Novel, homozygous *RAB3GAP1* c.2606 + 1G>A, p.Glu830ValfsTer9 variant and chromosome 3q29 duplication in a Turkish individual with Warburg micro syndrome

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Warburg micro syndrome (WARBM) is a rare, autosomal recessive, neurodevelopmental disorder characterized by microcephaly, cortical dysplasia, corpus callosum hypoplasia, congenital hypotonia leading to subsequent spastic quadriplegia, severe developmental delay and hypogenitalism. Ophthalmologic findings that may affect any ocular segment including characteristic, small, atonic pupils. WARBM is known to be caused by biallelic, pathogenic variants in at least five genes although additional genetic loci may exist. The *RAB3GAP1* c.748+1G>A, p.Asp250CysfsTer24 founder variant has been described in families of Turkish ancestry. We report the clinical and molecular findings in three, unrelated, Turkish families with WARBM. A novel c.974–2A>G variant causing WARBM in three siblings of Turkish descent was found. Functional studies of the novel, c.2606+1G>A variant in patients' mRNA revealed skipping of exon 22 which results in a premature stop codon in exon 23. However, the clinical consequences of this variant are blended given that the individual also had a maternally inherited chromosome 3q29 microduplication. ***Clin Dysmorphol*** **32: 55–61 Copyright © 2023 Wolters Kluwer Health, Inc. All rights reserved.**

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Introduction

Warburg micro syndrome (WARBM) is a rare, autosomal recessive, neurodevelopmental disorder first described by Warburg *et al*. (1993). Cardinal features are postnatal, progressive microcephaly, brain abnormalities including cortical dysplasia, corpus callosum hypoplasia, severe, global developmental delay and intellectual disability, congenital hypotonia with subsequent spastic quadriplegia and hypogonadism. Eye anomalies include microphthalmia, microcornea, congenital cataracts and optic atrophy. Dysmorphic features are deep-set eyes, ptosis, short- and up-slanting palpebral fissures, epicanthal folds, wide nasal bridge, prominent root of the nose, large ears, highly arched palate, small mouth, retro-micrognathia and facial hypertrichosis (Morris-Rosendahl *et al*., 2010).

The condition is genetically heterogeneous and is caused by pathogenic variants in at least five genes, all of which result in RAB18 deficiency (Handley and Sheridan, 2018). RAB3GAP regulates RAB3 activity and forms a heterodimeric complex with a 130-kD catalytic subunit which is encoded by *RAB3GAP1*, and a 150-kD noncatalytic subunit which is encoded by *RAB3GAP2*. Members of the RAB3 protein family regulate exocytosis of neurotransmitters and hormones (Aligianis *et al*., 2005). RAB3GAP is shown to affect the synaptic plasticity of hippocampal neurons and neuromuscular junctions (Muller *et al*., 2011). Previously reported pathogenic variants in these genes include splicing, frameshift and nonsense changes, suggesting that WARBM results from loss-of-protein-function as well as nonsense-mediated mRNA decay (NMD) (Handley *et al*., 2013).

We report the clinical features of three unrelated Turkish families with *RAB3GAP1* variants. The first family had a novel homozygous c.974–2A>G variant in three affected sisters. The second family had a homozygous c.748+1G>A founder variant. The third family had a novel homozygous c.2606+1G>A variant. Functional studies of c.748+1G>A and c.2606+1G>A variants in patients' mRNAs revealed skipping of exon 8 and *22*, respectively, which were confirmed by Sanger sequencing. Skipping of exon *22* resulted in premature stop codon (PSC) in exon 23. This patient also had microduplication of 3q29 identified by array comparative genomic hybridization (aCGH) inherited from a healthy mother.

Case reports

Informed written consent was obtained from the parents and included in the study.

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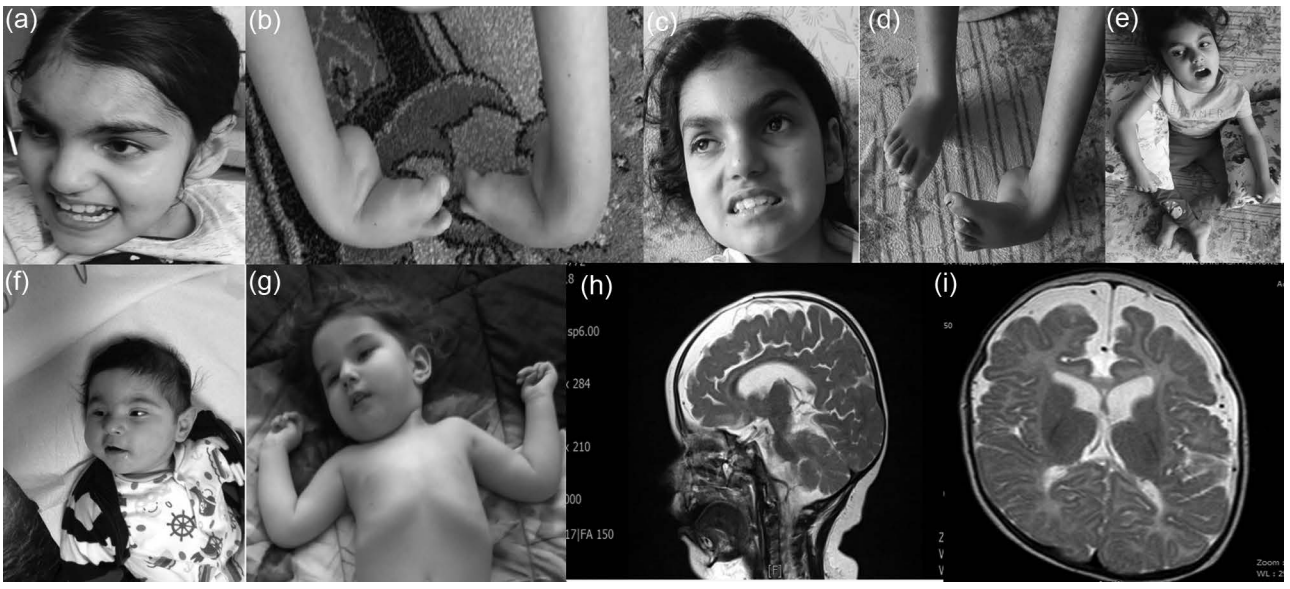
Family 1: Cases 1, 2 and 3

An 8-year-old girl was referred because of microphthalmia, congenital cataracts and severe intellectual disability. She was the first child of consanguineous parents and had two younger sisters with overlapping clinical presentations. She was born via spontaneous vaginal delivery at 38 s weeks of gestation. Her birth records were not available. She had been operated on for congenital cataracts. At the time of physical examination, her weight was 10 kg (−6.88 SD), height was 60.5 cm (−12.44 SD) and head circumference was 46 cm (−4 SD). She had a prominent, hirsute forehead, microphthalmia, deep-set eyes, ptosis, short- and up-slanting palpebral fissures, arched-thick eyebrows, prominent root of the nose, short philtrum, large, low-set ears, micrognathia, wide upper central incisors, overlapping toes, 4th toes clinodactyly and pes equinovarus. Flexion contractures of the knees were observed. She was unable to sit, walk or talk. Eye examination showed bilateral optic atrophy. Abdomen ultrasound and echocardiography were normal. Brain Evoked Response Auditory (BERA) had shown hearing impairment, but its report was not available and the test could not have been repeated. Brain MRI revealed diffuse cerebral atrophy and corpus callosum hypoplasia. Her two younger sisters (Cases 2 and 3) showed overlapping features (Fig. 1a–e).

Family 2: Case 4

A 6-month-old boy presented with hypotonia, microphthalmia, congenital cataracts, micropenis and cryptorchidism. He was the second child of second-cousin parents. His uncle and his grandmother's brother's two daughters had died due to congenital cataract, severe psychomotor retardation and epilepsy. He was born by cesarean section at term. His birth weight was 3000 g (−0.95 SD) and height was 50 cm (−0.06 SD). At the time of physical examination, his weight was 7.800 g (−0.28 SD), height was 64 cm (−1.43 SD) and head circumference was 41.5 cm (−1.72 SD). He was operated on for congenital cataracts at 2 months old. His physical examination showed a prominent forehead, depressed nasal bridge, deep-set eyes, ptosis, short- and up-slanting palpebral fissures, thick eyebrows, microphthalmia, V-shaped thin upper lip, micrognathia, long philtrum, low-set and large ears, hammertoes, hypoplastic nails, micropenis, hypospadias and bilateral cryptorchidism (Fig. 1f). Laboratory investigations (complete blood count, metabolic tests including tandem mass spectrometry, plasma amino acids, urine organic acids) were all normal. His hormone profile was follicle-stimulating hormone 0.43 mIU/ml, LH < 0.2 mIU/ml, E2 < 5 pg/ml, total testosterone < 0 07 ng/ml. Abdomen ultrasound, echocardiography and BERA were also normal. Eye examination evidenced glaucoma and optic atrophy. Brain MRI scan revealed diffuse cerebral atrophy

Fig. 1



(a) Case 1 showing a prominent forehead and hypertrichosis, microphthalmia, deep-set eyes, ptosis, short- and up-slanting palpebral fissures, arched-thick eyebrows, prominent root of nose, short philtrum, large anterior teeth. (b) Overlapping toes, pes equinovarus. (c) Case 2 younger female sibling of case 1 with similar dysmorphic features. (d) Overlapping toes, 4th toes clinodactyly, pes equinovarus. (e) Case 3 youngest female sibling of case 1 showing similar dysmorphic features, contractures of knees and hammer toes. (f) Case 4 at 4 months with a prominent forehead, deep-set eyes, ptosis, short- and up-slanting palpebral fissures, microphthalmia and micrognathia. (g) Case 5 with a prominent forehead, prominent root of nose, short neck, deep-set eyes, short- and up-slanting palpebral fissures, thick eyebrows, microphthalmia and ptosis, long eyelashes, micrognathia, V-shaped thin upper lip, long philtrum and large low-set ears. (h) Cranial MRI showing thin corpus callosum. (i) Enlarged ventricles, cortical and white matter atrophy predominantly on the frontotemporal regions.

predominantly of the frontotemporal regions, enlarged lateral and third ventricles and hypoplasia of corpus callosum. At the age of 3 years, his weight was 10 kg (–3.7 SD), height was 90 cm (–2.14 SD) and head circumference was 42.5 cm (–5.14 SD). He did not achieve sitting, speaking, walking or self-feeding.

Family 3: Case 5

A 3-year-old girl presented with hypotonia, dysmorphic features and congenital cataracts. She was the second child born to healthy, nonconsanguineous, 23-year-old mother and a 27-year-old father. She was born following an uncomplicated pregnancy by cesarean section at term. Her birth weight was 3700 g (+0.94 SD), height was 52 cm (+1.22 SD), head circumference was 35 cm (+0.36 SD). She was hospitalized for 18 days because of jaundice and difficulty in sucking and swallowing. She was operated for congenital cataracts at 40 days of age. She had feeding difficulties including gastrointestinal reflux and frequent urinary tract infections. At 4 months without any symptoms of epilepsy, she was started on valproate (250 mg) because of abnormal electroencephalogram (EEG) showing diffuse slowing of the background activity without epileptic activity. At 21 months, her head circumference was 44 cm (–2.61 SD); she had axial hypotonia and spastic diplegia. She was unable to hold her head, sit, walk or talk and did not make eye contact. Her weight was 12 kg (–1.35 SD), height was 90 cm (–1.35 SD) and head circumference was 48.5 cm (–0.13 SD). At 3 years and 10 months of age, she was diagnosed with focal epilepsy. At 4 years of age, her head circumference was 49 cm (–0.76 SD). On examination, she had a number of dysmorphic features including a prominent forehead, prominent root of nose, short neck, deep-set eyes, short- and up-slanting palpebral fissures, thick eyebrows, microphthalmia and ptosis, long eyelashes, highly arched palate, wide upper central incisors, micrognathia, V-shaped, thin, upper lip, long philtrum, large, low-set ears, prominent fingertip pads of third to fourth fingers, hammer toes, overlapping toes, fourth toes clinodactyly and sacral dimple (Fig. 1g). Eye examination showed bilateral optic atrophy. Biochemical, amino acids and organic acid tests were normal. Echocardiography, abdominal ultrasonography, skeletal survey, BERA and electromyography were all normal. Brain MRI revealed diffuse cerebral atrophy predominantly of the frontotemporal regions, enlarged ventricles and corpus callosum hypoplasia (Fig. 1h and i). Before 1 year of age, an EEG showed diffuse slowing of the background activity without epileptic activity. By 3 years and 10 months of age, the EEG was abnormal with bilateral, parietal and epileptic wave activity. She passed away at the age of 5 because of infection and sepsis.

Genetic investigations

DNA sequencing analysis

RAB3GAP1 sequencing analyses were performed for the patients. Genomic DNA was isolated from peripheral blood leucocytes using a QIAamp DNA Blood Mini QIAcube Kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's protocols. Sequencing was performed for all the 24 coding exons and their splice sites of *RAB3GAP1* which was carried out by PCR using custom-design primers. PCR amplicons were visualized by electrophoresis on a 2% agarose gel. For case 1, Sanger sequencing was performed using BigDye V1.1 (Life Technologies, California, USA) and capillary electrophoresis on 3130XL sequencer (Applied Biosystems, Massachusetts, USA). The sequencing data were analyzed using the ENSEMBL sequence ENST00000264158 as reference. For cases 4 and 5, all PCR products were sequenced via Miseq next-generation sequencing (NGS) platform (Illumina Inc., San Diego, California, USA). The libraries were prepared with a NexteraXT kit (Illumina Inc.), following the manufacturer's instructions. Alignment and variant detection were performed by Pepper, a proprietary basic algorithm of Sophia Genetics, according to the human genome reference hg19. The raw data were visualized with Integrative Genomics Viewer 2.4 (Broad Institute, California, USA) software. In order to evaluate the pathogenicity of the novel variants, we used in-silico prediction tools, mutation databases (Human Gene Mutation Database and Clinvar), allele frequency in population studies (1000 Genome, Genome Aggregation Database) and the American College of Medical Genetics and Genomics genetic variant classification criteria (Richards *et al.*, 2015).

Mutation analysis of the cDNA

To determine the effect of the identified splice site substitution on cDNA structure, total mRNA was extracted from peripheral blood in patients and their parents using the Qiagen RNeasy Plus Mini Kit (Qiagen GmbH) and reverse-transcribed using the QuantiTect Reverse Transcription Kit (Qiagen GmbH) following the manufacturer's protocol. For patient 4 following primer pair was designed in exons 7 and 9 of *RAB3GAP1*; 5′-TCAGGTGCCACTCTTTGTGC-3′ and 5′-CTAATAGGATCTTCGCAGGCACC-3′, and for patient 5 following primer pair was designed in exon 20 and 23; 5′-GCTGTACTCAAGGTAAAGGAAG-3′ and 5′-CTCTGGGCATTCACAAACAG-3′. Amplified products were analyzed via agarose gel electrophoresis and Sanger sequencing.

Array comparative genomic hybridization analysis

aCGH was completed using the whole-genome Affymetrix Cytoscan Human CGH Microarray 300K, SNP microarray. The platform-specific software copy number variation (CNV) call sets for the CytoScan array were obtained using the ChAS software (Affymetrix Inc., Santa Clara, California, USA). All CNVs were called and based on human assembly GRCh37 (hg19). Results were evaluated using current databases (Pubmed, Database of Genomic Variants, DECIPHER).

Results

DNA sequence analysis results

In cases 1, 2 and 3 Sanger sequencing revealed a homozygous splice-site variant (NM_012233.3: c.974–2A>G) in intron 11 of the *RAB3GAP1* gene. Her parents were heterozygous carriers for this variant (Fig. 2b and c). To the best of our knowledge, this variant has not been previously reported in the literature or in public databases including Human Genome Mutation Database, ClinVar, 1000 Genomes, Genome Aggregation Database and Exome Variant Server. It is reported as disease-causing by in-silico analysis (Mutation taster, Human Splicing Finder).

In case 4, NGS analysis revealed a homozygous, splice-site variant (NM_012233.3: c.748+1G>A) in intron 8 of the *RAB3GAP1* gene. The parents were heterozygous for this variant (Fig. 2d).

In case 5, NGS revealed a homozygous splice-site variant (NM_012233.3: c.2606+1G>A) in intron 22 of the *RAB3GAP1* gene (Fig. 2e). Sequencing analysis of the parents indicated that they are heterozygous carriers for this variant (Fig. 2f).

cDNA analysis results

In case 4, agarose gel electrophoresis of the cDNA samples reverse transcribed from RNA demonstrated that a mutated band (248 bp) was detected, a mutated band (248 bp) and a wild-type band (348 bp) were observed in his parents. Sequence analysis of the cDNA samples demonstrated that exon 8 is skipped in patients' mRNA resulting in the fusion of exon 7 to exon 9 and leading to a frameshift (Fig. 3a–c).

In case 5, agarose gel electrophoresis of the cDNA sample reverse transcribed from RNA demonstrated that a mutated band (232 bp) was detected whereas wild-type bands (347 bp) were observed in the controls. Sequence analysis of the cDNA samples demonstrated that exon 22 is skipped in patients' mRNA resulting in the fusion of exon 21 to exon 23 and leading to a frameshift and PSC in exon 23 (Fig. 4a–c).

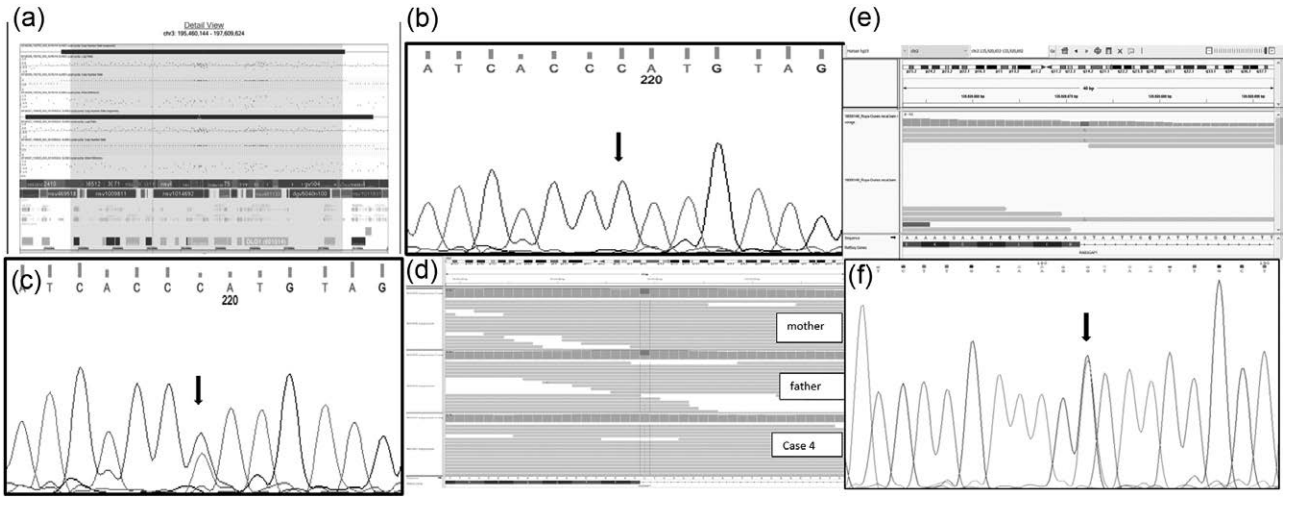
Array comparative genomic hybridization analysis result

In Case 5, 1.7 Mb microduplication 3q29 [arr(hg19) 3q29 (195 703 615–197 356 334)×3] was found. Her mothers' aCGH analysis showed 2.026 Mb microduplication 3q29 [arr(hg19) 3q29 (195 495 358–197 521 561)×3] (Fig. 2a).

Discussion

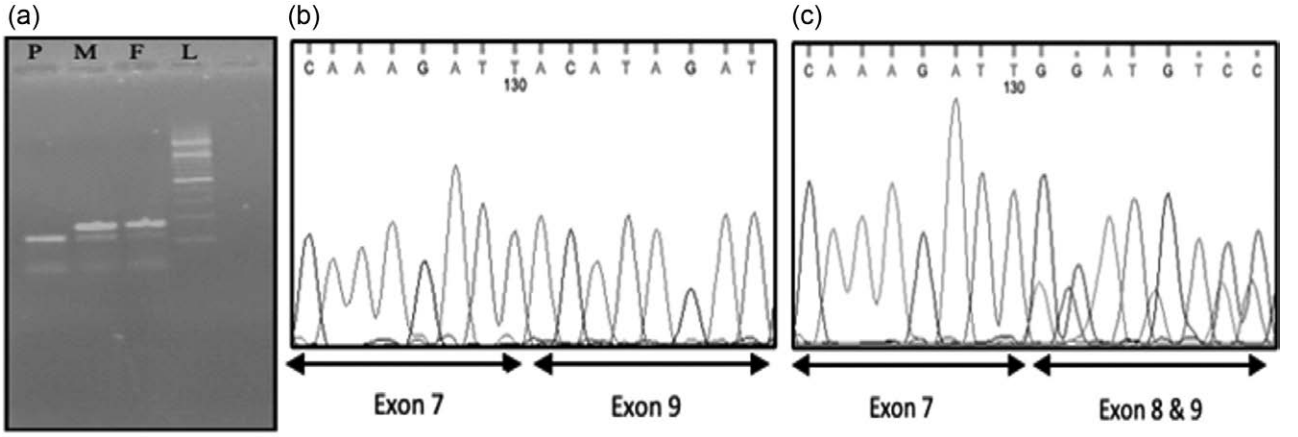
Our patients' clinical characteristics can be explained by the molecular confirmation of WARBM. Hearing impairment was present in Case 1. Her and her sisters' BERA results were not available. Although further genetic analysis of hearing loss was not investigated in our case, there was no family history of hearing impairment. Hearing is reported to be preserved in RAB18 deficiency patients (Handley *et al*., 2013; Mandarano *et al*., 2017). Hearing loss or impairment is an uncommon finding and has been reported in three patients with WARBM previously (Morris-Rosendahl *et al*., 2010; Imagawa *et al*., 2015).

Fig. 2



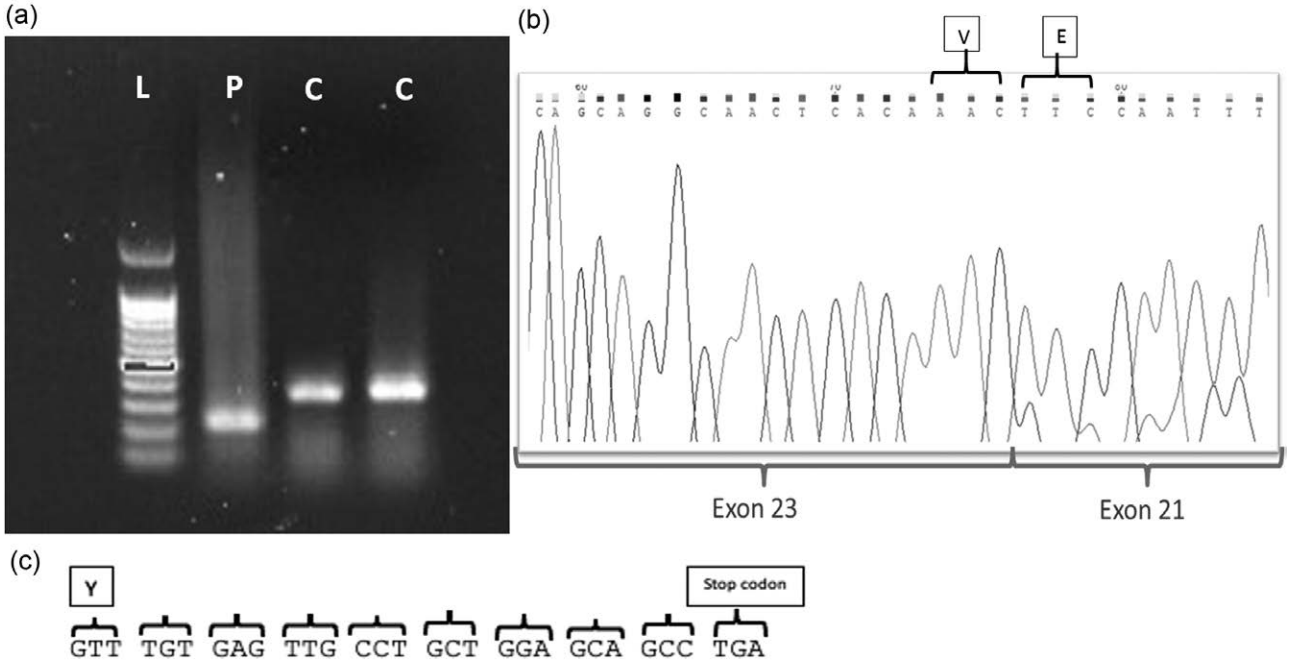
(a) aCGH analysis showed 1.7 Mb microduplication 3q29 (arr[hg19] 3q29(195 703 615–197 356 334)×3) mat. (b) RAB3GAP1 gene (NM_012233.3) c.974–2A>G homozygous mutation (reverse strand) in Cases 1, 2, and 3. (c) RAB3GAP1 gene c.974–2A>G heterozygous mutation (reverse strand) in the parents. (d) Integrative Genomics Viewer (IGV) sequence data of case 4 and his parents showing (NM_012233.3: c.748+1G>A) mutation. (e) IGV sequence data of case 5 and (f) (NM_012233.3: c.2606+1G>A) heterozygous mutation in case 5's parents. aCGH, array comparative genomic hybridization.

Fig. 3



(a) In cDNA analysis, a mutated band (248 bp) was detected in case 4, a mutated band (248 bp) and a wild-type band (348 bp) were observed in his parents. F, father; L, ladder; M, mother; P, patient. (b) Sanger sequencing showed exon 8 skipping in cDNA sequence of the patient. (c) Exons 8 and 9 were superposition in cDNA sequence of the parents.

Fig. 4



(a) In cDNA analysis, a mutated band (232 bp) was detected in case 5, whereas wild-type bands (347 bp) were observed in the controls. C, control; L, ladder; P, patient. (b) Sanger sequencing showed exon 22 skipping in cDNA sequence of the patient (reverse strand). (c) Frameship results in stop codon in exon 23.

RAB3GAP1 is located on chromosome 2 and has 24 exons. Until now about 200 variants have been reported in the ClinVar Database (https://www.ncbi.nlm.nih.gov/clinvar) involving *RAB3GAP1*. The majority of new *RAB3GAP1* variants reported are frameshift, splicing, or nonsense variations. In our three Turkish families, we found splicing variants. We found a novel homozygous c.974–2A>G splicing variant in family 1 of three affected siblings. Patients' mRNA studies were not available to show exon skipping.

In family 2, we found the previously described founder, homozygous c.748+1G>A, p.Asp250CysfsTer24 variant (Aligianis *et al.*, 2005; Yuksel *et al.*, 2007). cDNA samples

demonstrated that exon 8 is skipped in our patients' mRNA resulting in the fusion of exon 7 to exon 9 and leading to a frameshift.

In family 3, we found a homozygous c.2606 + 1G>A splice site variant in intron 22 (Geckinli *et al*., 2020; Albayrak *et al*., 2021). cDNA analysis of this patient showed that this variant causes skipping of exon 22 and fusion of exon 21 to exon 23 leading to a PSC in exon 23. We believe RAB3GAP1 protein function may be affected because of exon skipping and NMD. Koparir *et al.* reported a patient with homozygous c.2607–1G>C splice site variation in intron 22 in a Martsolf syndrome patient. Martsolf syndrome is characterized by a milder clinical phenotype than WARBM.

These patients have mild to severe intellectual disability and spasticity has a milder progression.

cDNA analysis showed that this variation caused the skipping of exon 23 and fusion of exon 22 to exon 24, which leads to a PSC in exon 24. Semi-quantitative reverse transcription PCR analysis of this patient showed that the RAB3GAP1 expression was significantly reduced in the peripheral blood sample (Koparir *et al*., 2019).

This individual was also found to have a chromosome 3q29 microduplication inherited from a healthy mother. Familial cases of 3q29 microduplication have been reported in the literature, with some cases with a mildly affected parent. The phenotype in these families reveals variable expressivity and reduced penetrance (Ballif *et al*., 2008; Goobie *et al*., 2008; Vitale *et al*., 2018).

3q29 microduplication syndrome (OMIM# 611936) presents with mild facial dysmorphisms and mild to moderate mental retardation. The clinical phenotype is heterogeneous and not specific, which variably includes hypotonia, generalized obesity, microcephaly, round face, bulbous nose, short- or down-slanting palpebral fissures, cleft palate, pes planus, conductive hearing loss and congenital heart disease (Ballif *et al*., 2008; Goobie *et al*., 2008; Blanquer *et al*., 2011; Quintela *et al*., 2015). In the majority of cases reported, the duplications ranged from 2.3 to 1.6 Mb, spanning from TFRC to BDH1 genes. Our patients' aCGH analysis showed 1.7 Mb microduplication of 3q29 [arr(hg19) 3q29(195 703 615–197 356 334) × 3] containing 18 OMIM genes: *TFRC* (OMIM# 190010), *SLC51A* (OMIM# 612084), *PCYT1A* (OMIM# 123695), *TCTEX1D2* (OMIM# 617353), *UBXN7* (OMIM# 616379), *RNF168* (OMIM#612688, *WDR53* (OMIM# 615110), *FBXO45* (OMIM# 609112), *NRROS* (OMIM# 615322), *CEP19* (OMIM# 615586), *PIGX* (OMIM# 610276), *PAK2* (OMIM# 605022), *SENP5* (OMIM# 612845), *NCBP2* (OMIM# 605133), *PIGZ* (OMIM# 611671), *MELTF* (OMIM# 155750), *DLG1* (OMIM# 601014), *BDH1* (OMIM# 60306). *PAK2*, *DLG1* (or *SAP97*) and *FBXO45* were implicated in mental retardation (Tada *et al*., 2010). It was reported that *TNK2* overexpression induces epilepsy in these patients (Hitomi *et al*., 2013). The chromosome duplication in this individual does not include the *TNK2* gene yet he has epilepsy. The wider availability of NGS showed that ~5% of individuals tested have more than one molecular diagnosis (Posey et al., 2017). Individuals with multiple genetic diagnoses can present with clinical features of either one or both conditions suggesting an apparent atypical or broader presentation of one of the conditions (Boycott and Innes, 2017; Posey et al., 2017). Given that the individual reported here with a double genetic diagnosis has severe, global, developmental delay and epilepsy, we believe that her clinical presentation, at the age of 3, is mostly compatible with WARBM.

In conclusion, we report a novel c.974–2A>G, p.Gly-324GlufsTer626 variant causing WARBM in three siblings of Turkish descent. The previously known founder c.748 + 1G>A variant was found in one family. In one family, we demonstrated c.2606 + 1G>A splice site variant resulting in the skipping of exon 22 and leading to a PSC in exon 23.

Acknowledgements

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Conflicts of interest

There are no conflicts of interest.

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