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DIAGNOSIS CHARACTERISTICS OF CONGENITAL DISORDERS OF GLYCOSYLATION OF 40 SUSPECTED PATIENTS FROM MOLDOVA

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REZUMAT

CARACTERISTICILE DIAGNOSTICULUI DEREGLĂRILOR CONGENITALE ALE GLICOZILĂRII LA 40 DE PACIENȚI SUSPECȚI DIN MOLDOVA

Introducere: Erorile în sinteza, asamblarea și / sau procesarea glicanilor provoacă o familie de patologii genetice grupate într-o unitate nosologică sub denumirea de Deraglări Congenitale ale Glicozilării (CDG), actualmente fiind descrise în jur de 150 de tipuri. În orice stare clinică inexplicabilă este necesar de suspectat CDG, în special în cazul afectărilor multisistemice cu implicare neurologică. Metoda obișnuită pentru diagnosticarea CDG este investigarea transferinei serice prin focalizare izoelectrică (IEF). **Scopul:** Diagnosticul CDG la pacienții suspecți cu simptome de afectare multisistemică, bazată pe screeningul transferinei serice prin focalizare izoelectrică.

Materiale și metode: În studiul prezent au fost utilizate probe de ser recoltate de la 40 de pacienți cu vârste variate (2 luni - 15 ani)suspectați pentru CDG, care aveau hipotonie, convulsii, retard psihoneuromotor, caracteristici dismorfice cu implicare multisistemică. Pentru diagnosticul CDG, IEF al transferinei serice a fost efectuat în colaborare cu RadboudUMC, Nijmegen, Olanda. În unele cazuri, s-a utilizat tratamentul cu neuraminidază pentru a detecta polimorfismul genetic al transferinei care poate imita structura anormală a glicanului. În plus, s-a efectuat spectroscopia RMN a urinei pacienților cercetați pentru diagnosticarea erorilor înnăscute de metabolism, care pot imita un profil caracteristic pentru CDG.

Rezultate: Ca urmare a screeningului selectiv, 37 de pacienți aveau un profil normal al transferinei, în timp ce 3 probe au fost identificate cu profil anormal, sugestiv pentru CDG I. Galactozemia, fructozemia, alcoolismul pot exprima același profil de IEF ca și pentru CDG I. Probele celor trei pacienți au fost analizate prin metode biochimice și molecular-genetice care au identificat că la un pacient paternul anormal IEF a transferinei a fost cauzat de galactozemie, în timp ce la un altul de fructozemie. În cazul celui de-al treilea pacient rezultatele sugerează prezența CDG I și necesită o analiză avansată a profilului glicomic prin spectroscopia de masă.

Concluzie: Focalizarea izoelectrică a transferrinei este instrumentul principal pentru diagnosticul CDG pentru multe laboratoare de screening datorită eficienței crescute la preț rezonabil, în comparație cu alte metode.

Cuvinte-cheie: Glicozilare, CDG, focalizare izoelectrică a transferinei, screening selectiv

РЕЗЮМЕ

ХАРАКТЕРИСТИКА ДИАГНОСТИКИ ВРОЖДЕННЫХ НАРУШЕНИЙ ГЛИКОЗИЛИРОВАНИЙ У 40 ПОДОЗРЕВАЕМЫЕ БОЛЬНЫХ ИЗ МОЛДОВЫ

Введение: Нарушения синтеза, сборки и/или процессинга гликанов являются причиной группы генетических патологий метаболизма, называемых врождёнными нарушениями гликозилирования (ВНГ), типов которых на данный момент описано около 150. ВНГ следует подозревать при любой необъяснённой клинической патологии, особенно с полиорганным поражением с вовлечением нервной системы. Обычный метод диагностики ВНГ – исследование трансферрина в сыворотке крови методом изоэлектрического фокусирования (ИЭФ).
Цель: Представление результатов ИЭФ трансферрина сыворотки крови 40 пациентов с полиорганными поражениями, с подозрением на ВНГ.

Материалы и методы: В представленном исследовании использовались сыворотки 40 педиатрических пациентов с подозрением на ВНГ, различного возраста (2 мес – 15 лет), с гипотонией, судорогами, задержкой психомоторного развития, признаками дизморфизма, нарушениями развития с полиорганной патологией. Для диагностики ВНГ ИЭФ трансферрина сыворотки производилось в сотрудничестве с RadboudUMC, Неймеген, Нидерланды. В некоторых случаях, использовалось лечение нейраминидазой с целью обнаружения генетического полиморфизма трансферрина.

Результаты: В результате селективного скрининга обнаружено, что у 37 пациентов с подозрением на ВНГ был нормальный профиль трансферрина, а в 3 образцах был обнаружен аномальный профиль, говорящий о возможности ВНГ I. К сожалению, анализ трансферрина методом ИЭФ имеет некоторые ограничения, в связи с фактом, что при галактоземии, фруктоземии и алкоголизме может обнаруживаться такой же профиль, как и при ВНГ I. Таким образом, данные пациенты были проанализированы с использованием биохимических и молекулярно-генетических методов, обнаруживших, что у одного пациента аномальный профиль ИЭФ трансферрина вызван галактоземией, а у другого пациента – фруктоземией. Последний пациент успешно прощёл данный тест, что говорит о наличии ВНГ I и необходимости определения профиля гликомики.

Заключение: ИЭФ трансферрина представляет собой основное средство диагностики ВНГ во многих лабораториях скрининга, в связи с экономической эффективностью по сравнению с другими методами

Ключевые слова: Гликозилирование, ВНГ, изоэлектрическое фокусирование трансферрина, селективный скрининг.

Introduction

Glycosylation represents a process of adding carbohydrate residues (glycans) to the protein molecule that physiologically occurs in approximately half of all proteins expressed in human cells. Formation of glycoproteins is characteristic for most extracellular proteins (ex. serum proteins), most membrane proteins and for several intracellular proteins like lysosomal enzymes. Depending on how the molecule of carbohydrate is linked to polypeptide chain we can distinguish two types of glycans: N-glycans where the sugar is linked to *Asn* aminoacid and O-glycans – in this case sugars bound to *Ser* and *Thr* [1].

Glycoproteins are widespread all other the human body and other living organisms realizing a crucial role for maintaining the life. They have a specific function that include glycoprotein targeting (e.g. most lysosomal enzymes) and adhesion of the cell to other cell or to extracellular matrix, and non-specific in which carbohydrate helps the protein in their correct folding obtaining its functional conformation, in increasing their secretion capability and in protection against proteolytic enzymes [2].

Process of glycosylation involves a vast variety of enzymes as glycosyltransferase family which catalyzes the glycan assembling and others that participate in formation of precursors and monomers used in formation of glycoproteins [2]. Therefore, errors in the synthesis assembly and/or processing of glycans due to defects in genes that encodes this enzymes provoke a family of metabolic genetic pathologies grouped together as Congenital Disorders of Glycosylations. From the moment of first description in 1980 until now, over 150 different types of Congenital Disorders of Glycosylation (CDG) have been described, most of them affecting the N-glycosylation pathway [3][4].

Clinical phenotype of CDG patients is very heterogeneous [5]. Therefore CDG should be considered in any unexplained clinical condition, particularly in multi-organ disease with neurological involvement [1][5]. First step in diagnosis of CDG is investigation of glycosylation state of serum transferrin by isoelectric focusing (IEF).

Although IEF of transferrin is not able to detect all types of CDG, it is widely used by many laboratories due to its cost efficiency compared with other methods making it accessible for most of screening laboratories [5][6]. Additionally in diagnostic algorithm it can be included also isoelectric focusing of serum apolipoprotein C-III which is only O-glycosylated and permits detection of some O-glycosylation disorders [7]. The final step confirming the diagnosis is performing of glycomics profiling using mass spectrometry and direct mutation analysis due to sequencing of panel of genes known to be involved in CDG or whole exome sequencing (WES) [1][5][6].

The aim: Identification of CDG through suspected patients with multi-organ involvement based on serum transferrin by isoelectric focusing screening.

Materials and methods

In the present study the serum of 40 suspected CDG pediatric patients with various ages (2mo–15y) was used. Selection of clinically suspected cases was made through medical-genetic consultation in Genetic Department of Institute of Mother and Child, Chisinau, Moldova. Patients with hypotonia, seizures, psihoneuromotor retardation, dimorphic features, failure to thrive with multisystem involvement were suspected for CDG and were included in this research.

Isoelectric focusing of serum transferrin was performed in collaborations with Translational Metabolic Laboratory, Radboudumc, Nijmegen, Netherlands as described by Wopereis [7][8]. Serum samples were incubated for 30 min with 10 mM ferric citrate and 0.5 mM sodium hydrogen carbonate (2:1) in a ratio of 10:3 (serum to solution) to saturate the transferrin with iron. The iron-saturated serum was diluted 5 times with water and applied to a hydrated precast gel (pH 4–7) on an Ultraphore system (Amersham Pharmacia Biotech).

After IEF, the transferrin isoforms were detected by adding rabbit anti-human transferrin antibody (Agilent, USA) and the gels were stained with Coomassie blue. The relative amounts of the transferrin isoforms were determined by scanning the stained gel using an Image master Labscan, Ver. 3.00 (Amersham Pharmacia Biotech).

In some cases, it was used neuraminidase treatment to detect transferrin genetic polymorphism that can mimic abnormal glycan. Human serum was incubated with neuraminidase (5 kU/L) from Clostridium perfringens (Sigma; 5U in 0.5 mL 0.1 mol/L Tris, pH 7.0) overnight at room temperature. Samples were analyzed for transferrin IEF as described above.

Results and discussions

As a result of selective screening by IEF transferrin method, 37 patients had a normal transferrin profile, while 3 samples were identified with abnormal profiles (tab.1).

During IEF method protein is segregated in a polyacrylamide gel with pH gradient by their isoelectric point. Serum transferrin being a glycoprotein has two fragments of glycan that end each with 2 moieties of sialic acids. A molecule of sialic acid is negatively charged, this greatly modifying the isoelectric point of transferrin molecule [5]. Normal transferrin IEF profile is represented by the presence of pronounced band characteristic to glycoprotein with 4 moieties of sialic acid (tetrasialotransferrin form). Additionally, sometimes can be seen weak band of protein with 3 (trisialotransferrin form) or 5 (pentasialo form) molecules of sialic acid. This fact shows that in normal condition, transferrin has a complete glycan but in the same time, low quantity of transferrin can be with reduced glycan structure [9].

When the same uncharacteristic bands appear associated with tri-, di-, mono- and asialotransferrin form, it means that a defect in glycosylation pathway has occurred. Due to the IEF analysis of transferrin, two big groups of CDG – I and II can be distinguished. For CDG I there is characteristic presence of disialo- and asialotransferrin forms, but at the same time for CDG II, trisialo- and monosialotransferrin forms may occur [5][9].

According to the obtained electrophoregram, three patients with abnormal IEF transferrin profile were diagnosed. In these samples a band of disialo- and asialotransferrin was identified that is suggestive for CDG I.

Table 1. Results of IEF of transferrin on a 40 CDG suspected patients

Patients code	IEFT Result	Patients code	IEFT Result	Patients code	IEFT Result	Patients code	IEFT Result
P1	normal	P11	normal	P21	normal	P31	normal
P2	normal	P12	normal	P22	normal	P32	normal
P3	normal	P13	normal	P23	normal	P33	normal
P4	normal	P14	normal	P24	normal	P34	normal
P5	normal	P15	normal	P25	normal	P35	normal
P6	normal	P16	Abnormal	P26	normal	P36	normal
P7	normal	P17	Abnormal	P27	normal	P37	normal
P8	normal	P18	Abnormal	P28	normal	P38	normal
P9	normal	P19	normal	P29	normal	P39	normal
P10	normal	P20	normal	P30	normal	P40	normal

Fig. 1. IEF of transferrin: 1-3 abnormal profiles suggestive for CDG I; 4-5 normal transferrin profile

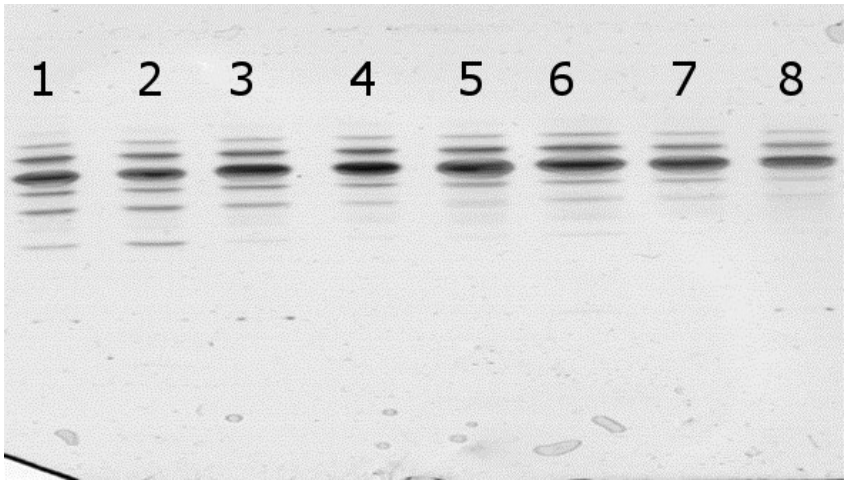
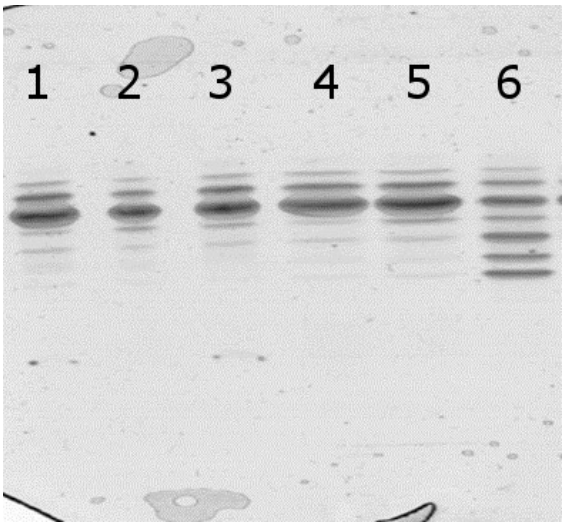


Fig. 2. IEF of transferrin: 1-5 normal transferrin profile; 6 – control sample with abnormal profile suggestive for CDG II.



Unfortunately, IEF analysis of transferrin has the same pitfalls due to the fact that galactosemia, fructosemia, alcoholism can express the same profile as for CDG I. Supplementary to this, in some cases, mutations that lead to amino acid changes in transferrin may alter the charge of the protein, thereby leading to shifts in the IEF pattern, which mostly resemble to either tri- or pentasialo- transferrin, resulting into transferrin polymorphism.

Therefore for confirmation of diagnosis for CDG I, the samples of these three patients were analyzed by biochemical and molecular genetics methods for galactosemia and fructosemia. In this order the samples of urine from suspected patients have been analyzed by ^{1}H NMR spectroscopy and the metabolites as galactose and galactitol have been identified in one patient, galactosemia being confirmed then by molecular analysis (P17). In the other one patient (P16) the molecular analysis obtained in accordance with clinical features revealed that abnormal transferrin IEF pattern was caused by fructosemia, in this cases CDG I was not confirmed. In contrast to those two patients the third one, successfully pass galactosemia and fructosemia test and even neuraminidase assay that could help in identification of amino acid changes in transferrin molecule. This fact suggests that this patient has a CDG I which requires analysis of glycomics profiling using mass spectrometry to establish the structure of the defective glycan that will help in determining the type of CDG. This investigation is undergoing in Radboudumc, Nijmegen, Netherlands.

Conclusion

- The CDG is relatively new discovered pathologies affecting many organs due to widespread all other the human body of different glycoprotein that have a crucial function in maintaining life.
- IEF of transferrin is main tool for diagnosis of CDG for many screening laboratories due to its cost efficiency compared with other methods.
- Before considering the positive result of transferrin IEF, should exclude the secondary abnormalities caused by Galactosemia, Fructosemia and other.
- Although IEF of transferrin is good screening test, the final diagnostic step includes analysis of the structure of the defective glycan by mass spectrometry and mutational assays using NGS platform.

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