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RESEARCH REPORT

Compound heterozygous *SLC19A3* mutations further refine the critical promoter region for biotin-thiamine-responsive basal ganglia disease

Whitney Whitford,[1,8] Isobel Hawkins,[1] Emma Glamuzina,[2] Francessa Wilson,[3] Andrew Marshall,[4] Fern Ashton,[5] Donald R. Love,[5] Juliet Taylor,[6] Rosamund Hill,[7] Klaus Lehnert,[1,8] Russell G. Snell,[1,8] and Jessie C. Jacobsen[1,8]

[1]School of Biological Sciences, The University of Auckland, Auckland 1010, New Zealand; [2]Adult and Paediatric National Metabolic Service, Starship Children's Hospital, Auckland 1023, New Zealand; [3]Department of Paediatric Radiology, Starship Children's Hospital, Auckland 1023, New Zealand; [4]Department of Paediatrics and Child Health, Wellington Hospital, Wellington 6021, New Zealand; [5]Diagnostic Genetics LabPLUS, Auckland City Hospital, Auckland 1023, New Zealand; [6]Genetic Health Service New Zealand, Auckland City Hospital, Auckland 1023, New Zealand; [7]Department of Neurology, Auckland City Hospital, Auckland 1023, New Zealand; [8]Centre for Brain Research, The University of Auckland, Auckland 1010, New Zealand

Abstract Mutations in the gene *SLC19A3* result in thiamine metabolism dysfunction syndrome 2, also known as biotin-thiamine-responsive basal ganglia disease (BTBGD). This neurometabolic disease typically presents in early childhood with progressive neurodegeneration, including confusion, seizures, and dysphagia, advancing to coma and death. Treatment is possible via supplement of biotin and/or thiamine, with early treatment resulting in significant lifelong improvements. Here we report two siblings who received a refined diagnosis of BTBGD following whole-genome sequencing. Both children inherited compound heterozygous mutations from unaffected parents; a missense single-nucleotide variant (p.G23V) in the first transmembrane domain of the protein, and a 4808-bp deletion in exon 1 encompassing the 5′ UTR and minimal promoter region. This deletion is the smallest promoter deletion reported to date, further defining the minimal promoter region of *SLC19A3*. Unfortunately, one of the siblings died prior to diagnosis, but the other is showing significant improvement after commencement of therapy. This case demonstrates the power of whole-genome sequencing for the identification of structural variants and subsequent diagnosis of rare neurodevelopmental disorders.

Corresponding author:
j.jacobsen@auckland.ac.nz

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INTRODUCTION

Thiamine metabolism dysfunction syndrome 2 (MIM: 607483) is an autosomal recessive neurometabolic condition, resulting from mutations in the solute carrier family 19, member 3 (*SLC19A3*, MIM: 606152) gene, which encodes the human thiamine transporter 2 (hTHTR2) (Subramanian et al. 2006a). The disease was first described in children of consanguineous parents, mainly of Saudi Arabian descent (Ozand et al. 1998). The causative mutation for the disease was subsequently mapped to the *SLC19A3* gene in 2005 (Zeng et al. 2005). The disease typically presents with recurrent bouts of subacute encephalopathy,

confusion, seizures, dysarthria, dysphagia, and dystonia, progressing to coma and death in early childhood to adolescence (Ozand et al. 1998; Tabarki et al. 2013); however, clinical manifestations in infancy have also been reported (Sremba et al. 2014; Ygberg et al. 2016).

Magnetic resonance imaging (MRI) analysis reveals lesions and necrosis in the caudate nucleus and putamen, with involvement of the infra and supratentorial brain cortex, and the brainstem (Ozand et al. 1998; Tabarki et al. 2013). Most individuals display normal biochemical and metabolic markers; however, increased levels of lactate and pyruvate in the serum and cerebrospinal fluid (CSF) and increased levels of amino acids in the serum and urine have been observed in a minority of cases (Ozand et al. 1998; Debs et al. 2010; Alfadhel et al. 2013; Fassone et al. 2013; Flønes et al. 2016; Gerards et al. 2013; Kevelam et al. 2013; Pérez-Dueñas et al. 2013; Tabarki et al. 2013; Ortigoza-Escobar et al. 2014, 2016; Schänzer et al. 2014; Sremba et al. 2014; Ygberg et al. 2016).

SLC19A3 belongs to a family of solute carrier genes that includes solute carrier family 19, member 1 (*SLC19A1*) encoding reduced folate transporter (RFC-1), and solute carrier family 19, member 2 (*SLC19A2*) encoding thiamine transporter 1 (hTHTR1) (Eudy et al. 2000). Together, *SLC19A2* and *SLC19A3* are responsible for transportation and homeostasis of thiamine, also known as vitamin B_1. Thiamine is not endogenously synthesized but is obtained from the diet via absorption through the small intestine. A number of thiamine-dependent enzymes are required for normal cellular function, including oxidative metabolism through the connection of glycolysis with the tricarboxylic acid (TCA) cycle via pyruvate dehydrogenase, the TCA cycle via the α-ketoglutarate complex, and the pentose phosphate shunt (Jhala and Hazell 2011). Although individuals with BTBGD have defective or nonfunctional versions of *SLC19A3*, they do not experience global thiamine deficiency, showing normal levels of thiamine in the blood; however, levels of free-thiamine in the CSF are often significantly reduced (Ortigoza-Escobar et al. 2016).

Expression of SLC19A3 is ubiquitous (The GTEx Consortium 2013; Uhlén et al. 2015), with expression in the brain restricted to the blood vessels. Specifically, the transporter is localized at the basement membrane and within the perivascular pericytes (Kevelam et al. 2013). In contrast, SLC19A2 is localized to the luminal side of the endothelial cells of the brain. This stark polarization of the two transporters in the brain differs from their localization in the peripheral tissues (namely, the intestines and kidneys), where SLC19A3 is found at the luminal apical side, whereas SLC19A2 is found both at the luminal and basolateral sides of these organs to maintain levels in the blood (Said et al. 2004; Ashokkumar et al. 2006; Subramanian et al. 2006b). The polarization of the two thiamine transporters in the brain but not the kidney or intestine suggests that both transporters are required for transport of thiamine across the blood brain barrier, and it may explain why individuals harboring *SLC19A3* gene mutations develop neurological symptoms and pathology, without having a systemic thiamine deficiency.

Ozand et al. (1998) reported that BTBGD patients responded to high doses of biotin with symptoms disappearing within days, with no further episodes of seizures, dystonia, confusion, or coma. Furthermore, clinical signs return to near baseline if treatment is initiated early enough (Ozand et al. 1998; Kono et al. 2009; Debs et al. 2010; Alfadhel et al. 2013; Tabarki et al. 2015). Early diagnosis is therefore essential, as permanent neurological sequelae including epilepsy, mental retardation, dystonia, and eventually death can occur if left untreated (Debs et al. 2010; Alfadhel et al. 2013; Tabarki et al. 2013; Flønes et al. 2016; Ygberg et al. 2016).

Biotin is not a substrate for the thiamine transporter (Subramanian et al. 2006a); thus the method of action for the response to biotin supplementation is unclear. The initial study by Ozand et al. reported no effect of thiamine alone on the clinical presentation of the patients; however, there are conflicting reports as to the effectiveness of biotin or thiamine alone (Kono et al. 2009; Debs et al. 2010; Yamada et al. 2010; Alfadhel et al. 2013; Gerards

et al. 2013; Sremba et al. 2014; Tabarki et al. 2015). Most current treatment protocols use a combination of both biotin and thiamine. There is limited physiological understanding as to the rationale behind this treatment approach. It has been suggested that thiamine and biotin act synergistically in the treatment of BTBGD (Ygberg et al. 2016), and there is evidence that biotin is required for the transcription of the *SLC19A3* gene (Vlasova et al. 2005). Thiamine-induced expression of an alternate thiamine transporter (encoded by the *SLC19A2* gene) has been suggested to underlie the clinical benefit seen in BTBGD patients (Kono et al. 2009; Gerards et al. 2013); however, this has not yet been supported by experimental evidence (Nabokina et al. 2013).

Disease-associated variants, both homozygous and compound heterozygous, have been reported throughout the gene (Table 1), and the effect on localization and activity of SLC19A3 has been investigated for a number of mutations. There are mutations that prevent the transport of the SLC19A3 protein to the cell surface, and others that reduce the affinity of the transporter for thiamine by either eliminating or reducing the functional capacity of the transporter (Subramanian et al. 2006a; Kono et al. 2009; Schänzer et al. 2014).

There is an apparent trigger for episodes of illness that can be identified in up to 50% of children with BTBGD (Tabarki et al. 2013). Bouts of decompensation are often preceded by febrile illness or mild trauma (Ozand et al. 1998; Tabarki et al. 2013; Ortigoza-Escobar et al. 2014; Ygberg et al. 2016). This indicates that there is a background level of thiamine that is transported in the cell with a dysfunctional SLC19A3 transporter that is able to support the energy needs of the brain during normal conditions. There is evidence to suggest the decline and clinical manifestation of the disorder may occur when the energy required exceeds the background levels and there is an inability of the mutant form of the *SLC19A3* gene to undergo the normal stress-induced expression and functional compensation (Schänzer et al. 2014; Ortigoza-Escobar et al. 2016).

Here we report two siblings who first presented with decompensation, encephalopathy, and seizures following febrile illness. They were subsequently diagnosed with BTBGD following whole-genome sequencing (WGS), which identified compound heterozygous inheritance of a previously reported missense mutation, p.G23V, and a novel 4808-bp promoter deletion. This deletion is the smallest promoter deletion reported for this gene to date, further defining the minimal promoter region for this gene in the context of the disease.

RESULTS

Clinical Presentation and Family History

The two cases are siblings, New Zealand–born from nonconsanguineous parents of mainly European descent. The proband (AM1005) died at 20 yr of age as a result of respiratory complications of severe dystonic spastic quadriplegia. He was born after a normal pregnancy, had Apgar scores of 5 at 1 min and 9 at 5 min and had 3 d in hospital for treatment of transient tachypnea of the newborn. He progressed normally in his development until he presented at 9 mo of age with seizures in the context of a viral illness. His brain MRI (not shown) revealed bilateral basal ganglia T2 hyperintensity and subdural hematomas. Subsequently he developed epilepsy and three further episodes of definite regression and encephalopathy. Follow-up brain MRI revealed involvement of the basal ganglia, caudate head, lentiform nuclei, and white matter atrophy. From approximately 12 yr of age he was essentially dependent for all activities of daily living but bright and aware of his surroundings. He had extensive neurometabolic workup in 2008 and had mild complex IV deficiency on muscle biopsy. All other investigations were normal and a tentative diagnosis of mitochondrial disease was made.

Table 1. Previously reported variants in *SLC19A3*

Chromosomal variant	Protein variant	Number of patients	Domain	Paper(s)
Amino acid substitutions				
c.68G>T	p.G23V	6	TM1	Kevelam et al. 2013; Ortigoza-Escobar et al. 2016; Pérez-Dueñas et al. 2013; Pronicka et al. 2016; Zeng et al. 2005
c.130A>G	p.K44E	2	Ext1	Kono et al. 2009
c.153A>G	p.I51M	1	Ext1	Ortigoza-Escobar et al. 2016
c.157A>G	p.N53D	1	Ext1	Ortigoza-Escobar et al. 2016
c.280T>C	p.W94R	2	TM3	Schänzer et al. 2014
c.337T>C	p.Y113H	3	TM4	Flønes et al. 2016
c.416T>A	p.V139G	1	Int3	Ferreira Whitehead & Leon 2017
c.517A>G	p.N173D	1	TM6	Fassone et al. 2013
c.527C>A	p.S176Y	1	TM6	Kevelam et al. 2013
c.541T>C	p.S181P	3	TM6	Flønes et al. 2016; Kevelam et al. 2013
c.958G>C	p.E320Q	2	TM8	Kono et al. 2009; Yamada et al. 2010
c.1154T>G	p.L385R	2	TM10	Kevelam et al. 2013
c.1196A>T	p.N399I	1	Int6	Kohrogi et al. 2015
c.1264 A>G	p.T422A	63	TM11	Alfadhel et al. 2013; Algahtani et al. 2017; Distelmaier et al. 2014; Ortigoza-Escobar et al. 2016; Tabarki et al. 2015, 2013; Zeng et al. 2005
c.1332C>G	p.S444R	1	TM12	Kevelam et al. 2013
Others				
Chr2: 228,568,440–228,613,489del		2		Flønes et al. 2016
c.20C>A	p.S7*	5		Gerards et al. 2013
c.74dup	p.S26fs	9		Sremba et al. 2014
c.81_82dup	p.M28fs	1		Sremba et al. 2014
c.507C>G	p.Y169*	1		Kevelam et al. 2013
c.1173–3992_1314 + 41del4175	p.Q393*fs	2		Schänzer et al. 2014
r.1173_1314del	p.E393*	1		Kevelam et al. 2013
c.895_925del	p.V299fs	2		Kevelam et al. 2013
c.980-14 A>G	p.Y327DfsX8	7		Debs et al. 2010; Ortigoza-Escobar et al. 2014; Ortigoza-Escobar et al. 2016; Serrano et al. 2012
c.982del	p.A328Lfs*10	2		Haack et al. 2014
c.1079dupT	p.L360Ffs*38	1		Ortigoza-Escobar et al. 2014
c.1403delA		2		Ygberg et al. 2016

TM, transmembrane domain; Ext, extracellular domain; Int, intracellular domain.

AM1006 is the proband's full sibling and is currently 7 yr of age. She was born after a normal pregnancy and had normal development until ~6 mo of age. By 2 yr she had a diagnosis of autism spectrum disorder. She could walk but was ataxic. At nearly 4 yr of age, after a period of steady development, she developed progressive problems with feeding and

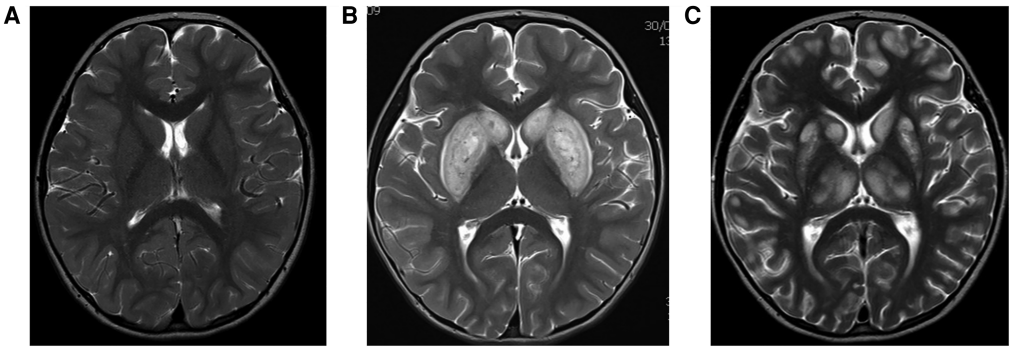

Figure 1. Magnetic resonance images taken from AM1006. (*A*) Axial T2 brain MRI taken at 3 yr and 5 mo of age prior to the first presentation. (*B*) Axial T2 brain MRI taken during first encephalopathy presentation at 3 yr and 9 mo of age: bilateral symmetrical T2 hyperintensity, swelling, and edema within the caudate nuclei and putamina sparing the globi pallidi and thalami. (*C*) Axial T2 brain MRI taken at 6 yr and 1 mo of age, during the second period of encephalopathy: bilateral symmetrical T2 hyperintensity within caudate nuclei, putamina, thalami, and subcortical white matter of the frontal lobes. There is resolution of basal ganglia swelling relative to the first degenerative episode (*B*).

encephalopathy in the context of a febrile illness. Brain MRI revealed acute restricted diffusion and swelling of the putamen and caudate nuclei (Fig. 1B). She was treated with a "mitochondrial cocktail" that included thiamine and improved. This was subsequently withdrawn after 6 mo as its effectiveness was not clear and pyruvate dehydrogenase deficiency was excluded. At 6 yr of age she had a prolonged period of neurological deterioration associated with a markedly abnormal brain MRI (Fig. 1C). She lost her ability to swallow and mobilize independently. Given the severity of her presentation she was again trialed on a short course of thiamine and improved. Prior to her diagnosis being made she was gastrostomy-fed, had excessive drooling, was unable to mobilize independently, was hypotonic, and was very irritable. Clinical features of both patients are summarized in Table 2.

Genomic Analyses

Genetic investigation was performed under the hypothesis that the affected siblings carry the same causative variant(s). The siblings were first assessed for copy-number variants using an Affymetrix CytoScan 750K Cytogenetics Array (analyzed using ChAS v.1.2/na32.1 software). Two shared regions of loss of heterozygosity (LOH) were identified; 3.5 Mb at 2p12p11.2 (hg19 Chr2:81,640,336–85,127,909) and 3.2 Mb at 19p13.2 (hg19 Chr19:9,917,692–13,157,540). The first region of LOH encompasses the succinate-CoA ligase, α-subunit (*SUCLG1*) gene that encodes the α-subunit of the enzyme succinate coenzyme A ligase, an enzyme involved in the TCA cycle. Autosomal recessive mutations in this gene result in mitochondrial DNA depletion syndrome 9 (MTDPS9, MIM: 245400) (Rouzier et al. 2010). The symptoms of MTDPS9 were similar to those observed in the two siblings; however, the lack of methylmalonic acid in the urine excluded the 2p12p11.2 LOH as the causative variant.

The second region of LOH contained only one gene: glutaryl CoA dehydrogenase (*GCDH*) which encodes a protein of the same name, involved in the metabolism of lysine, hydroxylysine, and tryptophan. Mutations in this gene result in glutaric acidemia type I (GA1, MIM: 231670) (Hedlund et al. 2006). GA1 was initially considered in the proband to be a strong candidate due to phenotypic similarities; however, urine organic acids, acylcarnitine profile, and enzymology in skin fibroblasts were normal, suggesting that the 19p13.2 was not the causative variant in the patients.

Table 2. Clinical summary of BTBGD in patients AM1005 and AM1006

	AM1005	AM1006	AM1006 post-treatment
Subacute encephalopathy			
Confusion	Yes	Yes	Nil further events
Dysarthria	Yes	Yes	Nil further events
Dysphagia	Yes	Yes	Nil further events
Reduced level of consciousness	Yes	Yes	Nil further events
Chronic symptoms			
Dysphagia	Yes	Yes	Resolved
Spasticity	Yes	No	No
Dystonia	Yes	No	No
Quadriparesis	Yes	No	No
Inability to walk	Yes	Yes	Improving
Inability to speak	Yes	Yes	Improving
Seizures	Yes	Yes	No
Severe gait ataxia	N/A—immobile	Severe	Moderate
Nystagmus	No	No	No
Ophthalmoplegia	No	No	No
Developmental delay	Yes	Yes	Improving
Biochemical studies of intermediary metabolism normal	Yes	Yes	N/A
Bacterial studies normal	Yes	Yes	N/A
Viral studies normal	Yes	Yes	N/A
Preceded by immune response to illness or trauma	Yes	Yes	N/A
Radiological features			
Swelling and T2 hyperintensity			
Basal ganglia	Yes	Yes	N/A
Thalami	No	Yes	N/A
Subcortical white matter	No	Yes	N/A
Cortex	No	Yes	N/A
Cerebral white matter	No	Yes	N/A
Cortex	No	Yes	N/A
Midbrain	No	Yes	N/A
Necrosis of			
Head of basal ganglia	Yes	Yes	N/A
Putamen	Yes	Yes	N/A
Novel clinical features			
	Subdural hematomas present on brain MRI	Autism spectrum disorder diagnosed preencephalopathic event with normal initial MRI scan, choreoathetoid movement disorder	

Table 3. Sequencing statistics for whole-genome analysis

Individual	Total read pairs	Total mapped reads	Average coverage	Reads at 25 plus coverage	Coverage at SNP	Average coverage over deletion
AM1005	541,331,895	806,382,669	38.8	99.2%	42	14.5
AM1006	569,003,503	765,493,391	36.1	98.8%	32	16.5

Whole-genome sequencing was performed on genomic DNA from both children, with sequencing statistics detailed in Table 3. 166,057 single-nucleotide and indel variants were discovered in the exonic and splice regions of both children. Synonymous variants, variants present in only one sibling, and variants exceeding a frequency of 0.01 in any of the ExAC, HapMap, or 1000 Genomes Project populations were excluded from further consideration. (The International Hapmap Consortium 2003; The 1000 Genomes Project Consortium 2015; Lek et al. 2016). Further filtering of the remaining 3481 variants was performed based on protein location, functional impact of the variant, its mode of inheritance (homozygous or compound heterozygous), as well as the biological relevance of the gene. This supervised exclusion of variants revealed compound heterozygous mutations in the *SCL19A3* gene. The first is a heterozygous missense mutation, c.68G>T (GRCh37 Chr2:228,566,967; NM_025243.3:c.68G>T) resulting in a glycine-to-valine change (p.G23V) (summarized in Table 4). The amino acid is conserved across species to *Drosophila melanogaster* and *Caenorhabditis elegans* (BLASTp, Gish and States 1993). The allele is also extremely rare, present at a frequency of 0.000008239 representing one individual of South Asian descent in the ExAC database (Lek et al. 2016), with no homozygotes present at this position. Confirmation of the single-nucleotide variant in both children and their parents was performed using polymerase chain reaction (PCR) and Sanger sequencing, which verified the mutation was inherited from the mother, who was also heterozygous (Fig. 2). The phenotypic presentation in the children corresponded to the description of BTBGD in the literature; however, no other candidate SNP or indels in *SLC19A3* were identified.

To investigate the *SLC19A3* locus for structural variants, the WGS read alignments were visualized with Integrated Genomics Viewer (IGV, 2.3.55) (http://software.broadinstitute.org/software/igv; Thorvaldsdóttir et al. 2013) and inspected for regions of altered coverage and alignment of discordant read pairs. This identified a region of reduced coverage, bordered by discordant reads encompassing exon 1 encoding the 5′ untranslated region (5′ UTR) (Fig. 3). The candidate deletion was also detected by copy-number variant detection algorithms: CNVnator (based on alterations in read depth) and BreakDancer (based on paired-end information) (Chen et al. 2009; Abyzov et al. 2011). The precise breakpoints were mapped by PCR and Sanger sequencing to GRCh37 Chr2:228,582,251–228,587,060 (NC_000002.11:g.228,582,251–228,587,060 del) with the insertion of a cytosine nucleotide (summarized

Table 4. SLC19A3 variant summary

Gene	Chromosome	HGVS DNA reference	HGVS protein reference	Variant type	Predicted effect	ClinVar ID	Parent of origin	Break points
SLC19A3	2	NM_025243.3	Gly23	Missense	Gly>Val	SCV000574715	Mother	
SLC19A3	2	NC_000002.11		Deletion	Critical promoter deleted	SCV000574716	Father	228,582,251–228,587,060

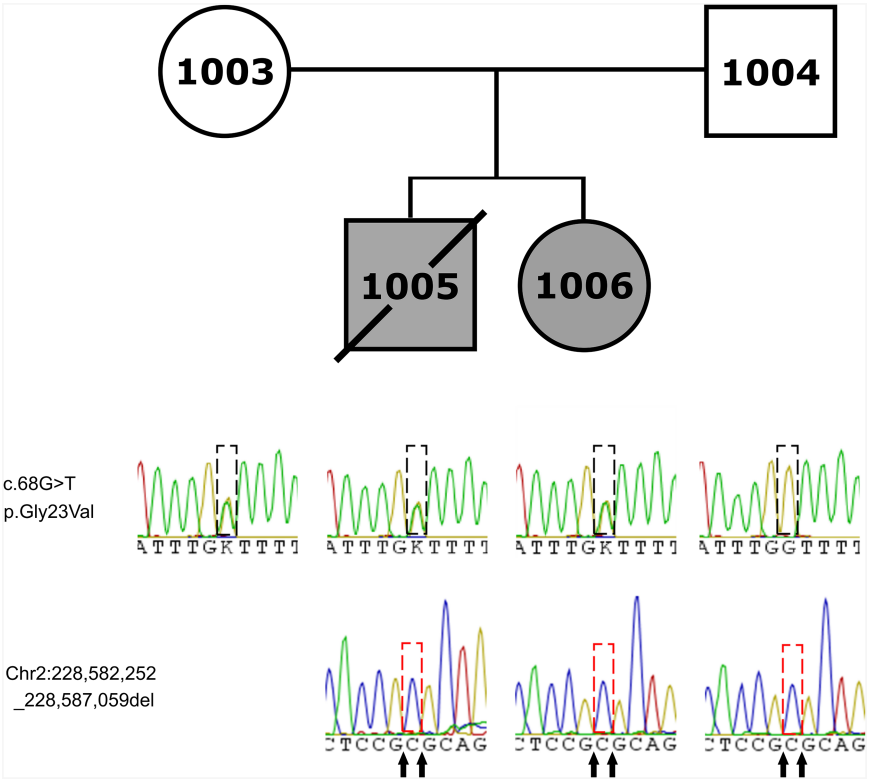

Figure 2. Family pedigree and corresponding Sanger sequencing electropherograms showing the transmission of the c.68G>T mutation and Chr2: 228,582,252–228,587,059 deletion in the *SLC19A3* gene. The breakpoints for the deletion are indicated by the black arrows, with an insertion of a single base, cytosine, at the breakpoint indicated by the red box. No PCR product was amplifiable from the mother who did not contain the deletion as the PCR was optimized for the 1481-bp deleted product.

in Table 4). This 4808-bp deletion is present in both children and PCR analysis confirmed inheritance from the father. We hypothesize that this deletion abolishes the activity of the promoter, thus eliminating expression from this allele. RNA-seq analysis of RNA from fibroblasts of the surviving child, AM1006, and three unrelated controls did not detect any reads

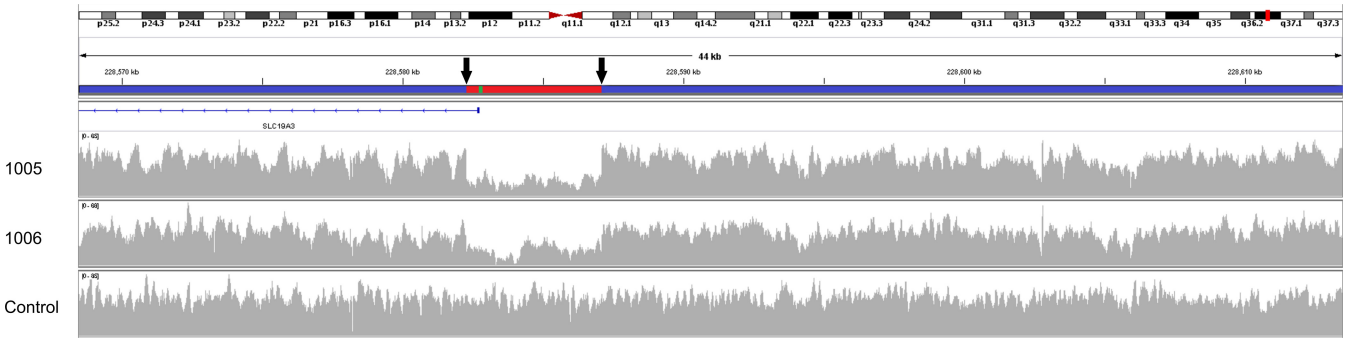

Figure 3. Deletion in *SLC19A3*. Integrated Genomics Viewer showing depth of coverage of WGS reads across the 4808-bp deletion (red bar) for both siblings and an unrelated control. The arrows denote the breakpoints for this deletion. The blue bar denotes the 45,049-bp deleted region reported by Flønes et al. (2016), whereas the green bar denotes the minimal promoter region defined by Nabokina and Said (2004).

mapping to the *SLC19A3* gene; in contrast *SLC19A1* and *SLC19A2* were well expressed in all samples. Thus, we were unable to categorically determine the effect of the deletion on *SLC19A3* gene expression.

Thus, the two cases harbor compound heterozygous inherited mutations in the *SLC19A3* gene; a maternally inherited heterozygous p.G23V and a paternally inherited 4808-bp heterozygous deletion in the 5′ region between Chr2:228,582,251 and Chr2:228,587,060, confirming the diagnosis of BTBGD.

Treatment Outcomes

Following the detection of the *SLC19A3* gene mutations, AM1006 was commenced on high-dose (20-mg/kg/day) thiamine and (15-mg/kg/day) biotin at the age of 6 yr and 9 mo. Ten months post commencement of therapy she is now running independently, eating a full diet, and no longer requiring gastrostomy for nutrition. She has had no further encephalopathic events and is communicating with noises and making excellent cognitive progress. She remains intellectually disabled and has autism but is more engaged and interactive and making weekly gains in her development.

DISCUSSION

Analysis of WGS data resulted in a molecular diagnosis of BTBGD for two siblings with progressive neurodegeneration. This diagnosis led to successful biotin/thiamine treatment for the surviving child. Both cases harbor compound heterozygous mutations in the *SLC19A3* gene, one inherited from each unaffected parent.

One of the mutations had previously been reported for multiple families with BTBGD, c.68G>T, p.G23V (Table 1), whereas the other is a novel 4808-bp deletion encompassing the first exon and predicted promoter region. This deletion is within the much larger 45-kb deletion recently reported by Flønes et al. (2016) and includes the minimal promoter region for the *SLC19A3* gene (Fig. 3; Nabokina and Said 2004). The deletion reported in this study is currently the smallest promoter deletion in this gene identified, further defining the critical promoter region. Taken together, these data suggest that the deletion results in reduced or no expression from this allele. From a molecular diagnostic perspective, the deletion is too small to be detected by aCGH and would have been missed by typical WES approaches (for review, see Tan et al. 2014).

The other mutation (p.G23V) lies within the first transmembrane domain of hTHTR2 (Fig. 4), a position that is highly conserved through to invertebrates. The p.G23V mutant SLC19A3 protein has been shown to have minimal effect on targeting of the protein to apical surface of cells, but effectively abolishes the ability of the protein to transport thiamine across the cell membrane (Subramanian et al. 2006a). Based on crystallographic and experimental data of other transporter proteins, the transmembrane domain 1 of *SLC19A3* is one of the four tilted helices that are crucial for the shape and activity of the central hydrophobic pore (Tamura et al. 2001; Hirai et al. 2002; Abramson et al. 2003; Huang et al. 2003; Glaeser et al. 2010). There is an overrepresentation of glycine in membrane spanning proteins where the helices interact (Javadpour et al. 1999), and substitutions with larger amino acids such as valine in this case may result in steric hindrance in the mutated protein, resulting in conformational changes (Subramanian et al. 2006a). The detrimental effect of glycine-to-valine substitutions has been reported for multiple other solute carrier transporters, resulting in decreased transporter function (Zhou et al. 2004; Li et al. 2012).

Confirming previous reports, the surviving patient responded positively to thiamine and biotin treatment. The exact mechanism through which biotin and thiamine ameliorate the symptoms of BTBGD is unknown. The minimal promoter region for both the *SLC19A2*

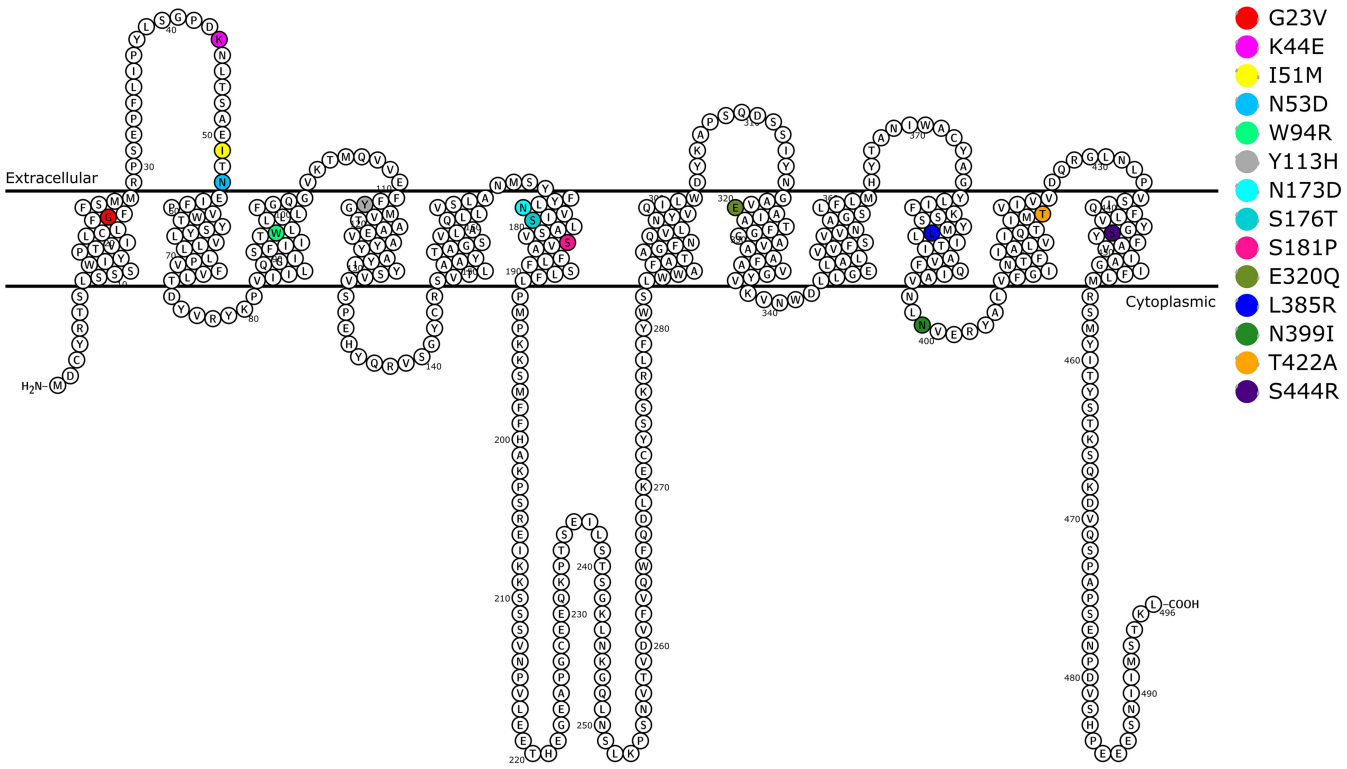

Figure 4. Topography of the hTHTR2 protein, consisting of 12 transmembrane domains. Point mutations reported in the literature are indicated as per the legend. The pG23V mutation identified in this study is indicated in red. The topography map is generated from Protter (Omasits et al. 2014).

and *SLC19A3* genes has been previously identified (Reidling et al. 2002; Reidling and Said 2003; Nabokina and Said 2004). Both promoters contain binding sites for the transcription factor Specificity Protein 1 (SP1), and binding increases the transcription of both genes. Specificity Protein 3 (SP3) binding to the minimal promoter of *SLC19A3* also increases transcription of the gene (Nabokina and Said 2004). Biotin supplementation of cells in culture increases the nuclear abundance of both SP1 and SP3, as well as their association with the respective DNA binding sites, with consequential increases in transcription (Griffin et al. 2003). Therefore, biotin's therapeutic effect may be through an indirect transcriptional activation of the *SLC19A2* and *SLC19A3* genes via SP1 and SP3. This proposed method of action for biotin treatment of BTBGD is in agreement with the hypothesis that the disease is caused by an inability of neurons to undergo a stress-induced increase in *SLC19A3* gene expression (Schänzer et al. 2014). An increase in SP1 expression has been shown to result in the differential up-regulation of *SLC19A3* gene transcription and not *SLC19A2* (Nabokina et al. 2013). Therefore, supplementation of biotin may result in increased transport of thiamine through a semifunctional *SLC19A3* allele (Kevelam et al. 2013).

Critically, the application of WGS was required to detect the small promoter deletion, which lay below the thresholds of detection using conventional high-resolution array-based genome analysis and WES. The identification of the variants reported here resulted in a refined and precise molecular diagnosis and subsequent treatment for the surviving child via supplementation of biotin and thiamine. Early detection and treatment of BTBGD is critical for the long-term survival and outcome for patients. Therefore, these results provide strong support for the expeditious use of WGS in the diagnosis of rare neurodevelopmental disorders.

METHODS

DNA Extraction

DNA was extracted from peripheral blood EDTA samples using Gentra Puregene DNA Extraction kit (QIAGEN), according to the manufacturer's instructions.

aCGH Analysis

Genome-wide copy number and SNP analysis was undertaken using an Affymetrix CytoScan 750K Array, according to the manufacturer's instructions. Regions of copy-number change and LOH were determined using the Affymetrix Chromosome Analysis Suite software (ChAS) v.1.2/na32.1 and interpreted with the aid of the UCSC genome browser (http://genome.ucsc.edu/; Human Feb. 2009 GRCh37/hg19 assembly).

Whole-Genome Sequencing

WGS services were provided by The Kinghorn Centre for Clinical Genomics (KCCG) at the Garvan Institute using the Illumina HiSeq X Ten instrument. Reads were mapped with Burrows–Wheeler aligner (v 0.7.12) (Li and Durbin 2009) to the 1000 Genomes human genome reference sequence (GRCh37.p13), sequencing statistics in Table 3. After removal of optical and PCR duplicates, indels were realigned and base quality scores were recalibrated using the Genome Analysis Toolkit (v. 3.4-0) (DePristo et al. 2011). The alignments for the two children were combined with alignments from 14 unrelated individuals for joint variant discovery with the GATK's HaplotypeCaller and GenotypeGVCFs tools, followed by variant quality score recalibration according to the Broad Institute's Best Practices (DePristo et al. 2011).

Population frequency of SNV and indel variants were obtained from the Exome Aggregation Consortium (ExAC) (Lek et al. 2016), HapMap (The International Hapmap Consortium 2003), and 1000 Genomes Projects (The 1000 Genomes Project Consortium 2015). Bioinformatic identification of CNVs was performed using default parameters of BreakDancer (v 1.4.5) (Chen et al. 2009) and CNVnator (v 0.3) (Abyzov et al. 2011).

WGS alignments and regional variation in read coverage were viewed using the Integrated Genomics Viewer (IGV, 2.3.55) (http://software.broadinstitute.org/software/igv; Thorvaldsdóttir et al. 2013). Discordant read pairs (pairs mapped further apart than the average library fragment size) were identified with IGV.

Sanger Sequencing

Confirmation of variants was performed using PCR and Sanger sequencing. The forward primer 5′ TTGAGGGAAGCCCTGTATCC 3′, and the reverse primer 5′ GCAGTTCCT GGATTTACCCC 3′ generated a 204-bp product using the Expand High Fidelity PCR System (Roche) with the following thermocycler conditions: 2 min initial denaturation at 94°C, with 30 cycles of 15-sec denaturation at 94°C, 30 sec of annealing at 50°C, and 45 sec of extension at 72°C, followed by 7 min of final extension at 72°C. This 205-bp product includes the C>G missense mutation. The forward primer 5′ TTGCGGCAGCGACATTGATT 3′, and the reverse primer 5′ TGTGCTCGTCTACACATCTTCC 3′ were designed to generate a 6289-bp product in the wild-type allele, and a 1481-bp product in the deleted allele. Thermocycler conditions were optimized so that only the deleted copy was exponentially amplified; 2 min of initial denaturation at 94°C, with 30 cycles of 15-sec denaturation at 94°C, 30 sec annealing at 50°C, and a 3-min extension at 72°C, followed by a 7-min final extension at 68°C. Sanger sequencing was performed by the Genomics Centre, Auckland Science Analytical Services, The University of Auckland, New Zealand. Sanger sequencing data were viewed using Geneious (8.1.5) (http://www.geneious.com; Kearse et al. 2012).

ADDITIONAL INFORMATION

Data Deposition and Access

Consent was not obtained to deposit full variant calling data publicly; however, mutations have been deposited to ClinVar (https://www.ncbi.nlm.nih.gov/clinvar) under the accession numbers SCV000574715 and SCV000574716.

Ethics Statement

The genetic analysis and de-identified publication of variants was performed under the approval of the New Zealand Northern B Health and Disability Ethics Committee (12/NTB/59), and parents provided written informed consent.

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Author Contributions

W.W. and I.H. performed DNA-based laboratory experiments; W.W., I.H., J.C.J., and K.L. performed data and bioinformatics analysis. E.G. was the metabolic specialist doctor responsible for the care of the patients and contributed to the manuscript. F.W. was the radiologist who collected and reported on the magnetic resonance images. A.M. was the pediatrician responsible for the long-term medical care of the cases. F.A. performed the array-based analysis and identification of shared LOH regions. D.R.L. contributed to sample preparation and clinical confirmation of the variants. J.T. provided genetic counseling for the family following revised molecular diagnosis. R.H., R.G.S., K.L. and J.C.J. conceived the study. W.W. wrote the initial manuscript, and J.C.J. critically reviewed the manuscript. All authors edited and reviewed the final manuscript.

Competing Interest Statement

The authors have declared no competing interest.

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