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Novel *RAB3GAP1* Mutation in the First Tunisian Family With Warburg Micro Syndrome

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Background and Purpose Warburg Micro syndrome (WARBM) is a rare autosomal recessive genetic disease characterized by ocular, neurologic, and endocrine anomalies. WARBM is a phenotypically and genetically heterogeneous syndrome caused by mutations in *RAB3GAP1*, *RAB3GAP2*, *RAB18*, and *TBC1D20*. Here we present the clinical and genetic characterization of a consanguineous Tunisian family with a WARBM phenotype presenting two pathogenic variations, one of which is on *RAB3GAP1*.

Methods We applied whole-exome sequencing (WES) to two affected young males presenting a WARBM-compatible phenotype.

Results We reveal a new variation in *RAB3GAP1* (NM_012233.3: c.297del, p.Gln99fs) and another variation in *ABCD1* (NM_000033: c.896A>G, p.His299Arg). Each of these mutations, which in silico predictions concluded as being pathogenic variations, affects a critical protein region. Both affected males presented a WARBM-compatible phenotype, with severe intellectual disability, severe developmental delay, postnatal growth delay, postnatal microcephaly, congenital bilateral cataracts, general hypotonia, and a thin corpus callosum without a splenium. However, intrafamilial clinical heterogeneity was present, since only the oldest child had large ears, microphthalmia, foot deformities, and a genital anomaly, and only the youngest child had microcornea. Despite the mutation identified in *ABCD1*, our patients did not have any X-linked symptoms of adrenoleukodystrophy disorder that are usually caused by *ABCD1* mutations, which prompted our interest in clinical monitoring.

Conclusions WES analysis of a consanguineous Tunisian family with WARBM revealed a novel variation in *RAB3GAP1* (NM_012233.3: c.297del, p.Gln99fs) that is most likely pathogenic and allowed us to confirm the diagnosis of WARBM.

Keywords Warburg Micro syndrome; RAB3GAP1 protein, human; ABCD1 protein, human; mutation; whole exome sequencing.

INTRODUCTION

Warburg Micro syndrome (WARBM), also known as Micro syndrome, is a genetically heterogeneous, autosomal recessive disease. It is extremely rare, but its true incidence is unknown.[1] WARBM was first described by Warburg et al.[2] in 1993 in a consanguineous Pakistani family with two affected siblings and their cousin. The use of the "Micro" term by Warburg et al.[2] was inspired by the morphologic traits of the affected patients: microphthalmia, microcornea, microcephaly, and micrognathia. In that report they distinguished it from similar syndromes such as cerebro-oculo-facio-skeletal syndrome (MIM #214150), Cockayne syndromes A (MIM #216400) and B (MIM #133540), and Martsolf syndrome (MIM #212720).[2] All of these diseases are characterized by intellectual disability, postnatal growth deficiency, microcephaly, cataract, contractures, and hypothalamic hypogonadism. In

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WARBM, intellectual disability is more severe and cranial magnetic resonance imaging (MRI) usually shows cortical dysplasia, in particular hypoplasia or agenesis of the corpus callosum. The congenital bilateral cataracts are not isolated in all of these syndromes, and is associated with microphthalmia, optic atrophy, and microcornea in WARBM.[1-22] WARBM is also characterized by facial dysmorphia, with a hairy forehead, large anteverted ear, broad nasal root, and micrognathia.[2-4,10,11,13,17,19,20]

WARBM is classified into four subtypes based on the mutated gene: WARBM1 (MIM #600118), WARBM2 (MIM #614225), WARBM3 (MIM #614222), and WARBM4 (MIM #615663). These four subtypes are respectively caused by biallelic mutations in *RAB3GAP1* (RAB3 GTPase-Activating Protein Catalytic Subunit; MIM *602536; 2q21.3), *RAB3GAP2* (RAB3 GTPase-Activating Protein Noncatalytic Subunit; MIM *609275; 1q41), *RAB18* (Ras-Associated Protein RAB18; MIM *602207; 10p21.1) and *TBC1D20* (TBC1 Domain Family Member 20; MIM *611663; 20p13).[1,5,7,9,23-26] It has thought that WARBM can be caused directly by the loss of function of the RAB18 protein, or indirectly by the loss of function of the RAB3GAP complex, or TBC1D20 protein.[14,27]

RAB3GAP1 consists of 24 exons and encodes for the catalytic subunit (p130) of the heterodimeric RAB3GAP complex. The noncatalytic subunit (p150) of this complex is encoded by *RAB3GAP2*.[5,28-31] The RAB3GAP complex is a GTPase-activating protein (GAP) that regulates the Ca^{2+}-mediated exocytosis of neurotransmitters and hormones[5] by switching Rat Sarcoma-Associated Binding-Related Protein 3 (RAB3) between the RAB3-GTP "active form" and the RAB3-GDP "inactive form".[28,29,32] Indeed, the RAB3 subfamily of RAB proteins, which are small G proteins belonging to the rat sarcoma superfamily,[32] are required for normal eye and brain development.[5] The RAB3GAP complex also functions as a guanine nucleotide exchange factor for RAB18 protein,[33] and TBC1D20 protein exhibits modest GAP activity toward RAB18 protein.[34]

Since there are no hotspot mutations causing WARBM, the genetic analysis of the entire RAB3GAP1 gene as well as all of the other genes is necessary to identify the genetic cause of the syndrome.[35] *RAB3GAP1* mutations were the first reported and are now the most frequently (40%) reported in WARBM patients.[1,3,5,25,36] More than 70 different pathogenic variations, which are commonly homozygous, have been identified in this gene[1,3,5,11,17,20,25,27,31,35-47] (Supplementary Fig. 1 in the online-only Data Supplement).

Here we present the clinical and genetic characterization of a consanguineous Tunisian family with a WARBM phenotype presenting two pathogenic variations, one of which is on *RAB3GAP1*.

METHODS

This project was approved by our local ethics committee (no. CEBM/45/2021). Informed consent was obtained from the parents for molecular genetic analysis and for publication of the clinical and molecular data.

DNA extraction

Standard protocols were used to extract genomic DNA from peripheral leukocytes of the two patients as well as their healthy brother and parents.

Whole-exome sequencing

Whole-exome sequencing (WES) was performed for the two affected brothers. Exonic DNA libraries were prepared using the Illumina DNA Prep with Enrichment kit (Illumina, San Diego, CA, USA). The prepared libraries were quantified using qPCR according to the Illumina qPCR Quantification Protocol Guide. A standard curve of fluorescence readings and library sample concentrations was generated using Roche's Rapid library standard quantification solution and calculator (F. Hoffmann-La Roche AG, Basel, Switzerland). The prepared libraries were sequenced on an Illumina NovaSeq 6000 system with 150 paired-end reads.

Exome sequence analysis

The paired-end sequence reads were aligned to the human genome GRCh38.p13 using Bowtie2 algorithm.[48] The aligned reads were processed using the Picard SortSam algorithm (http://broadinstitute.github.io/picard/) to create and sort an alignment binary file, the Picard AddOrReplaceReadGroups algorithm to insert size information, and the Picard MarkDuplicates algorithm to remove PCR duplicates. After duplication removal, the depth of coverage of targeted exome regions was calculated using the Picard BuildBamIndex algorithm. The variations were called using the GATK HaplotypeCaller algorithm, and the variation calling file was filtered using the GATK VariationFiltration algorithm.[49] The variations were then annotated using Annovar software.[50]

Suspected pathogenic variations were identified using VarAFT software.[51] We first selected the autosomal recessive and X-linked inheritance modes, and then filtered out synonymous and noncoding variations before selecting variations with a minor allele frequency of ≤0.01 in the dbSNP150, Exome Aggregation Consortium, gnomAD, 1000 Genomes Project, KaViar (Known Variations Project), and HRC (Haplotype Reference Consortium) databases, as well as in our local database. The pathogenicity of variations was analyzed using the following in silico tools: Provean,[52] PolyPhen-2,[53] MutationTaster,[54] UMD-Predictor,[55] SNPs&GO,[56] PredictSNP,[57]

CADD,[58] DANN,[59] FATHMM,[60] and GWAVA[61] for nonsynonymous amino acid changes; MutationTaster for stop codons and in-frame insertions/deletions in coding regions; and Human Splicing Finder[62] and ESEfinder (version 3.0)[63] for splice-site variations. Pathogenic variations were retained if at least one in silico tool predicted them as pathogenic.

We interpreted the filtered variations according to the American College of Medical Genetics and Genomics (ACMG) guidelines,[64] which classify variations into the following five categories using a criteria-based scoring system: pathogenic, likely pathogenic, uncertain significance, likely benign, or benign.

Suspected mutations were visually inspected using the Integrative Genomics Viewer.[65]

Sanger sequencing

The sequences of exon 5 of *RAB3GAP1* and exon 1 of *ABCD1* (ATP-binding cassette D1 subtype) were analyzed using PCR with the primer pairs listed in Supplementary Table 1 (in the online-only Data Supplement). PCR amplicons were then analyzed by direct DNA sequencing. DNA sequencing reactions were performed using the BigDye Terminator cycle sequencing kit (version 3.1, Life Technologies, Thermo Fisher Scientific, Carlsbad, CA, USA) and capillary electrophoresis on the ABI 3500 sequencer (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA). Sequence data were analyzed using Sequencing Analyzed Software (version 6.0; Thermo Fisher Scientific, Waltham, MA, USA) and Applied Biosystems SeqScape (version 3.0) software. The NCBI sequences NM_012233.3 and NM_000033 were used as reference sequences for *RAB3GAP1* and *ABCD1*, respectively.

RESULTS

Clinical features

The analyzed family included three children: two affected males (the oldest child [V-1] and the youngest child [V-3]) and a healthy male child (V-2). The parents were second cousins, and both were healthy. There was no relevant abnormality in the family history (Fig. 1).

The affected brothers (V-1 and V-3) were referred to the Department of Congenital and Hereditary Diseases at Charles Nicolle Hospital for multiple congenital anomalies. They had been born via spontaneous vaginal deliveries at term after uncomplicated pregnancies. The neonates had normal birthweights, heights, and head circumferences.

The oldest child (V-1) died at 10 years following a lung infection. He had a severe intellectual disability, and was unable to sit unaided, walk, or speak. His congenital bilateral cataracts were operated on at 4 months old. A physical examination performed at the age of 5 years revealed height, weight, and head circumference at -3.5 SDs, -5.1 SDs, and -5.4 SDs, respectively, as well as facial dysmorphia, with an elongated face, rarefied eyebrows, microphthalmia, beaked nose, small mouth, high arched palate, and protruding large ears. Moreover, he had hypoplastic scrotum, overlapping toes, and general hypotonia (Table 1 and Fig. 2).

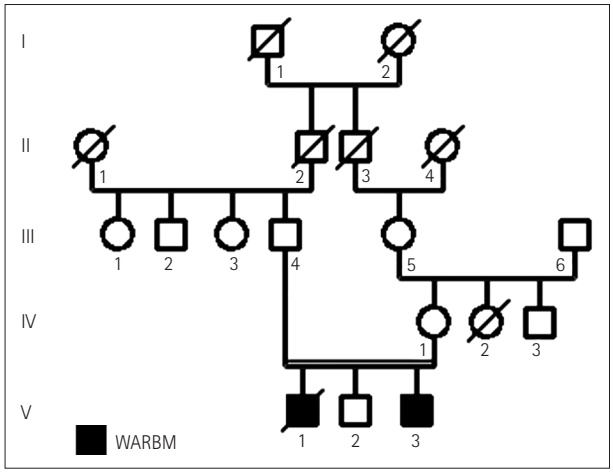

Fig. 1. Familial pedigree. Black and white symbols are affected and unaffected subjects, respectively. WARBM, Warburg Micro syndrome.

The youngest child (V-3) was an 8-year-old male. Like his older brother, he had severe intellectual disability and developmental delay. He had also received an operation for congenital bilateral cataracts, at 1 month old. A physical examination performed at the age of 5 years revealed growth delay (height=-4.7 SDs and weight=-2.7 SDs), microcephaly (head circumference=-4 SDs), general hypotonia, and dysmorphic features such as an elongated face, high forehead, strabismus, microcornea, tented upper lip, and thin lower lip (Table 1).

Cranial magnetic resonance imaging results

Cranial MRI of V-1 and V-3 at the ages of 8 and 7 months, respectively, revealed a thin corpus callosum without a splenium. No other cerebral anomalies were detected in either child.

Chromosome analysis

The two brothers had a normal male karyotype (46, XY).

Molecular findings

The analysis of the WES data of our two patients revealed the homozygous deletion c.297del (p.Gln99fs) in exon 5 of *RAB3GAP1* (2q21.3) that has not been reported previously in the literature or in the public databases, and the hemizygous missense mutation c.896A>G (p.His299Arg) in exon 1 of *ABCD1* (Xq28).

Table 1. WARBM clinical features of our patients (V-1 and V-3) and literature data

	V-1	V-3	WARBM[1-5,10,11,13,17,19,20,25,27,35,36,38,40-42,44,46,47,69,70]*
Age (yr)	Died at 10 years old following a lung infection	8	
Growth			
Normal length and weight at birth	+	+	(15/19)
Postnatal growth delay	+ (height=-3.5 SDs/ weight=-5.1 SDs)	+ (height=-4.7 SDs/ weight=-2.7 SDs)	(35/55)
Head and neck			
Postnatal microcephaly	+ (head circumference=-5.4 SDs)	+ (head circumference=-4 SDs)	(85/91)
Congenital cataract	+	+	(89/93)
Microphthalmia	+	-	(79/88)
Microcornea	-	+	(50/80)
Facial dysmorphism	Elongated face, beaked nose, rarefied eyebrows, protruding large ears, and high arched palate	Elongated face, curved and high forehead, strabismus, tented upper lip, and thin lower lip	Hairy forehead, large anteverted ear, broad nasal root, and micrognathia
Skeletal			
Foot deformities	+ (overlapping toes)	-	(9/38)
Neurologic			
Intellectual disability	Severe	Severe	Severe to profound (93/93)
Optic atrophy	-	-	(43/59)
Hypotonia	+	+	(50/75)
Spastic diplegia	-	-	(59/66)
Seizure	-	-	(17/54)
Sitting	-	-	(29/33)
Walking	-	-	(50/53)
Speech	-	-	(43/52)
Cranial MRI			
Abnormal corpus callosum	Thin corpus callosum without a splenium	Thin corpus callosum without a splenium	Hypoplasia or agenesis (80/86)
Cerebral atrophy	–	–	(26/69)
Polymicrogyria	–	–	(33/71)
Pachygyria	–	–	(18/62)
Enlarged sylvian fissures	–	–	(6/65)
Cerebellar hypoplasia	–	–	(14/73)
Demyelination	–	–	(31/73)
Others symptoms			
Genital abnormalities	+ (hypoplastic scrotum)	–	(49/71)

*Pairs of values in round bracket indicate (a/b): a. number of cases with this clinical abnormality; b. total number of cases in which this feature was analyzed.
WARBM, Warburg Micro syndrome; +, present; -, absent; SD, standard deviation.

The c.297del (p.Gln99fs) variation resulted in a truncated protein without its C-terminal catalytic domain (Fig. 3A). In silico prediction using MutationTaster concluded that this was a disease-causing variation. Sanger sequencing of exon 5 of *RAB3GAP1* confirmed the presence of this variation in a homozygous state in both affected brothers and in a heterozygous state in the parents and their healthy child (Fig. 3B). According to the ACMG guidelines,[66] we classified the c.297del variation as pathogenic, since 1) it was predicted to be a null variation in *RAB3GAP1*, where a loss-of-function variation is a known mechanism of WARBM (PVS1), 2) it is absent from population databases (PM2), 3) it changes the RAB-3GAP1 protein length (PM4), 4) it is predicted to be deleterious by in silico prediction tools (PP3), 5) it cosegregated with WARBM in two of the affected family members (PP1), and 6) the phenotype of the patients was highly specific for *RAB-*

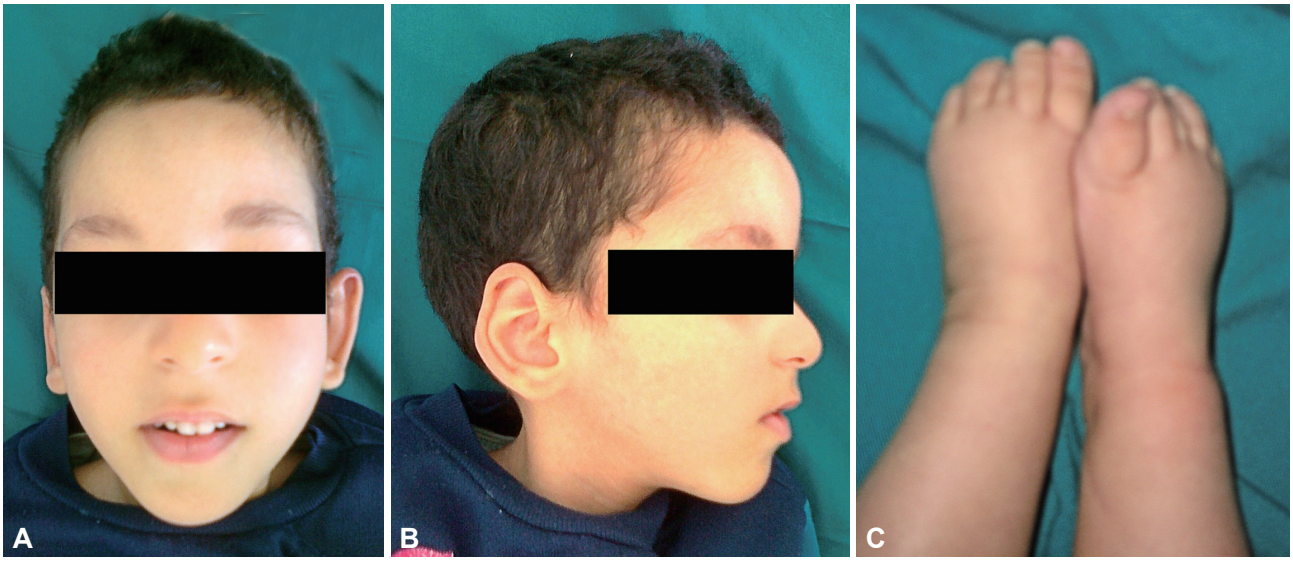

Fig. 2. Dysmorphic features noted in case of the oldest child (V-1). Facial dysmorphism of case V-1: frontal (A) and profile (B). These photographs show an elongated face, a beaked nose, rarefied eyebrows, and protruding large ears. (C) Foot deformities in case V-1. This photograph shows overlapping toes.

3GAP1 (PP4). This variation has been submitted to the ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar/; c.297del variation ID: SCV001519035).

The c.896A>G (p.His299Arg) variation was predicted to be deleterious using in silico prediction tools (Supplementary Table 2 in the online-only Data Supplement). According to the NCBI SmartBLAST (https://blast.ncbi.nlm.nih.gov/smartblast/)[67] and NCBI CD-search (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi)[68] tools, the 299th histidine residue is localized in a highly conserved domain (Peroxysomal Fatty Acyl CoA Transporter Family protein 1-681aa) (Fig. 4A). Moreover, Human Splicing Finder and ESEfinder (version 3.0) showed that the c.896A>G variation created a new donor splicing site. The Sanger sequencing of exon 1 of *ABCD1* confirmed the presence of this variation in a hemizygous state in the two children (V-1 and V-3) and in a heterozygous state in their mother (Fig. 4B).

DISCUSSION

WARBM is a rare autosomal recessive disease[1] characterized by ocular, neurologic, and endocrine abnormalities[1-5,10,11,13,17,19,20,25,27,35,36,38,40-42,44,46,47,69,70]. It is a phenotypically and genetically heterogeneous syndrome caused by mutations in *RAB3GAP1*, *RAB3GAP2*, *RAB18*, and *TBC1D20*[1,5,7,9,23-26]. In addition to WARBM, loss of function of the RAB3GAP complex is also associated with Martsolf syndrome[9,25] that shares many of the characteristics of WARBM, although it is less frequently reported and less severe with only moderate intellectual disability and developmental delay, a long life expectancy, and pronounced cerebral anomalies in only rare cases.[6,8-10,12,13,15,16,21,22,47] Amorphic mutations of *RAB3GAP1* and *RAB3GAP2* cause WARBM, while hypomorphic mutations could result in Martsolf syndrome.[9] Only one homozygous RAB3GAP1 frameshift mutation was identified in two siblings with a moderate Martsolf phenotype. This mutation occurred so close to the transcriptional start site that translation of an alternative transcript might be initiated from the next in-frame ATG (start codon), resulting in a protein with reduced product level that lacks the first 50 amino acids of its N-terminal domain and has its entire C-terminal catalytic domain.[25]

Our two patients had a phenotype more compatible with WARBM than Martsolf syndrome, as they presented with severe intellectual disability, severe developmental delay, postnatal growth delay, postnatal microcephaly, congenital bilateral cataracts, general hypotonia, a thin corpus callosum without a splenium, and a loss-of-function frameshift mutation in *RAB3GAP1*, which results in a truncated protein without its C-terminal catalytic domain.

We noted an intrafamilial clinical heterogeneity, since only the oldest child (V-1) who died at 10 years had large ears, microphthalmia, foot deformities (overlapping toes), and a genital anomaly (hypoplastic scrotum), which are specific clinical features of WARBM. Moreover, microcornia, which is one of the characteristic symptoms of WARBM, was detected only in the youngest child (V-3). Certain WARBM anomalies were absent from our two patients, such as optic atrophy, micrognathia, and spastic diplegia[1-5,10,11,13,17,19,20,25,27,35,36,39-42,44,46,47,69,70] (Table 1 and Fig. 2). In WARBM, the hypoplasia or agenesis of the corpus callosum is often associated with one or more

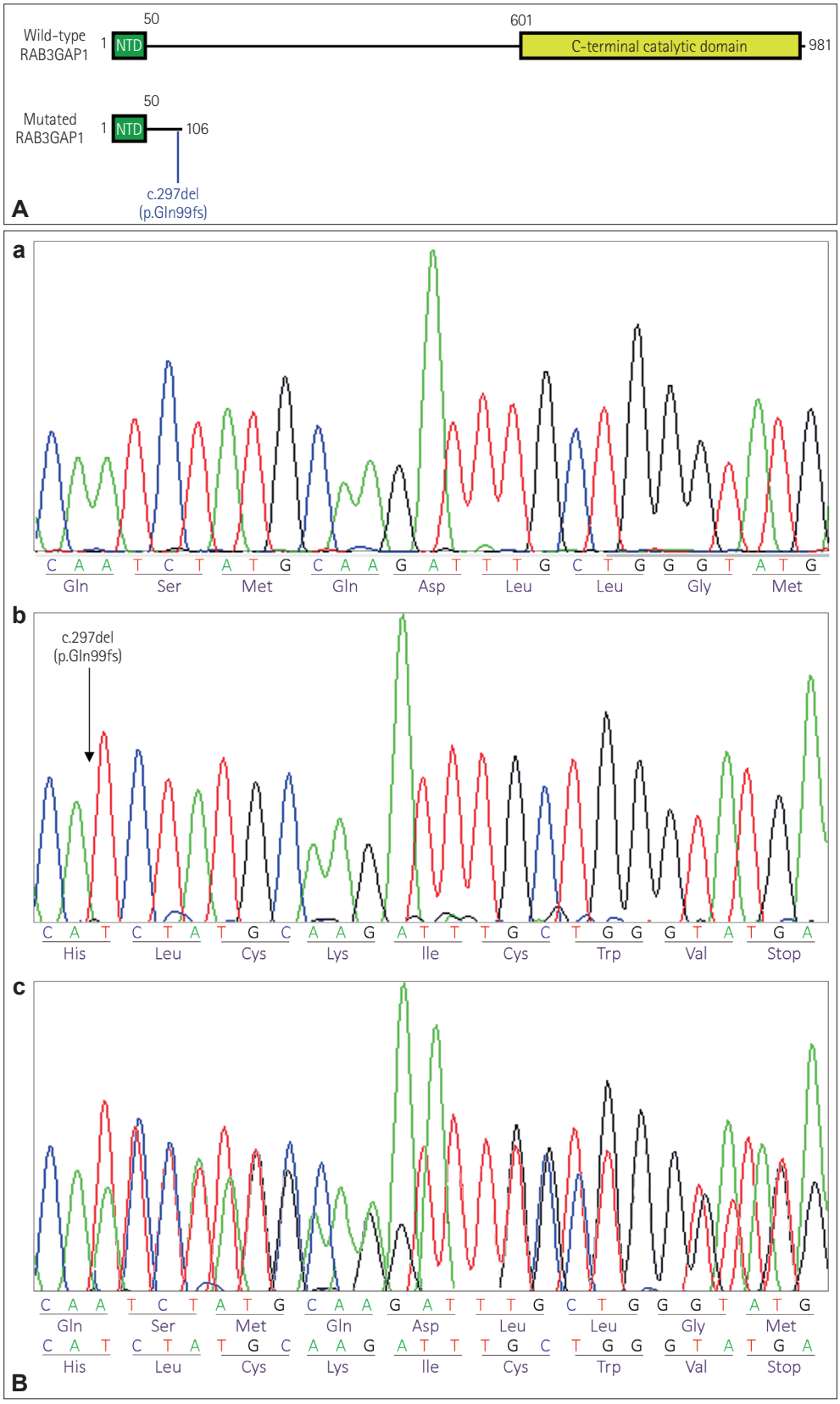

Fig. 3. Illustration of RAB3GAP1 protein (wild type and mutated) and Sanger sequencing validation of the *RAB3GAP1* mutation. A: Wild-type and mutated RAB3GAP1 protein. B: Sanger sequencing validation of the *RAB3GAP1* mutation. (a) Wild-type electropherogram. (b) Electropherogram of the two affected brothers. (c) Electropherogram of the parents and the unaffected son.

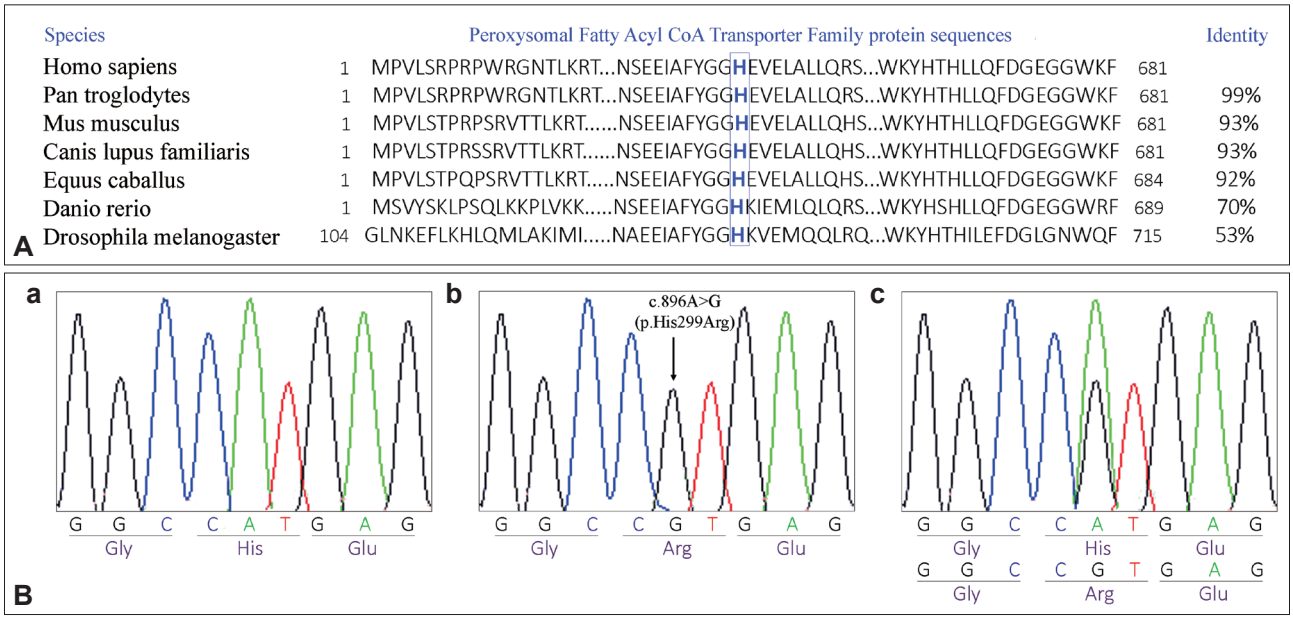

Fig. 4. Protein alignment of ABCD1 orthologs and Sanger sequencing validation of the ABCD1 mutation. A: Protein alignment of seven ABCD1 orthologs showing conservation of histidine (in blue) from human to fruit fly. B: Sanger sequencing validation of the *ABCD1* mutation. (a) Wild-type electropherogram. (b) Electropherogram of the two affected brothers. (c) Electropherogram of the mother.

of the following cerebral anomalies: cerebral atrophy, polymicrogyria, pachygyria, enlarged sylvian fissures, cerebellar hypoplasia, and abnormal myelination.[1,25] In our patient, cranial MRI showed only a thin corpus callosum without a splenium.

Molecular analysis of the two affected children using WES revealed a new frameshift mutation in *RAB3GAP1* (c.297del, p.Gln99fs), which created an early stop codon that resulted in a truncated protein without its C-terminal catalytic domain. Indeed, most *RAB3GAP1* mutations are predicted to produce a truncated protein either before or within the C-terminal catalytic domain, resulting in a mutant protein without GAP activity.[5,17,71] Inactivation of the RAB3GAP1 GTPase results in the accumulation of unlipidated LC3-I (microtubule-associated protein light chain 3−form I), impaired lipidation of Atg8 (autophagy-related protein 8) family members, and reduced SQSTM1 (sequestosome 1) protein levels, causing a disturbed autophagosome formation that influences the WARBM phenotype.[26,72,73] Patients with truncated proteins before the catalytic domain show normal growth, postnatal microcephaly, severe intellectual deficiency and developmental delay, optic atrophy, spastic cerebral palsy, hypotonia, cerebral anomalies (hypogenesis of the corpus callosum with agenesis of the splenium, and decreased myelination of white matter), ocular anomalies (congenital cataracts, microphthalmia, and microcornia), and genital anomalies (micropenis, bifid scrotum, and cryptorchidism).[5,17] These clinical features differ slightly from those found in our patients, confirming the clinical heterogeneity of WARBM.

The exome analysis also revealed another mutation (c.896A >G, p.His299Arg) in *ABCD1* (MIM *300371), which was previously described in the ClinVar archive as a variation of uncertain significance (variation ID: VCV000834266) associated with X-linked adrenoleukodystrophy disorder (MIM #300100). This disease, which affects the nervous system and the adrenal glands, can appear at an advanced age.[74,75] Since our patients were still young, clinical monitoring was necessary to diagnose this disease.

Supplementary Materials

The online-only Data Supplement is available with this article at https://doi.org/10.3988/jcn.2022.18.2.214.

Availability of Data and Material

The datasets generated or analyzed during the study are available from the corresponding author on reasonable request.

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Conflicts of Interest

The authors have no potential conflicts of interest to disclose.

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None

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