Adult-Onset Presentation of a Hyperornithinemia-Hyperammonemia-Homocitrullinuria Patient Without Prior History of Neurological Complications

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Abstract The Hyperornithinemia-Hyperammonemia-Homocitrullinuria (HHH) syndrome is a disorder of the urea cycle and ornithine degradation pathway caused by mutations in the mitochondrial ornithine transporter, ORNT1 (SLC25A15). In general, the majority of patients with HHH syndrome come to medical attention during infancy or early school years with symptoms such as learning disabilities, changes in cognitive development, spasticity, or liver dysfunction. In this report, we describe a 35-year-old male of Indian descent who was diagnosed with HHH syndrome after he presented to the emergency room with gastroenteritis, disorientation, and slurred speech. Molecular analysis revealed that this patient was heterozygous for two ORNT1 mutations, p.[Gly220Arg(+)Arg275X] (c.[658G>A(+)+823C>T]) that had been previously reported in homozygous probands who presented during the first year of life. Cellular studies revealed that the ORNT1 p.Gly220Arg mutation was nonfunctional but targeted to the mitochondria. Given that this patient was a successful college graduate on a vegetarian diet without a

prior history of learning or neurological impairment, additional factors such as gene redundancy, environmental, and epigenetic factors may have contributed to the delay in onset of presentation and lack of any previous symptoms. To the best of our knowledge, this is the first reported case of an adult-onset HHH syndrome presentation without a prior history of neurological or cognitive deficiency.

Introduction

The Hyperornithinemia-Hyperammonemia-Homocitrullinuria (HHH) syndrome (OMIM # 238970) is an autosomal recessive disorder of the urea cycle and ornithine degradation pathway caused by mutations in the mitochondrial ornithine transporter, ORNT1 (SLC25A15) (Valle and Simell 2001). In spite of the impermeability of the inner mitochondrial membrane to amino acids, the great majority of patients with nonfunctional ORNT1 mutations have a later onset and milder clinical course when compared to patients with ornithine transcarbamylase deficiency with no enzymatic activity (Brusilow and Horwich 2001). Since ORNT1 deficiency does not completely abolish ornithine transport across the inner mitochondrial membrane in HHH patients' liver and fibroblasts, we hypothesized that additional transporters with overlapping function must exist. Our hypothesis was confirmed by the isolation of two additional mitochondrial ornithine transporters, the retroposon ORNT2 (SLC25A2) and, more recently, ORNT3 (SLC25A29), a carrier protein that also functions as a carnitine/acyl-carnitine-like transporter (CACT-like) (Camacho and Rioseco-Camacho 2009). A salient feature of HHH syndrome is phenotypic variability in patients with the same mutation and a lack of correlation between ORNT1 mutant genotype and

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the clinical phenotype in terms of onset and severity. Presenting symptoms of patients with ORNT1 deficiency include liver dysfunction, coagulopathy, unexplained seizure activity, developmental delay, academic difficulties, cognitive impairment, stroke-like lesions on MRI and/or gait disturbances (Debray et al. 2008; Tessa et al. 2009). The cognitive development of patients with ORNT1 deficiency can range from normal intelligence to severe impairment. In some instances, patients suffer from significant neurological deficits without any evidence of cognitive impairment. Moreover, HHH patients commonly develop spastic paraparesis. Classically, patients are characterized by chronic elevation in plasma ornithine, episodic or postprandial hyperammonemia and urinary excretion of homocitrulline and orotic acid. In general, patients are maintained on a protein-restricted diet and supplemented with special formulas, citrulline and sodium phenylbutyrate.

In this report, we describe the case of a previously asymptomatic 35-year-old male who was diagnosed with HHH syndrome after he presented to the ER with a gastroenteritis, disorientation, slurred speech, and mild elevations in plasma ammonia, glutamine and ornithine. Molecular analysis revealed that the patient carries two ORNT1 mutations, p.[Gly220Arg(+)Arg275X] (c.[658G>A(+)823C>T]), which have been previously described in HHH homozygous probands presenting during infancy and the early childhood period (Al-Hassnan et al. 2008; Torisu et al. 2006).

Methods and Patient Information

Patient Data

All medical records, blood for DNA and lymphocyte cell lines were obtained with institutional IRB approval. For this report, only the proband was available for blood draw since his mom had recently died due to chronic lung disease and his father is frail at the age of 80. RS is a 35-year-old college-educated male of Indian descent who, prior to the onset of symptoms, never had a history of learning disabilities, liver disease, psychiatric illness, or neurological deficits. The patient was born to a previously healthy 29-year-old G1A0P1 after an uncomplicated pregnancy. He had a healthy and active life and always did well in his schoolwork. The patient went to college and started to work in his native country until all of his family moved to the USA 4 years prior to the onset of symptoms. The patient's sister (only sibling) and her two children are all in good health. There is no family history of genetic or metabolic disorders.

The patient states he never smoked, used alcohol or drugs. Nutritional history was remarkable for self-restriction of

protein products since childhood (pre-adolescence years) given that they would make him feel sick or induce vomiting. Although, on occasion, he would eat eggs, he avoided milk because of lactose intolerance. The patient also reported avoiding seafood because of a prior history of severe allergic reaction that caused respiratory compromise. The patient's diet consisted mostly of rice, lentils, nuts, fruits, and vegetables. The calculated daily protein intake prior to the onset of symptoms was about 47 g per day (0.48 g/kg/day). Approximately 4 years prior to the onset of symptoms, the patient reported getting overweight and was diagnosed with asthma that required bronchodilator therapy. Currently, at 6 ft tall, he weighs 227 lbs and has a BMI of 30.7. The patient states that for the last 10 years he has slowly been gaining weight.

Five weeks prior to the initial onset of symptoms, his mother died from chronic lung disease. This family loss led RS to start consuming nonhealthy foods in large quantities and, in part, abandon his vegetarian diet. The patient was brought to the ER by his brother-in-law with a 3-day history of vomiting, diarrhea, dizziness, and confusion. Upon examination, the patient was noted to be disoriented, had slurred speech, and could not read or recall his home telephone number. No evidence of GI bleeding was found, and an abdominal CT scan was normal. His plasma ammonia was elevated at 114 μ M (normal range 25–50 μ M), but the rest of his blood chemistries and blood count were normal. During the patient's brief stay at the ER, he received IV fluids with dextrose and electrolytes that stabilized his condition (ammonia went down to 43 μ M). He was discharged home the same day on Lactulose. No specific diagnosis was given.

Two days after discharge from the ER, the patient returned with a similar set of symptoms. On this occasion, his plasma ammonia level was 140 μ M. Patient was admitted to the hospital and a genetic consult was called. Although his blood chemistries and blood count were again within normal limits, his biochemical profile was suggestive of HHH syndrome. These values included elevations in plasma ornithine at 292 μ M (normal range 30–110 μ M) and glutamine at 1,322 μ M (normal range 400–900 μ M) with normal levels of lysine at 82 μ M (normal range 80–250 μ M), arginine at 48 μ M (normal range 20–120 μ M), and citrulline at 20 μ M (normal range 13–45 μ M). His urine organic were normal and his urine amino acids did not show increased excretion of cationic amino acids (lysine, ornithine, arginine), citrulline or arginosuccinic acid. No orotic aciduria was observed nor increases in methionine (with normal plasma levels) in the urine amino acid profile, thus suggesting no urinary excretion of homocitrulline given that the peaks of these two amino acids overlap. This observation on our patient contrasts sharply with a group of five previously reported Kaiser-Permanente HHH patients, who consistently showed

a urinary pattern suggestive of homocitrullinuria (Camacho et al. 2006). The patient's plasma carnitine profile was normal. CT scan of his brain and liver did not reveal any abnormalities. The patient was subsequently followed 1 month later at the Human Genetics Clinic where he was found to be neurologically asymptomatic and feeling back to normal. After evaluation by a nutritionist, the patient was placed on a protein-restricted diet of 50–60 g/day (~0.5 g/kg/day), citrulline and sodium phenylbutyrate.

In general, this patient's plasma levels of ornithine (327–704 μM) and glutamine (1,043–1,435 μM) have always remained elevated. On several occasions, values for lysine and phenylalanine were decreased in the range of 47–60 μM (normal range 80–250 μM) and 28–30 μM (normal range 40–100 μM), respectively. Finally, the patient reported suffering from a severe asthma attack that required the use of intravenous anti-inflammatory steroids. This treatment modality caused a low-grade episode of hyperammonemia that lasted 4 days and caused mental confusion, forgetfulness, and generalized body weakness. After the patient recovered, he returned to the Human Genetics Clinic where he was found to have a normal physical exam. A follow-up brain MRI and a recent psychometric testing were normal.

Mutation Analysis of Human ORNT1 and ORNT2

Genomic DNA (blood) and total RNA (EBV-transformed lymphocytes) were used for mutation analysis utilizing previously published protocols (Torisu et al. 2006; Camacho et al. 2006). We identified two mutant *ORNT1* alleles: c.658G>A (p.Gly220Arg) in Exon 6 and c.823C>T (p.Arg275X) in Exon 7. To confirm that this patient was heterozygous for the c.823 C > T mutation, we used forward primer 5'-GCATCCTTCTATGACTTGTTG-3' and backward primer 5'-AATAGAGGTTCTTCCATAAAG-3' to amplify a 431 bp fragment that when digested with Taq I would generate a wild-type and heterozygous pattern. Finally, we amplified and sequenced human ORNT2 as previously detailed (Camacho et al. 2003).

Expression Constructs, Transfections, Ornithine Transport Assay and Indirect Immunofluorescence

For our studies, we used previously described N-myc-tagged wild-type mouse *Ornt1* (*Slc25a15*) and *Ornt3* (*Slc25a29*) constructs as positive controls (Camacho and Rioseco-Camacho 2009). Using a wild-type ORNT1-N-myc-tagged construct as a backbone, we generated the *ORNT1* mutation p.Gly220Arg as previously reported (Camacho et al. 2006). We performed electroporation (transient transfection), the ornithine transport assay, and immunofluorescence as previously described (Camacho

and Rioseco-Camacho 2009). For transient transfection studies, we assayed three to six wells per transfected plasmid and expressed results as mean $\pm$ SD. We expressed the ability to incorporate ^{14}C -ornithine into protein as glutamate and proline relative to total protein synthesis (^3H -leucine) as a $^{14}\text{C}/^3\text{H}$ ratio. We analyzed data using the unpaired *t* test and presented our results in graphs as percent increase compared to control cells.

Results

Molecular Studies

Initial amplification by RT-PCR revealed two mutations. The first mutation, c.658G>A (p.Gly220Arg), was previously reported in a homozygous HHH proband who presented with hypotonia, liver dysfunction, and stroke-like lesions on MRI at the age of four (onset of symptoms by 12 mo) (Al-Hassnan et al. 2008). The second mutation, c.823C>T (p.Arg275X), was previously reported in a homozygous HHH proband who became symptomatic at the age of 5 months and who, by the age of 6 years, had a phenotype which included deafness, significant cognitive impairment, and mild spastic paraplegia (Torisu et al. 2006).

Genomic PCR confirmed that our proband was heterozygous for the *ORNT1* c.[658G>A(+823C>T) alleles (Fig. 1). Given that the stop codon mutation, c.823C>T, eliminated a Taq I site, we amplified a smaller PCR product from genomic DNA and subjected it to enzymatic digestion in order to further demonstrate that this patient was heterozygous for this allele. Results shown in Fig. 1b demonstrate the presence of three fragments in the patient's lane corresponding the proband's wild-type (250-bp and 181-bp fragments) and mutant, undigested (431-bp fragment) alleles.

Finally, we examined the sequence of the second mitochondrial ornithine transporter, ORNT2, for any potential polymorphisms that could affect its function. Results of our search demonstrated that the patient has the c.676G>A (p.Val226Iso) change, which has been annotated in the NCBI-SNP database (RefSNP ID: rs3749780) and which has a frequency that varies from 0.058 to 0.237 in several geographical and ethnic groups. Contrary to two previously reported ORNT2 protein polymorphisms, p.Val226Iso does not appear to affect this carrier's mitochondrial targeting or transporting ability (unpublished observations) (Camacho et al. 2003).

Functional Studies

We thought of further investigating the functional consequences of the Gly²²⁰ to Arg²²⁰ change given that, in the past, several reports have demonstrated that ORNT1

Fig. 1 Human ORNT1 c.[658G>A(+)]823C>T] (p.[Gly220Arg(+)]Arg275X]) mutations. **(a)** Patient was found to have two previously known ORNT1 mutations, p.[Gly220Arg(+)]Arg275X]. *Left* and *right* panel shows the control and mutant sequences, respectively. **(b)** *Top* panel: Undigested PCR products. *Bottom* panel: Genomic PCR

amplification of part of intron 6 and exon 7 that contains a single Taq I (TCGA) site that was abolished by the c.823C>T mutation. Approximately 20 μ l of Taq I-digested patient genomic PCR product was run on a 7% acrylamide gel

mutations that appeared to be nonfunctional have residual function (Camacho et al. 2006). Overexpression studies shown in Fig. 2a clearly demonstrate that the p.Gly220Arg change completely eliminates ORNT1 transporting capacity when compared to both mouse (*Mm*) Ornt1 and Ornt3. The level of ornithine transporting ability of the cells expressing the human (*Hs*) N-myc-ORNT1-Gly220Arg construct is the same as those expressing only the empty pcDNA3.1 vector and nontransfected HHH fibroblasts expressing the non-functional ORNT1 p.Phe188del mutation. We also performed immunofluorescence studies using previously published protocols to determine whether the p.Gly220Arg change affected the mitochondrial targeting pattern of ORNT1 (Camacho and Rioseco-Camacho 2009). Our results also demonstrate that the N-myc-*Hs*ORNT1-Gly220Arg transporter has a normal mitochondrial-targeting pattern similar to the wild-type ORNT1 construct (Fig. 2b).

Discussion

In this report, we describe a patient with HHH syndrome who was initially diagnosed at the age of 35, although he carries two nonfunctional ORNT1 mutations, p.[Gly220Arg(+)]Arg275X]. This late-onset presentation and lack of any

prior neurological or hepatic clinical findings is in sharp contrast to two previously reported HHH probands who were homozygous for the same mutations and who presented at the age of 5 (p.Arg275X) and 12 (p.132Gly220Arg) months (Al-Hassnan et al. 2008; Torisu et al. 2006). The lack of changes in MRI and CT scan and a negative medical history of cognitive or neurological impairment suggest that this patient had not suffered from the chronic effects of elevated ammonia and glutamine or from severe potential abnormalities in ornithine, lysine, and arginine metabolism. This is the first case, to the best of our knowledge, of an adult onset HHH patient who does not exhibit any previous form of neurological or cognitive deficit. After nearly 35 years without any neurological symptoms or cognitive deficiency, it is difficult to ascertain the triggering event(s) that caused this patient's symptoms, though deviating from a vegetarian diet may certainly have precipitated this crisis.

The incomplete metabolic triad exhibited by this HHH patient, viz. the absence of a urinary amino acid profile suggestive of homocitrullinuria, is particularly interesting. Given that our patient had a very mild phenotype, it is conceivable that his redundant ornithine transport was enough to prevent a significant intramitochondrial carbamylation of lysine. In fact, orotic acid excretion, which in HHH syndrome reflects the accumulation of carbamyl

Fig. 2 Transient transfection studies of N-myc-*HsORNT1*-G220R construct does not restore ornithine metabolism in HHH cultured fibroblasts cells. **(a)** Fibroblasts carrying the nonfunctional *ORNT1*-F188Δ allele were electroporated with an N-myc-*HsORNT1*-G220R, N-myc-*MmOrnt1*, N-myc-*MmOrnt3* or empty pcDNA3.0 vector using previously described conditions. The ornithine transport assay is

performed at the nonsaturating ornithine concentration of 2.1 μM. * $p < 0.05$ vs. HHH+vector. **(b)** Immunofluorescence studies of wild-type N-myc-*Ornt1* and N-myc-*ORNT1*-G220R mutant constructs. Transfection efficiency for these experiments was ~15–20%. Ornithine transport assays and immunofluorescence studies are representative of three experiments

phosphate and, thus, its intramitochondrial underuse (Valle and Simell 2001), was also absent further validating the lack of homocitrulline in the urine. Moreover, the patient's history of a low protein (vegetarian) diet may have reduced the amount of homocitrulline since there is a correlation between homocitrullinuria and the level of protein intake in *ORNT1* deficiency (Valle and Simell 2001).

It is well known that the toxic effects of elevated levels of ammonia and glutamine on the brain astrocyte are in part responsible for the symptoms of patients with urea cycle disorders including HHH syndrome (Sofroniew and Vinters 2010). However, given that *ORNT1* also functions as a cationic amino acid transporter, it is reasonable to propose that the pathophysiology of HHH syndrome may also be dependent on the interruption of physiological/biological functions of *ORNT1*, which include a role in mitochondrial protein synthesis, the metabolism of arginine and lysine, plus alterations in polyamine metabolism (Palmieri 2008). Another factor that may also contribute to the mechanisms

of disease in HHH syndrome relates to the synthesis of creatine given that ornithine negatively regulates the activity of L-arginine:glycine amidinotransferase, the enzyme that catalyzes the first step in the synthesis of creatine (Valle and Simell 2001; Humm et al. 1997). Interestingly, mitochondrial dysfunction as a putative disease mechanism in HHH syndrome was previously posited in two siblings homozygous for the *ORNT1* c.446delG mutation (Korman et al. 2004). Lastly, recent work suggests that both ornithine and homocitrulline may directly induce oxidative stress, affect cellular bioenergetics and cause mitochondrial dysfunction (Viegas et al. 2011).

Synopsis

Presentation of a 35-year-old HHH patient without prior history of neurological complications.

Authors' Contributions

Kamer Tezcan and Kristal T. Louie – Main persons taking care of the patient and contributed to the writing and editing of the manuscript.

Yong Qu – Performed and interpreted all clinical biochemical analysis and contributed to the writing and editing of the manuscript.

Jorge Velasquez – Performed the site-directed mutagenesis experiments, sequenced and purified all constructs, conducted immunofluorescence studies and editing of the manuscript.

Frank Zaldivar – Responsible for establishing the B-lymphocyte cell lines of the patient and contributed to editing of the manuscript.

Natalia Rioseco-Camacho – Performed DNA and RNA isolation, all tissue culture related experiments, assisted in the design of experiments and contributed to the writing and editing of the manuscript.

José A. Camacho – Contributed to patient care, ORNT1 sequencing, experimental design, main manuscript writing and editing. In addition, will assume all the responsibility as guarantor for this manuscript.

Competing Interest Statement

All authors declare that the answers to all questions on the JIMD competing interest form are No, and therefore they have nothing to declare.

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Ethics Approval

This study was conducted under the Institutional IRB #2002-2608.

Patient Consent

No patient identifiers were used. Patient consented to the review of his medical records, laboratory studies, scans and the publication of his case.

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