Molecular Genetics and Metabolism Reports 17 (2018) 46–52

Contents lists available at ScienceDirect

Molecular Genetics and Metabolism Reports

journal homepage: www.elsevier.com/locate/ymgmr

The first pediatric case of glucagon receptor defect due to biallelic mutations in *GCGR* is identified by newborn screening of elevated arginine

Hong Li[a,b,*,1], Lihua Zhao[c,1], Rani Singh[a,b], J. Nina Ham[d], Doris O. Fadoju[d], Lora J.H. Bean[a,e], Yan Zhang[f], Yong Xu[f], H. Eric Xu[c,g,2], Michael J. Gambello[a,b,2]

[a] Department of Human Genetics, School of Medicine, Emory University, Atlanta, GA, United States
[b] Department of Pediatrics, School of Medicine, Emory University, and Children's Healthcare of Atlanta, Atlanta, GA, United States
[c] VARI-SIMM Center for Structure and Function of Drug Targets and the CAS Key Laboratory of Receptor Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 201203, China
[d] Division of Pediatric Endocrinology, Department of Pediatrics, School of Medicine, Emory University, Children's Healthcare of Atlanta, Atlanta, GA, United States
[e] EGL Genetic Diagnostics, Tucker, GA, United States
[f] Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou, 510530, China
[g] Laboratory of Structural Sciences, Center for Structural Biology and Drug Discovery, Van Andel Research Institute, Grand Rapids, MI, United States

ARTICLE INFO

Keywords:
Glucagon receptor
Newborn screening
GCGR mutation
Hyperaminoacidemia
Mahvash disease
Pancreatic α cell hyperplasia (ACH)
Pancreatic neuroendocrine tumor (PNET)

ABSTRACT

Glucagon receptor (GCGR) defect (Mahvash disease) is an autosomal recessive hereditary pancreatic neuroendocrine tumor (PNET) syndrome that has only been reported in adults with pancreatic α cell hyperplasia and PNETs. We describe a 7-year-old girl with persistent hyperaminoacidemia, notable for elevations of glutamine (normal ammonia), alanine (normal lactate), dibasic amino acids (arginine, lysine and ornithine), threonine and serine. She initially was brought to medical attention by an elevated arginine on newborn screening (NBS) and treated for presumed arginase deficiency with a low protein diet, essential amino acids formula and an ammonia scavenger drug. This treatment normalized plasma amino acids. She had intermittent emesis and anorexia, but was intellectually normal. Arginase enzyme assay and *ARG1* sequencing and deletion/duplication analysis were normal. Treatments were stopped, but similar pattern of hyperaminoacidemia recurred. She also had hypercholesterolemia type IIa, with only elevated LDL cholesterol, despite an extremely lean body habitus. Exome sequencing was initially non-diagnostic. Through a literature search, we recognized the pattern of hyperaminoacidemia was strikingly similar to that reported in the $Gcgr^{-/-}$ knockout mice. Subsequently the patient was found to have an extremely elevated plasma glucagon and a novel, homozygous c.958_960del (p.Phe320del) variant in *GCGR*. Functional studies confirmed the pathogenicity of this variant. This case expands the clinical phenotype of GCGR defect in children and emphasizes the clinical utility of plasma amino acids in screening, diagnosis and monitoring glucagon signaling interruption. Early identification of a GCGR defect may provide an opportunity for potential beneficial treatment for an adult onset tumor predisposition disease.

1. Introduction

The glucagon receptor (GCGR) is a G-protein-coupled receptor expressed mainly in the liver and kidney. Upon glucagon binding, it activates the stimulatory G protein (Gs) and increases cAMP level, subsequently transducing glucagon signaling involved in glucose, amino acids and lipid metabolism [1]. Mahvash disease is the only reported human phenotype associated with glucagon receptor defect. It is an autosomal recessive hereditary pancreatic neuroendocrine tumor (PNET) syndrome caused by biallelic inactivating mutations in *GCGR* gene [2]. Since first reported in 2008, 11 cases have been described [3,4]. All are adult patients with variable age at diagnosis (25–74 years old); no pediatric cases have been reported, nor is there much known about the pediatric medical histories of the affected adults. The typical

* Corresponding author at: Division of Medical Genetics, Department of Human Genetics, School of Medicine, Emory University, 1365 Clifton Rd. NE, Building B, Suite 2200, Atlanta, GA, 30322, United States.
E-mail address: Hong.Li@emory.edu (H. Li).
[1] Both authors contributed equally to this work
[2] Co-senior authors.

https://doi.org/10.1016/j.ymgmr.2018.09.006
Received 17 July 2018; Received in revised form 18 September 2018; Accepted 18 September 2018

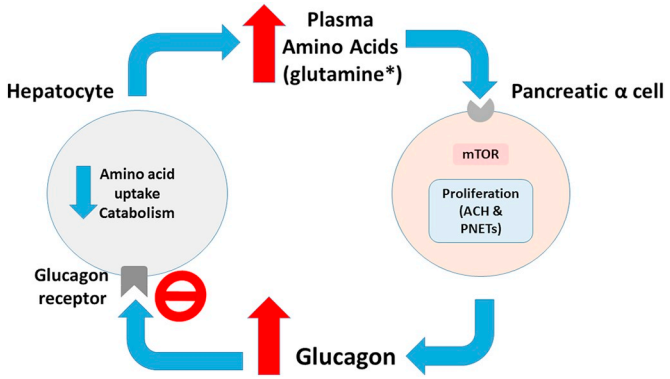

Fig. 1. The hepatic α cell axis feedback loop underlies the pathogenesis of glucagon receptor defect in *Gcgr*$^{-/-}$ mice. Interrupted glucagon signaling secondary to glucagon receptor defect in hepatocytes leads to decreased hepatic amino acids uptake and catabolism, and increased plasma amino acids. The hyperaminoacidemia, especially glutamine (*), activates pancreatic islet α cell proliferation partially through mTOR dependent mechanisms. This activation results in pancreatic α cell hyperplasia (ACH) with or without pancreatic neuroendocrine tumors (PNETs), and increases glucagon production (hyperglucagonemia). mTOR: mechanistic target of rapamycin. Adapted from [5,6].

presentation is non-specific abdominal pain and subsequent abdomen imaging study identifies pancreatomegaly with or without clear masses. The pathological findings are characterized by diffuse pancreatic α cell hyperplasia (ACH) with or without PNETs. Although patients have extreme hyperglucagonemia, there is no evidence of glucagonoma syndrome, such as skin rash, stomatitis, hyperglycemia or weight loss, because of the dysfunctional GCGR.

In the murine *Gcgr*$^{-/-}$ model, the complete block in glucagon signaling reduces hepatic uptake and catabolism of amino acids by altering hepatic gene expression of amino acid transporters and catabolic enzymes. As a consequence, circulating plasma amino acids are elevated [5,6]. Hyperaminoacidemia, especial elevated L-glutamine, stimulates mTOR signaling leading to pancreatic ACH and increased glucagon production, thus revealing a hepatic α cell axis as the basis of strong negative feedback mechanism in *Gcgr*$^{-/-}$ mice [5,6], which is outlined in Fig. 1. Hyperaminoacidemia was also reported in a patient with Mahvash disease [7], however, it is unknown if hyperaminoacidemia is a feature of all cases of GCGR defect, even presymptomatically.

Here, we describe the first pediatric case of glucagon receptor defect due to biallelic mutations in the *GCGR,* uniquely identified by positive newborn screening (NBS) for elevated arginine. Although expanded NBS has allowed the identification of many inborn errors of metabolism, GCGR defect associated with hyperargininemia or hyperaminoacidemia has never been considered or reported though NBS [8]. The similar pattern of plasma amino acids in the *Gcgr*$^{-/-}$ mouse and our case, previously unrecognized, may represent an early opportunity to detect asymptomatic Mahvash patients.

2. Patients and methods

2.1. Subject

The patient's information was all collected clinically in order to make the diagnosis and guide the management and surveillance. Informed consent was obtained from parents for the use of this information. Clinical exome analysis was performed (EGL Genetic Diagnostics, Tucker GA) using the V5Plus exome capture method (Agilent, Santa Clara, CA), NextGene alignment (SoftGenetics, State College, CA) and analyzed using an in-house bioinformatics pipeline. The NM_000160.3:c.958_960del (p.Phe320del) variant was confirmed by Sanger sequencing in the proband and both parents. Functional studies proved its pathogenicity.

2.2. Elective fasting hypoglycemia test

To investigate our patient's fasting tolerance, a hospital-based controlled fasting study was done clinically.

2.3. Functional studies of the novel variant in GCGR

2.3.1. Plasmid construction

Human glucagon receptor (GCGR) without the predicted signal peptide coding region was synthesized by Genewiz (Beijing, China). The encoded fragments were digested with *Bam*HI and *Not*I restriction endonucleases, then they were inserted into a modified pcDNA6 expression vector which encodes a fusion protein consisting of an N-terminal human IgG leader (MGWSCIILFLVATATGVHSE) for targeting the protein into the cell membrane. It also has a FLAG tag (DYKDDDD) at the C-terminus for detection by immunoblotting. Fused GCGR represents self-activation construct which tethers the glucagon peptide hormones (GCG) to the full-length receptor with a FLAG-GSA5 linker and constitutively activates receptor signaling; full-length (FL) GCGR needs glucagon binding to activate (Fig.4A). For the GCGR localization assays, we added sfGFP at the N-terminal of the constructs above [9]. All of these constructs contain the same IgG leader, as described above.

2.3.2. cAMP assay

Wild-type and mutant (p.Phe320del) GCGR were transiently expressed in HEK293 suspension cells. Site-directed mutagenesis experiments were carried out using the QuikChange method (Agilent) and all plasmid constructs were confirmed by DNA sequencing (Genewiz). Cells were transfected using Lipofectamine reagent (Life Technologies) with 200 ng CRE-driven fly luciferase reporter, 10 ng TK promoter driven renilla luciferase and 50 ng cDNA of GCGR. TK was used as an internal transfection controls. After three hours transfection, the cells were treated with 1 μM doxycycline for 24 h, then glucagon with concentration of 0 nM and 500 nM were added to cells for 4 h incubation. We assessed GCGR activity by measuring the cAMP signal, which is the activity of cAMP-responsive CRE-driven fly luciferase reporter relative to renilla luciferase activity (RLU) from different constructions of GCGR. This activity was measured by the EnVision plate reader (PerkinElmer), according to the manufacturer's instructions for the Dual-luciferase reporter assay system from Promega [9]. GCG dose response curves of FL and fused human GCGR WT and p.phe320del mutant were conducted.

2.3.3. GCGR localization assay

Confocal microscopy imaging was performed to monitor the location of GCGR in live cells. The HEK293 suspension cells (1×10^5) were seeded in a 35 mm glass bottom dish and transfected with a modified pcDNA6 expression vector, pcDNA6-sfGFP-GCGR and pcDNA6-sfGFP-GCG(1-29)-FLAG-5GSA-GCGR, including Wild-type GCGR and p.Phe320del. We confirmed the expression and location of GCGR by confocal microscopy imaging. (Cell Observer SD, Zeiss, Germany).

2.3.4. Western blot analysis

The HEK293 suspension cells were harvested by centrifugation, then their pellets solubilized in cell lysis reagent (CelLyticTM M, Sigma) supplemented with 1 mM PMSF and centrifuged at 16,000 ×*g* for 30 min. The supernatants were subjected to SDS-PAGE and transferred to PVDF membranes, and the membranes were blocked with 5% milk in TBST (20 mM Tris-HCl pH 8.0, 150 mM NaCl and 0.05% Tween-20), then incubated with anti-FLAG M2 antibodies from mouse (Sigma) or monoclonal anti-β-actin antibody from mouse clone AC-15 (Sigma), followed by anti-mouse HRP antibodies in 5% milk in TBST. The images were collected using ChemiDocTM XRS + imager (BIO-RAD) [9].