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CLASSICAL GALACTOSEMIA- A CASE REPORT

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SUMMARY

Key Words: Galactosemia, GALT gene, classical galactosemia, inborn error of metabolism.

Background: Galactosemia is an autosomal recessive metabolic error which is caused by deficiency in four enzymes coded by GALT, GALK, GALE, and GALM genes. Accumulation of galactose and its metabolites causes nervous system injuries, cataracts, kidney and liver damage, etc. The form of galactosemia characterized by the most severe manifestations is classic galactosemia associated with mutations in GALT gene. The current report is based on a case of a 9 month old patient manifesting hepatosplenomegaly, failure to thrive and incipient cataract, suggesting galactosemia, inducing the process of diagnosis.

The aim: Presentation of a galactosemia case and association of the biochemical parameters with the molecular genetics results in order to find a correlation between the genotype and the phenotype.

Material and methods: Quantification of the total blood galactose was performed on dry blood spots at *CytoGenomic* laboratory in Bucharest, Romania. Urine galactose and galactitol levels were determined using NMR spectroscopy of body fluids at the "Petru Poni" Institute of Macromolecular Chemistry Iași, Romania. The DNA was obtained from patient's whole blood samples using salt- out method. The identification of p.Q188R mutation in GALT gene was done using PCR/RFLP technique with visualization in polyacrylamide and agarose gel. The second mutation, p.K285N, was established using Sanger Sequencing of all GALT gene exons.

Results: The DBS analysis revealed an elevated level of total galactose- 40.34 mg/dL. The further step was NMR Spectroscopy of body fluids revealed high levels of urine galactose- 10255 mmol/mol creatinine on the first day of diagnosis and 25287 mmol/mol creatinine on the second day. The galactitol value on the first day was 9244 mmol/mol creatinine, on the second day- 8777 mmol/mol creatinine, and on the day 62 after the beginning of the treatment- 714 mmol/mol creatinine. The molecular genetics analysis revealed that the patient's GALT gene genotype is p.Q188R/p.K285N, presenting a classical form of galactosemia.

Conclusion: Galactosemia is a serious inborn error of galactose metabolism that needs fast and efficient biochemical diagnosis through neonatal screening and molecular genetics study for an effective treatment for prevention of the multisystem complications that the disease may cause.

REZUMAT

GALACTOZEMIA CLASICĂ- RAPORT DE CAZ

Cuvinte cheie: Galactozemia, gena GALT, galactozemia clasică, eroare înnăscută de metabolism.

Introducere: Galactozemia este o eroare metabolică autozom-recesivă, care este cauzată de deficiența a patru enzime codate de genele GALT, GALK, GALE și GALM. Acumularea galactozei și a metaboliților săi provoacă afecțiuni ale sistemului nervos, cataractă, leziuni ale rinichilor și ficatului, etc. Forma de galactozemie caracterizată prin cele mai severe manifestări este galactozemia clasică asociată cu mutațiile din gena GALT. Raportul actual se bazează pe un caz al unui pacient de 9 luni, cu hepatosplenomegalie, dificultăți de dezvoltare **și** manifestări incipiente de cataractă, care au sugerat galactozemia, inducând procesul de diagnostic.

Scopul: Prezentarea unui caz de galactozemie şi asocierea parametrilor biochimici cu rezultatele geneticii moleculare pentru a elucida o corelaţie între genotip şi fenotip.

Materiale şi metode: Cuantificarea galactozei totale din sânge a fost efectuată din DBS la laboratorul *CytoGenomic* din Bucureşti, România. Nivelurile de galactoză şi galactitol în urină au fost determinate utilizând spectroscopia RMN a fluidelor corporale la Institutul de Chimie Macromoleculară „Petru Poni" Iaşi, România. ADN-ul a fost obţinut din probele de sânge ale pacientului, folosind metoda salt- out. Identificarea mutaţiei p.Q188R în gena GALT s-a realizat folosind tehnica PCR / RFLP cu vizualizare în poliacrilamidă şi gel de agaroză. A doua mutaţie, p.K285N, a fost stabilită utilizând secvenţierea Sanger a tuturor exonilor genei GALT.

Rezultate: Analiza DBS a relevat un nivel crescut de galactoză totală - 40,34 mg/dL. Pasul următor a fost spectroscopia RMN a fluidelor corporale a relevat niveluri ridicate galactoză **în urină**- 10255 mmol / mol creatinină în prima zi de diagnostic şi 25287 mmol / mol creatinină în a doua zi. Valoarea galactitolului în prima zi a fost de 9244 mmol/ mol creatinină, în a doua zi - 8777 mmol/mol creatinină şi în ziua 62 după începerea tratamentului- 714 mmol/mol creatinină. Analiza molecular - genetică a demonstrat faptul că genotipul genei GALT a pacientului este p.Q188R / p.K285N, prezentând o formă clasică de galactozemie.

Concluzii: Galactozemia este o eroare înnăscută gravă a metabolismului galactozei care necesită un diagnostic biochimic rapid şi eficient prin screening neonatal şi studiu molecular- genetic pentru stabilirea unui tratament eficient **şi** prevenirea complicaţiilor multisistemice pe care boala le poate provoca.

РЕЗЮМЕ

КЛАССИЧЕСКАЯ ГАЛАКТОЗЕМИЯ- ОПИСАНИЕ КЛИНИЧЕСКОГО СЛУЧАЯ

Ключевые слова: Галактоземия, ген GALT, классическая галактоземия, врожденное нарушение метаболизма.

Введение: Галактоземия - это аутосомно-рецессивное нарушение метаболизма, вызванное дефицитом четырех ферментов, кодируемых генами GALT, GALK, GALE и GALM. Накопление галактозы и ее метаболитов вызывает нарушения нервной системы, катаракту, повреждение почек и печени и т. д. Наиболее тяжелой формой галактоземии является классическая галактоземия, вызванная мутациями в гене GALT. Данный отчет основан на случае девятимесячного пациента с галактоземией, у которого была выявлена гепатоспленомегалия, задержка развития и начальная форма катаракты, предполагая галактоземию и повлекая за собой процесс диагностики.

Цель: Презентация случая галактоземии и ассоциация биохимических параметров с результатами молекулярной генетики с целью выявления корреляции между генотипом и фенотипом.

Материалы и методы: Определение общего содержания галактозы в крови проводилось на сухих пятнах крови в лаборатории *CytoGenomic* в Бухаресте, Румыния. Уровни галактозы и галактитола в моче были определены с помощью ЯМР-спектроскопии жидкостей организма в Институте химии высокомолекулярных соединений им. Петру Пони, Яссы, Румыния. ДНК была получена из образцов крови пациента с помощью технологии salt- out. Идентификация мутации p.Q188R в гене GALT была произведена с помощью метода ПЦР/ ПДРФ с визуализацией в полиакриламидном и агарозном геле. Вторая мутация, p.K285N, была установлена с использованием секвенирования всех экзонов гена GALT по Сэнгеру.

Результаты: Анализ DBS выявил повышенный уровень общей галактозы - 40,34 мг/дл. Следующим шагом была ЯМР-спектроскопия биологических жидкостей, которая показала высокий уровень галактозы в моче- 10255 ммоль/моль креатинина в первый день диагностики и 25287 ммоль/моль креатинина на второй день. Показатель галактитола в первый день составил 9244 ммоль/моль креатинина, на второй день- 8777 ммоль/моль креатинина, а на 62-й день после начала лечения- 714 ммоль/моль креатинина. Молекулярно- генетический анализ показал что генотип гена GALT пациента p.Q188R/p.K285N представляет собой классическую форму галактоземии.

Заключение: Галактоземия - серьезное врожденное нарушение метаболизма галактозы, которое требует быстрой и эффективной биохимической диагностики с помощью неонатального скрининга и молекулярно-генетического исследования для эффективного лечения и предотвращения множественных осложнений, которые может вызвать болезнь.

Introduction

Galactosemia is an autosomal- recessive error of galactose metabolism. Galactose is a monosaccharide that together with glucose makes up lactose. In normal metabolism, galactose is processed into glucose-1-phosphate by a series of enzymes, representing Leloir pathway (fig.1).

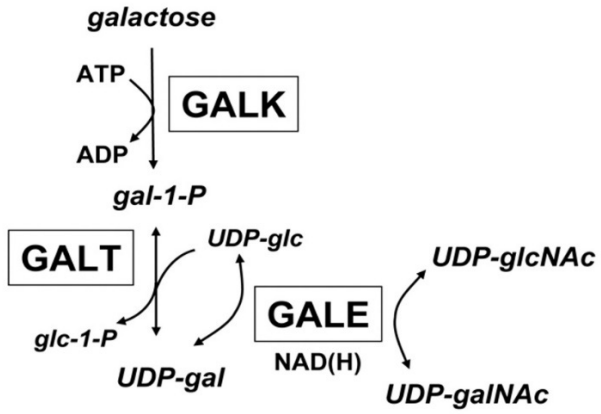

Fig.1 ***The Leloir pathway of galactose metabolism. K. Schulpis. 2017***

In case of a pathology, metabolic reactions are disrupted on a certain step depending on the responsible enzyme: 1-phosphate uridylyltransferase (GALT), galactokinase (GALK), and UDP-galactose 4'-epimerase (GALE). The disease represents a considerable threat for newborns whose main source of nutrition is breast milk, rich in galactose. The essence is that in galactosemia, the processing of galactose into glucose is not possible, inducing serious consequences of toxic accumulation of galactose and its metabolites[1]. Additionally, in cases of galactosemia unrelated to those enzymes, another recently discovered enzyme is inspected- galactose mutarotase (aldose -1-epimerase), encoded by GALM gene that transforms α- D-galactose to ß-D-galactose[2].

Materials and Methods

We report on a 8 months old girl case - the first child, born from the first pregnancy in a non-consanguineous Moldovan couple. She was born at term with body weight of 2,900 grams and Apgar Score 3/5. The child was hospitalized with mild hepatosplenomegaly, elevated transaminases, vomiting, compensated acidosis, and severe hypoglycemic symptoms (3.3-2.9 mmol/L). Because of the liver involvement and metabolic manifestations, metabolic work- up (DBS, urine, plasma, blood serum) have been collected and galactosemia was considered. No abnormalities were identified in amino acids analysis and lactate status. The quantification of total galactose was performed at CytoGenomic laboratory in Bucharest, Romania. The measurement of urine galactose and galactitol were performed using NMR spectroscopy of body fluids at the "Petru Poni" Institute of Macromolecular Chemistry Iași, Romania.

Extraction of the DNA from whole blood samples was performed using salt-out method as described in the practical guide elaborated in the laboratory. The DNA was stored in *TE-Buffer* at -20 degrees Celsius. The PCR method was used for amplification of exon 6 from GALT gene, necessary for identification of the common p.Q188R mutation. The reagents used for this reaction include: dNTPs (0.2 mM each nucleotide), MgCl2 (20 mM), *DreamTaq Polymerase* (0.6-0.7 U), primer F and R (0.25 μM), *DreamTaq Buffer*, and genomic DNA 0.5-1 g. Using the amplification program (Denaturation 95 degrees Celsius - 30 sec. Annealing, 62 degrees Celsius - 30 sec, Elongation, 72 degrees Celsius - 30 sec, repeated in 35 cycles) we acquired a fragment of 348 bp of exon 6 and addiacent intron sequence. The obtained fragment (10μL of amplicon) was digested using *MspI* enzyme from Thermo Fischer Scientific, USA and *Tango Restriction Buffer*. The electrophoresys analysis in 7.5% PAAG gel was used for separation of DNA fragments and their visualisation. In case of presence of the mutation, the fragment is digested in two parts: 184 bp and 164 bp. In case when PCR/RFLP method did not allow identification of the full pathological genotype, Sanger Sequencing of all exons of GALT gene and their adjacent intronic region (10-20 bp) was performed. The sequencing reaction was made using *BigDye Terminator 3.1* (Applied Byosystems, USA), purification using *BigDye Xterminator* (Applied Byosystems, USA), and capillary electrophoresis was done on Genetic Analyzer 3500 DX (Applied Byosystems, USA). The analysis workflow was performed as described in the practical guide of the laboratory.

Results and Discussions

Clinical Background

At the moment of hospitalization (9 months), the patient already manifested some of the characteristic symptoms such as hepatosplenomegaly, vomiting, and poor growth, but there were no obvious signs of cataract - a common complication in galactosemia that has a irreversible character. It is caused by galactitol accumulation in cells, this respectively provokes hyperosmotic and oxidative stress in eye tissues. Additionally, untreated galactosemia can induce irreversible nervous system issues such as mental retardation, developmental perturbations, learning and speaking disabilities, and memory issues. In addition, those individuals are susceptible to certain psychological disorders such as those from the autistic spectrum, obsessive - compulsive disorder, depression, etc. Not to mention the effect of the disease on female gonads that has a high prevalence in female patients. They experience serious problems such as primary ovarian insufficiency and delayed pubertal development[3]. The manifestations that the patient presented suggested disorders of galactose metabolism and DBS sample was collected to quantify the total galactose in blood. In the reviewed case, the primary analysis from DBS showed an elevated level of total galactose (Tgal= 40.34 mg/dL) that suggested further monitoring of galactose parameters and other metabolites.

Urine metabolic profile was used in order to confirm the diagnosis, particularly paying attention to galactose and galactitol values. High levels of urine galactose and galactitol are distinct biomarkers of galactose metabolism defects. Galactose is one of the first substances that newborn is exposed to because it is one of the core elements of breast milk that serves as the main source of nutrients for the newborn. As galactose enters the cell, the process of its metabolism begins. First, galactose is processed to galactose-1-phosphate by the enzyme encoded by GALK gene with use of ATP. The following step is performed by GALT enzyme, transforming galactose-1-phosphate into UDP-galactose and UDP-glucose into glucose-1-phosphate. The role of the third enzyme, GALE, is to convert uridine diphosphate N-acetylgalactosamine into uridine diphosphate N-acetylglucosamine[3]. The process of galactose metabolism is called Leloir pathway and it is the mechanism that offers information about the possible issues in the process of galactose metabolism by separation of each phase accordingly to the responsible enzyme. By pointing out the steps taking place in the Leloir Pathway and alternative galactose metabolism pathways, it is possible to highlight the causes of clinical manifestations of galactosemia, represented mainly by defected enzyme, indicating elevated levels of galactose and other metabolites in patient's urine in comparison with the normal values. In a study it was described that normal levels of urine galactose vary between 0.0- 33.0 μmol/mmol Crea in infants[4]. In another study, normal levels of galactitol for newborns is 0-30 μmol/mmol Crea[5].

There are several forms of galactosemia: type I galactosemia, the most frequent is related to GALT gene (1-phosphate uridylyltransferase), type II galactosemia related to GALK gene (galactokinase), and type III galactosemia, linked to GALE gene (UDP-galactose 4'-epimerase)[6]. The recently discovered type IV of galactosemia, caused by biallelic variants in GALM gene affecting the galactose mutarotase enzyme[7].

In type I and II galactosemia, high levels of urine galactitol can serve as a biochemical marker. Galactitol is a toxic product of alternative galactose reductive pathway that induces oxidative stress in the affected cells[8]. Another toxic metabolite is galactonate- a product of oxidative pathway of galactose that is detected in abnormally high concentration in urine (>130 umol/mmol creatinine)[9]. Thus, the monitoring of galactitol and galactonate concentration are necessary and useful in terms of galactosemia type detection and diet management. A crucial element in prevention of the severe complications of galactosemia is a prompt diagnosis of the disorder in order to proceed to the immediate dietary plan. That said, at the moment, the only solution that may help galactosemia patients is a coordinated low-galactose diet along with regular monitoring of biochemical parameters that indicate the level of galactose and its metabolites in the blood and urine. After the diagnosis, the patient from the current report proceeded to the diet therapy. The child was treated by lactose free diet and therefore, blood galactose level normalized. Those steps are important because the diet therapy is individual and to understand what is suitable for every individual patient, genetic and biochemical aspects must be taken into account. In addition, complementary target - oriented treatments may be necessary to ameliorate cataracts[10], development delays, hepatomegaly, and any other emerging manifestations of galactosemia on the organism[11]. It is essential to follow the diet and observe the dynamics of the disease as long as new symptoms may emerge or the present ones are evolving. As a potential treatment for type I galactosemia, pharmacological chaperones are considered. This method lacks research evidence or efficiency, making it a prospective solution for patients with severe forms of galactosemia[12].

GALT deficiency can be stratified in three categories, based on the residual enzyme activity of 1-phosphate uridylyltransferase, The most severe is classic galactosemia with residual enzymatic activity of GALT less than 1%. The clinical variant is characterized by increased enzyme activity (1-10%) in other organs: brain, liver, intestines. Respectively, the biochemical (Duarte) form has values between 10% and 35% of enzyme residual activity and in some cases can be considered as a benign type. The severity of the metabolic disorder directly depends on the activity of 1-phosphate uridylyltransferase enzyme, thus it an important aspect that provides details about the severity of galactosemia the patient has[13]. In the current case, multiple galactose and galactitol quantification analysis were performed that confirmed the malfunction of the metabolic process of breaking down galactose and for the confirmation of the diagnosis, analysis of GALT gene was done (tab.1), assuming that the clear majority of galactosemia cases are caused by GALT deficiency[14].

Tab. 1

Results of NMR Spectroscopy: Galactose and galactitol levels in urine.

Probe number	Date interval	Material	Galactose Value	Galactitol Value	Treatment
1	Day 1 9 months	Urine	10255 mmol/mol creatinine	9244 mmol/ mol creatinine	Before treatment
2	Day 2 9 months	Urine	25287 mmol/mol creatinine	8777 mmol/ mol creatinine	Before treatment
3	Day 64 10 months	Urine	-	714 mmol/ mol creatinine	Long-term lactose-free diet

During long – term treatment, the monitoring of urine galactose and galactitol by NMR spectroscopy revealed that galactose disappeared completely on diet, contrarily to galactitol level which diminished a lot by more than 10 fold times, but did not disappear entirely.

As the first step of molecular genetic testing, PCR/RFLP analysis was performed that confirmed the presence of

the p.R188Q mutation in exon 6 of GALT gene. Given that, for the detection of the second mutation Sanger Sequencing was done. As result, the second mutation was detected- p.K285N that formed a complete picture of the patient's genotype. That said, GALT deficiency was confirmed. The first mutation, p.Q188R induces reduced activity of the enzyme, thus its presence has a serious impact on the protein structure. The second mutation- p.K285N presents lower impact on the functionality of the enzyme[15]. The combination of these mutations lead to type I galactosemia, but with a milder phenotype because one of the mutations has a lower coefficient of pathogenicity than the other. As a consequence, there was supposed that the cataract was not present at the moment of diagnosis due to the second milder mutation[10].

Conclusions

In the case described in this report, an efficient combination of clinical biochemical and molecular genetics methods allowed diagnosis of galactosemia. As the main diagnosis tools, high levels of galactose in blood, analyzed in DBS and high levels of galactose and galactitol in urine obtained by NMR spectroscopy were used. Using those tools permits an efficient long - term management of the disease and treatment.

Molecular genetics methods were used to confirm GALT deficiency. The identified mutations (p.Q188R and p.K285N) can help to understand the pathway of clinical manifestations according to genotype - phenotype association.

Galactosemia is a serious inborn error of galactose metabolism that needs fast and efficient biochemical diagnosis through neonatal screening and molecular genetics study for an effective treatment for prevention of the multisystem complications that the disease may cause.

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BIBLIOGRAPHY

1. Timson DJ. The molecular basis of galactosemia — Past, present and future. *Gene*. 2016;589(2):133-141. doi:10.1016/j.gene.2015.06.077
2. Wada Y, Kikuchi A, Arai-Ichinoi N, et al. Biallelic GALM pathogenic variants cause a novel type of galactosemia. *Genet Med*. 2019;21(6):1286-1294. doi:10.1038/s41436-018-0340-x
3. Demirbas D, Coelho AI, Rubio-Gozalbo ME, Berry GT. Hereditary galactosemia. *Metabolism*. 2018;83:188-196. doi:10.1016/j.metabol.2018.01.025
4. (). Geigy Scientific Tables, 8th Rev edition, pp. 130. Edited by C. Lentner, West Cadwell, N.J.: Medical education Div., Ciba-Geigy Corp. Basel, Switzerland c1981-1992.. . *Geigy Sci Tables*.
5. Embade N, Cannet C, Diercks T, et al. NMR-based newborn urine screening for optimized detection of inherited errors of metabolism. 2019;(May):1-9. doi:10.1038/s41598-019-49685-x
6. Schulpis KH, Thodi G, Chatzidaki M, Iakovou K, Molou E. Letter to the Editor Rare cases of galactose metabolic disorders : identification of more than two mutations per patient. 2017:6-7.
7. Iwasawa S, Kikuchi A, Wada Y, et al. The prevalence of GALM mutations that cause galactosemia: A database of functionally evaluated variants. *Mol Genet Metab*. 2019;126(4):362-367. doi:10.1016/j.ymgme.2019.01.018
8. Coelho AI, Rubio-gozalbo ME, Vicente JB, Rivera I. Sweet and sour : an update on classic galactosemia. 2017:325-342. doi:10.1007/s10545-017-0029-3
9. Yager CT, Chen J, Reynolds R, Segal S. Galactitol and galactonate in red blood cells of galactosemic patients. 2003;80:283-289. doi:10.1016/j.ymgme.2003.08.021
10. Cotlier E. CURRENT j. 1988;32(5).
11. Rubio-Gozalbo ME, Haskovic M, Bosch AM, et al. The natural history of classic galactosemia: Lessons from the GalNet registry. *Orphanet J Rare Dis*. 2019;14(1):1-11. doi:10.1186/s13023-019-1047-z
12. Mccorvie TJ, Timson DJ. *Galactosemia : Opportunities for Novel Therapies*. Elsevier Inc.; 2020. doi:10.1016/B978-0-12-819132-3/00011-7
13. Le B, Gt B, Metab JI. Galactosemia. 2021;40(October 2019).
14. Ii LJE, Lai K. The molecular biology of galactosemia. 1991.
15. Gene G, Tyfield L, Reichardt J, et al. Classical Galactosemia and Mutations at the Galactose-1-Phosphate Uridyl Transferase. 1999;430(February).