrina sérica para el diagnóstico de defectos congénitos de la N-glicosilación

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ABSTRACT

Introduction: transferrin is a glycoprotein produced in the liver, whose function is to transport iron to the tissues. It has been used mainly for the differential diagnosis of anemias as a biomarker. There are different isoforms due to the difference in their glycosylation patterns. This microheterogeneity has allowed its use as a biomarker for Congenital Disorders of Glycosylation; genetic diseases as a result of mutations in genes that encode the enzymes of the post-translational mechanism of protein glycosylation.
Objective: to evaluate the microheterogeneity of serum transferrin for the diagnosis of Congenital Disorders of Glycosylation in Cuba
Methods: a descriptive and cross-sectional study was developed at the National Center for Medical Genetics in the period from 2016 to 2022. The analytical method used was the transferrin isoelectric focusing with immunofixation described by Van Eijik et al in 1983. Serum samples from 26 patients with multisystem clinical symptoms and suspicion of having a genetic disease without a definitive diagnosis were processed
Results: the IEF with immunofixation used allowed us to determine the glycosylation pattern of serum Tf. An altered Tf glycosylation pattern was found in four samples, two of type I and two of type II.
Conclusions: the IEF method to evaluate Tf glycoforms allowed the positive diagnosis in four patients, thus demonstrating the presence of Congenital Disorders of Protein N-glycosylation in Cuba

Keywords: Transferrin; Biomarker; Diagnosis; Congenital Disorders of Glycosylation.

RESUMEN

Introducción: la transferrina (tf) es una glicoproteína producida en el hígado, cuya función es transportar el hierro hacia los tejidos. Como biomarcador se ha empleado fundamentalmente para el diagnóstico diferencial de anemias. Existen diferentes isoformas debido a la diferencia en sus patrones de glicosilación. Esta microheterogeneidad ha permitido su empleo como biomarcador de los Trastornos Congénitos de la Glicosilación; enfermedades genéticas debido a mutaciones en genes que codifican las enzimas del mecanismo postraduccional de glicosilación de proteínas.
Objetivo: evaluar la microheterogeneidad de la transferrina sérica para el diagnóstico de los Trastornos Congénitos de la Glicosilación en Cuba.
Métodos: en el Centro Nacional de Genética Médica se desarrolló un estudio descriptivo y transversal en el período comprendido entre el 2016 al 2022. El método analítico empleado fue el isoelectroenfoque (IEF) de transferrina con inmunofijación descrito por Van Eijik y cols en 1983. Se procesaron muestras de suero procedente de 26 pacientes con síntomas clínicos multisistémicos y sospecha de presentar alguna enfermedad genética sin diagnóstico definitivo.
Resultados: el IEF con inmunofijación empleado permitió determinar el patrón de glicosilación de la Tf sérica. En cuatro muestras se encontró un patrón de glicosilación de Tf alterado dos de tipo I y dos de tipo II.
Conclusiones: el método IEF para evaluar las glicoformas de Tf permitió el diagnóstico positivo en cuatro pacientes, demostrando así la presencia de Trastornos Congénitos de la N-glicosilación de proteínas en Cuba

Palabras clave: Transferrina; Biomarcador; Diagnóstico; Trastornos Congénitos de Glicosilación.

INTRODUCTION

Mutations in genes encoding enzymes involved in the glycosylation of proteins and lipids are the molecular basis of Congenital Disorders of Glycosylation (CDG).1 In 1980, Jaeken described the first case, and since then, more than 170 subtypes have been identified; within these, an important group is defects in N-glycosylation. 2 -4 N-glycosylation is the most complex co- and post-translational modification that proteins undergo. The first stage of the pathway occurs in the cytosol and the rough endoplasmic reticulum, where the synthesis and transfer of a core oligosaccharide to the side chain nitrogen of an asparagine residue of the nascent protein. The order of the monosaccharide residues and the type of glycosidic bond between them is defined by the specific action of the glycosyltransferases involved in this pathway. During the second stage, specific processing of the N-glycan core occurs in the Golgi apparatus. Monosaccharides are eliminated and added according to the type of glycoprotein formed by combining highly specific glycosyltransferases and glycosidases. Depending on the stage where the affected enzyme acts, we are in the presence of a type I or II N-glycosylation disorder.

The organic alterations of CDG can be expressed at different stages of life involving any system. However, the broad clinical spectrum involves mostly the central and peripheral nervous, digestive, musculoskeletal, hematologic, immune, and integumentary systems.1,5 The diagnosis is purely biochemical and molecular. The algorithm begins with identifying defects in protein glycosylation and the affected stage. To confirm the specific subtype, further studies are required to identify the defective enzyme and the corresponding genotype.

The primary biochemical marker is serum transferrin (Tf), an iron-transporting glycoprotein in plasma.6 Tf is synthesized mainly in hepatocytes and is present in the blood in high concentration. It

comprises a polypeptide chain of 679 amino acids and an isoelectric point (pI) that can vary between 5.4 and 5.9. Each molecule consists of two lobes of similar structure for iron binding. The glycosidic group comprises two complex N-glycan chains linked to residues Asn 413 and Asn 611. The isoforms are due to the composition of amino acid sequences, the differential iron content, and the composition of the carbohydrate chains. These chains vary in their degree of branching, and each can have up to four antennae. Suppose the two stages of the protein N-glycosylation process proceed without defects. Each antenna will terminate in a sialic acid residue (negatively charged), and the major isoform will be tetrasialotransferrin (tetrasialoTf). It has four sialic acid termini, and its pI is 5.4. The next isoforms in concentration are pentasialotransferrin (pentasialoTf) and trisialotransferrin (trisialoTf). In addition, small amounts (less than 2.5 %) of glycoforms with less than three sialic acid residues circulate. These are generically referred to as carbohydrate-deficient Tf (CDT) and correspond to the isoformsasialoTf (pI 5.9), monosialotransferrin (monosialoTf, pI 5.8) and disialotransferrin (disialoTf, pI 5.7). Thus, the sialic acid content can vary from 0-8, which determines the microheterogeneity of the molecule.7-9

In patients with impaired N-glycosylation, there is an increase in the concentration of TDCs and triviality with characteristic patterns for each of the two stages. This forms the theoretical basis for diagnosing CDGs of the N-glycosylation pathway, provided that genetic polymorphisms and Iron content are considered.10 Several techniques have been developed to assess the microheterogeneity of Tf; among them are high-performance liquid chromatography, capillary electrophoresis, and mass spectrometry. The most widely used analytical method is isoelectrofocusing (IEF) with immunofixation.11 An early diagnosis of CDG is crucial to offer treatment for the symptoms that are part of the natural history of each type.12

Until 2016, no case with this diagnosis had been registered in Cuba due to the lack of an analytical method to evaluate the microheterogeneity of Tf. For this reason, the present research aimed to introduce serum Tf as a biomarker for identifying congenital disorders of protein N-glycosylation in Cuba by evaluating its microheterogeneity using IEF.

METHODS

An observational, descriptive case series study was conducted at the National Center of Medical Genetics from 2016 to 2022. The study population consisted of patients who attended clinical genetics consultations in Cuba, with suspicion of suffering from an inborn error of metabolism not elucidated using the methods protocolized in that institution. The sample consisted of 26 patients after applying a purposive sampling technique and carrying out the informed consent process, considering the following criteria.

Inclusion criteria

Patients with multisystemic manifestations have evidence of hematological, immunological, hepatic, gastrointestinal, or neurological alterations of unknown etiology.

Exclusion criteria

Patients with less than three months of life.

Patients who received a blood transfusion 10 days prior to the study.

Exit criteria

Patients whose biological samples did not meet the quality parameters to be processed in the laboratory.

The biological sample was serum obtained from each patient after centrifugation (3000 g, 5 min) of whole blood with 10% EDTAK2 as an anticoagulant. The Tf concentration of each was determined by an immunoturbidimetric method in a Mindray BS200E chemical analyzer and normalized to 80g/L using 0.9%

sodium chloride solution. The same was pre-treated to achieve saturation of serum Tf with iron under alkaline conditions. For this purpose, five µL of sodium hydrogen carbonate (0.5mmol/L) and five µL of iron (III) chloride (20mmol/L) were added to 100 µL of serum.

The analytical method employed was Tf isoelectrofocusing with immunofixation described by de Jong and van Eijik in 1988.13 In parallel, we proceeded as described in the standard operating procedure and instruction manual of the BIORAD model 111 Mini IEF Cell kit model 111 Mini IEF Cell.

A polyacrylamide gel was prepared as a support (T: 12.5%) using ampholytes with a pH range of 4-7. A polyclonal anti-human Tf antibody was used for immunofixation. Development was performed by staining with Coomassie blue after removing the rest of the serum proteins and washing with 0.9% sodium chloride solution for 24 hours. The electrophoretic run proceeded as follows:

Stage 1(migration of the ampholytes): 15 min at 100 V;

Stage 2 (alignment of the sample proteins): 15 min at 200 V,

Stage 3 (separation of Tf isoforms): 1 h at 450 V.

The result of the IEF technique was analyzed qualitatively by visualization of patterns suggestive of stage I or II defects in N-glycosylation compared with international standards. For this analysis, a negative control and two positive controls (for both patterns) were provided by the Centro de Diagnóstico de Enfermedades Moleculares de Madrid, an internationally certified institution for diagnosing these entities. To guarantee the reliability of the results, repeatability and reproducibility tests were performed.

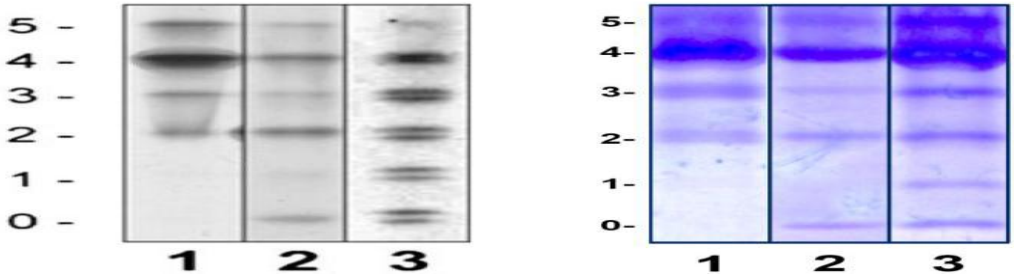
Four variables were defined to achieve the proposed objective. Table 1 defines the data corresponding to these variables.

The research is part of an institutional research project. The principles outlined in the Declaration of Helsinki of the World Medical Association, which establishes the ethical principles for research on human beings, were respected for its development.14

RESULTS

The immunofixation Tf EIF method used in the laboratory reproduced the normal, type I and II patterns of serum Tf microheterogeneity (Figure 1).

Figure 1. Electrophoretic patterns of Tf glycoforms established internationally and obtained in the laboratory.



Fuente: elaboración propia.

Note: Left image: internationally established Tf EIF patterns. Right image: Tf EIF patterns obtained in the investigation. Vertical numbers: 0 (asialoTf band); 1 (monosialoTf band); 2 (disialoTf band); 3 (trisialoTf band); 4: (tetrasialoTf band); 5: (pentasialoTf band). Horizontal numbers: lane 1 (negative control); lane 2 (positive control standard I); lane 3: (positive control standard II).

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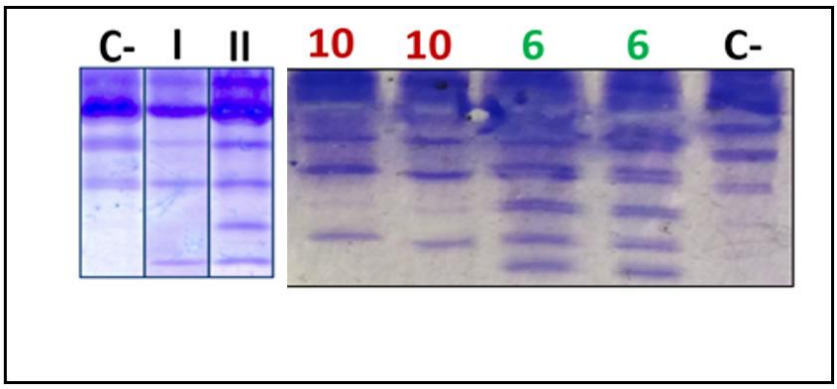
Of the 25 patient samples evaluated, four showed an altered electrophoretic pattern. Two of these corresponded to a type I pattern and two to a type II pattern. In the rest of the samples, patterns similar to the negative control were obtained.

The data for each of them are described below.

Patient 6: 56-month-old male with severe neurological and cardiovascular damage, coagulopathy, hypotonia, apnea, hepatomegaly. Type II PIT-TfE pattern (Figure 2). Diagnosis: congenital disorder of the second stage of protein N-glycosylation.

Patient 10: 23-month-old female with failure to thrive, hepatic dysfunction, hypotonia, fascial dysmorphism, cardiomyopathy, inverted mammary glands, lipodystrophy and immunodeficiency. Type I PID-TfD pattern (Figure 2). Diagnosis: congenital disorder of the first stage of protein N-glycosylation, possible PMM2-CDG (CDG Ia).

Figure 2. Electrophoretic patterns of Tf glycoforms from patients 6 and 10. Left image: internationally established Tf EIF patterns.



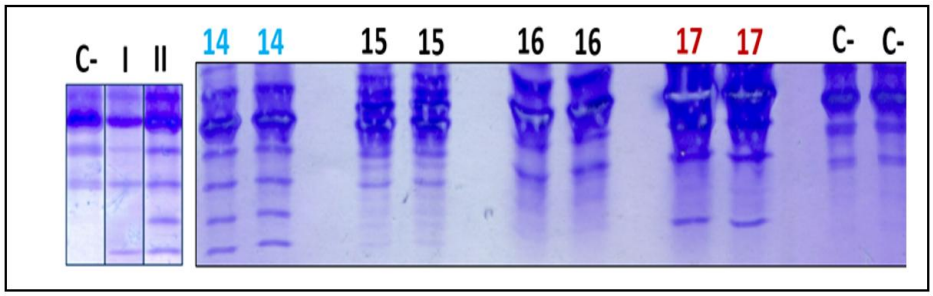
Source: own elaboration.

Note: Right image: EIF patterns of Tf obtained in the investigation. Lane C- (negative control); lane I (positive control pattern I); lane II: (positive control pattern II); lane 10 (patient serum 10); lane 6 (patient serum 6).

Patient 14: 18-month-old male with heart disease, anemia, neurological disorders, episodes of hypothermia, immunodeficiency. Type II PIT-TfE pattern (figure 3). Diagnosis: congenital disorder of the second stage of N-glycosylation of CDG-II proteins.

Patient 17: 8-month-old male with protein-losing enteropathy, chronic diarrhea, coagulopathy, recurrent vomiting, signs of cortical and cerebellar atrophy, hypoglycemia, anemia, edema. Pattern of type I PIT-TfE (Figure 3). Diagnosis: congenital disorder of the first stage of N-glycosylation of CDG-I proteins, possible MPI-CDG (CDG Ib).

Figure. 3 Electrophoretic patterns of Tf glycoforms from patients 14, 15, 16 and 17.



Source: own elaboration.

Note: Left image: internationally established Tf EIF patterns. Right image: Tf EIF patterns obtained in the investigation. Lane C- (negative control); lane I (positive control standard I); lane II: (positive control standard II); lane 14 (patient serum 14); lane 15 and 16 (negative sera); lane 17 (patient serum 17).

CONCLUSIONS

In this work we introduced the IEF method to evaluate Tf glycoforms and thus use it as a first biomarker for CDG identification of N-glycosylation of proteins in the country. This allowed the positive diagnosis in four patients, thus demonstrating the presence of these diseases in Cuba. In order to confirm the specific subtype, the introduction of molecular biology methods is recommended. With this, the knowledge of these diseases can be expanded and the arsenal of diagnostic methods for inborn errors of metabolism in the national territory can be increased.

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FINANCING

None.

CONFLICT OF INTEREST

None.