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Absence of mutations in *HCRT*, *HCRTR1* and *HCRTR2* in patients with ROHHAD

Sarah F. Barclay[a,*], Casey M. Rand[b], Paul A. Gray[c], William T. Gibson[d], Richard J.A. Wilson[e], Elizabeth M. Berry-Kravis[f], Diego Ize-Ludlow[g], N. Torben Bech-Hansen[a,1], Debra E. Weese-Mayer[b,h,1]

[a] *Department of Medical Genetics, Cumming School of Medicine, Alberta Children's Hospital Research Institute, University of Calgary, Calgary, AB, Canada*
[b] *Center for Autonomic Medicine in Pediatrics (CAMP) in Stanley Manne Children's Research Institute and in Department of Pediatrics, Ann & Robert H. Lurie Children's Hospital of Chicago, Chicago, IL, USA*
[c] *Department of Anatomy and Neurobiology, Washington University of Medicine, St. Louis, MO, USA*
[d] *Department of Medical Genetics, University of British Columbia and Child & Family Research Institute, Vancouver, BC, Canada*
[e] *Department of Physiology and Pharmacology, Alberta Children's Hospital Research Institute and Hotchkiss Brain Institute, Cumming School of Medicine, University of Calgary, Calgary, AB, Canada*
[f] *Departments of Pediatrics, Neurological Sciences, and Biochemistry, Rush University Medical Center, Chicago, IL, USA*
[g] *Department of Pediatrics, Division of Pediatric Endocrinology at University of Illinois at Chicago, Chicago, IL, USA*
[h] *Northwestern University Feinberg School of Medicine, Chicago, IL, USA*

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ABSTRACT

Background and objectives: Rapid-onset obesity with hypothalamic dysfunction, hypoventilation, and autonomic dysregulation (ROHHAD) is a rare pediatric disease of unknown cause. Here, in response to a recent case report describing a ROHHAD patient who suffered from secondary narcolepsy confirmed by an absence of hypocretin-1 in the cerebrospinal fluid, we consider whether the ROHHAD phenotype is owing to one or more mutations in genes specific to hypocretin protein signalling.
Methods: DNA samples from 16 ROHHAD patients were analyzed using a combination of next-generation and Sanger sequencing to identify exonic sequence variations in three genes: *HCRT*, *HCRTR1*, and *HCRTR2*.
Results: No rare or novel mutations were identified in the exons of *HCRT*, *HCRTR1*, or *HCRTR2* genes in a set of 16 ROHHAD patients.
Conclusions: ROHHAD is highly unlikely to be caused by mutations in the exons of the genes for hypocretin and its two receptors.

1. Introduction

Though extremely rare, with fewer than 100 cases reported in the literature, Rapid-onset Obesity with Hypothalamic Dysfunction, Hypoventilation, and Autonomic Dysregulation (ROHHAD) is a devastating pediatric disease (Fishman et al., 1965; Ize-Ludlow et al., 2007; Katz et al., 2000). Initially described 50 years ago, the disease, previously termed "late-onset central hypoventilation syndrome with hypothalamic dysfunction", remains poorly understood (Fishman et al., 1965; Ize-Ludlow et al., 2007; Katz et al. 2000). Following the initial symptom of rapid-onset obe-

* Corresponding author.
E-mail addresses: sarah.barclay@ucalgary.ca (S.F. Barclay), CRand@luriechildrens.org (C.M. Rand), pgray@pcg.wustl.edu (P.A. Gray), wtgibson@cfri.ubc.ca (W.T. Gibson), wilsonr@ucalgary.ca (R.J.A. Wilson), Elizabeth_Berry-Kravis@rush.edu (E.M. Berry-Kravis), diegoize@uic.edu (D. Ize-Ludlow), ntbech@ucalgary.ca (N.T. Bech-Hansen), D-Weese-Mayer@Northwestern.edu (D.E. Weese-Mayer).
[1] Joint senior authors.

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sity (an otherwise healthy 2–7 year-old child will gain 20–30 pounds over a 3–6 month period), additional signs of hypothalamic and autonomic malfunction progressively manifest, as do perturbations of respiratory control, (Bougneres et al., 2008; De Pontual et al., 2008; Ize-Ludlow et al., 2007). In addition, about 40% of ROHHAD patients will develop benign neural crest tumours (Bougneres et al., 2008; De Pontual et al., 2008; Ize-Ludlow et al., 2007). In the absence of a clear understanding of the etiology of the disease, or of a definitive diagnostic marker, a ROHHAD diagnosis is often delayed or missed, potentially leading to worsening hypoventilation, impaired neurocognitive development, and even cardiorespiratory arrest.

Multiple theories about the pathological mechanisms of ROHHAD have been proposed, including that it is an autoimmune disease (Chow et al., 2015; Sartori et al., 2014); that it is a paraneoplastic condition (Ouvrier et al., 1995; Paz-Priel et al., 2011; Sirvent et al., 2003); and, as ROHHAD shares some features of other genetic neurocristopathies, such as Congenital Central Hypoventilation Syndrome (CCHS) (Weese-Mayer et al., 2010), that there is a genetic basis (De Pontual et al., 2008; Ize-Ludlow et al., 2007; Rand et al., 2011). Several studies have attempted to identify genetic mutations causing ROHHAD, either using a candidate gene approach (De Pontual et al., 2008; Ize-Ludlow et al., 2007; Rand et al., 2011) or whole exome sequencing (Barclay et al., 2015; Thaker et al., 2015), but a clear answer remains elusive.

A recent case report described a 7-year old girl diagnosed with ROHHAD who also suffered from narcolepsy with cataplexy, confirmed by an absence of hypocretin-1 in her cerebrospinal fluid (Dhondt et al., 2013). Many features of the described phenotype of the child as well as the disordered sleep/wake symptoms of this patient are not routinely characteristic of ROHHAD (see Table 1) and, to our knowledge, this is the first report of co-morbid ROHHAD and narcolepsy. Nonetheless, the co-occurrence of ROHHAD (or a ROHHAD-like phenotype) and narcolepsy in one patient may present a clue to the underlying cause of ROHHAD spectrum, with one obvious possibility being that the disease is caused by genetic mutations in the gene encoding hypocretin-1.

Also known as orexins, the hypocretins (hypocretin-1 and hypocretin-2) were first discovered in 1998 (de Lecea et al., 1998; Sakurai et al., 1998) and are the mature products of the hypocretin neuropeptide precursor, which is encoded by the *HCRT* gene. Hypocretin-1 and hypocretin-2 bind two receptors, encoded by *HCRTR1* and *HCRTR2*, with different affinity. In addition to hypocretin's role in sleep/wake regulation, hypocretin signalling also has important roles in energy balance, and the control of breathing (Chemelli et al., 1999; Li and Nattie, 2014). Indeed, hypothalamic orexinergic neurons have long been implicated in energy balance (Sellayah and Sikder, 2013), and exhibit exquisite CO_2 sensitivity (Williams et al., 2007). They also project to the brainstem retrotrapezoid nucleus, a key site regulating ventilatory responses to changes in blood gases with neurons that also exhibit remarkable CO_2 sensitivity (Lazarenko et al., 2011). Thus, abnormalities in one or more hypocretin signalling system genes might be expected to produce a ROHHAD spectrum phenotype.

We have assessed the coding sequence of *HCRT*, *HCRTR1* and *HCRTR2* in a substantial cohort of rigorously evaluated patients with ROHHAD to assess whether mutations in any of these three hypocretin-related genes are possibly one of the underlying genetic causes for ROHHAD.

2. Patients and methods

2.1. Criteria for patient diagnosis and selection

Ize-Ludlow et al. (2007) published the basic criteria for consideration of the diagnosis of ROHHAD in 2007 and introduced the acronym. Briefly, the key features include: (1) onset of rapid and extreme weight gain after age 1.5 years (typically 2–7 years) in a previously non-obese and seemingly normal child, (2) evidence of hypothalamic dysfunction, (3) central alveolar hypoventilation, and (4) features of autonomic dysregulation. The Center for Autonomic Medicine in Pediatrics (CAMP) at Ann & Robert H. Lurie Children's Hospital of Chicago and the Stanley Manne Children's Research Institute is a Center of Excellence for the study of ROHHAD. Medical records for each proband referred to CAMP were reviewed to confirm ROHHAD characteristics, with most of the patients (13/16) being evaluated clinically in the CAMP laboratory. Patients who met strict diagnostic criteria for ROHHAD were offered inclusion to the IRB-approved ROHHAD Genetic Inquiry project (Ann & Robert H. Lurie Children's Hospital of Chicago IRB, study ID: 2009-13904; and the University of Calgary Conjoint Health Research Ethics Board, study ID: REB13-0164_REN2), and written informed consent was obtained from all who agreed to participate. All 16 patients included in this study demonstrated characteristic features of the ROHHAD phenotype including rapid-onset obesity, hypothalamic dysfunction, central alveolar hypoventilation requiring artificial ventilation, and autonomic dysregulation (Table 1). None of the 16 patients had atypical findings such as those described in Dhondt et al. (2013) or Thaker et al. (2015), especially the pre-existing obesity, food obsession, and delayed neurocognition that predated the rapid-onset weight gain.

2.2. Sample collection and DNA extraction

Genomic DNA was isolated from peripheral blood samples, using the Puregene reagent kit (Qiagen).

2.3. Next-generation sequencing

The exonic regions of the three genes of interest were first examined in data from whole exome sequences of 16 ROHHAD patients. Exome captures were completed using the Agilent SureSelect capture kit, 38 MB, V4, or V5 + UTRs, or the Illumina All Exon 65 MB kit. Massively parallel sequencing was performed on either a SOLiD or Illumina platform, at one of four institutions, and sequences were aligned to the human reference genome (GRCh37) using BWA 0.5.9 (Li and Durbin, 2009), Lifescope Genomic Analysis Software 2.5 (Life Technologies), NovoAlign 2.07.13 (Novocroft), or Bowtie 0.12.7 (Langmead et al., 2009). All aligned sequences were then analyzed at a single site using the following pipeline. Variants were called using Samtools (Li et al., 2009) and annotated for filtration and prioritization using ANNOVAR (Wang et al., 2010). Candidate mutations were identified as novel or rare (Minor allele frequency [MAF] <0.005, according to 1000 Genomes Project (Abecasis et al., 2012), the Exome Variant Server [EVS; http://evs.gs.washington.edu/EVS/] and the Exome Aggregation Consortium [ExAC; http://exac.broadinstitute.org]), exonic or splice site (within 2 bp of an exon) variants in *HCRT*, *HCRTR1* or *HCRTR2*.

2.4. Sanger sequencing

All exons not covered at least 10-fold in the next-generation sequences were analyzed by Sanger sequencing. We used the NCBI Primer Blast tool to design site-specific primers in order to amplify the regions of interest. Amplicons were purified using a spin purification protocol (Omega Biotek) and sequenced using the fluorescent dideoxy terminator method (Sanger method). Amplicon sequences were compared to the reference genome (GRCh37) using Mutation Surveyor (SoftGenetics) in order to identify any variant positions within the exons or canonical splice sites. As noted above, candidate mutations were identified as novel or rare