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Warburg micro syndrome type 1 associated with peripheral neuropathy and cardiomyopathy

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Abstract

The Warburg micro syndrome (WARBM) is a genetically heterogeneous syndrome linked to at least 4 loci. At the clinical level, WARBM is characterized by microcephaly, microphthalmia, microcornea, congenital cataracts, corpus callosum hypoplasia, severe mental retardation, and hypogonadism. In some families additional clinical features have been reported. The presence of uncommon clinical features (peripheral neuropathy, cardiomyopathy) may result in misdirected molecular diagnostics. Using the next generation sequencing approach (NGS), we were able to diagnose WARBM1 syndrome by detection of a new mutation within the RAB3GAP1 gene. We have detected some DNA variants which may be responsible for cardiomyopathy. We did not find any obvious pathogenic mutation within a set of genes known to be responsible for hereditary motor and sensory neuropathy (HMSN). We conclude that: (i) in clinically delineated syndromes, a classical single-gene oriented approach may be not conclusive especially in the presence of rare clinical features, (ii) peripheral neuropathy and cardiomyopathy are rare additional symptoms coexisting with WARBM1, (iii) a pleiotropic effect of a single point mutation is sufficient to be causative for WARBM1 and (iv) more WARBM-affected patients should be reported to delineate a complete phenotype.

Key words: *Warburg syndrome, whole-exome sequencing, RAB3GAP1.*

Introduction

The Warburg micro syndrome (WARBM) is a very rare genetic disorder with an unknown frequency reported in a small group of patients (less than 100) around the world.

For the first time in 1993, WARBM was reported in a consanguineous Pakistani marriage in three affected children with mental retardation [18]. In 2005, in turn, WARBM1 was linked to the 2q21.3 region, in which inactivating mutations within the *RAB3GAP1* gene were detected [2]. In recent years, access to molecular genetic analysis has made possible the delineation of the WARBM phenotype. WARBM manifests with microcephaly, microphthalmia, microcornea, congenital cataracts, *corpus callosum* hypoplasia, hypogonadism and severe mental retardation [6]. Two WARBM-affected sisters were also found to manifest peripheral neuropathy [11].

The vast majority of WARBM patients originate from consanguineous Muslim families [1,2,14].

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To date, four genes coding for RAB-family proteins have been reported to be mutated in WARBM syndrome, i.e. the *RAB3GAP1* gene on chromosome 2q21.3 (MIM# 602536), *RAB3GAP2* (MIM# 609275) on chromosome 1q41, the *RAB18* gene (MIM# 602207) located in the 10p12.1 region and finally the *TBC1D20* gene (MIM#611663) linked to the 20p13 *locus*. At the clinical level, WARBM1-4 subtypes are indistinguishable.

To date, the analysis of WARBM-involved genes has been directed by the presence of typical clinical features. In fact, in previous studies, only a single gene was analyzed and no data from the rest of the genome were available.

A question thus arises as to whether the whole clinical manifestation of WARBM is related to a single mutation in one of the 4 RAB genes.

In the work described here, we performed for the first time a whole-exome sequencing analysis in a Polish six-generational consanguineous WARBM pedigree.

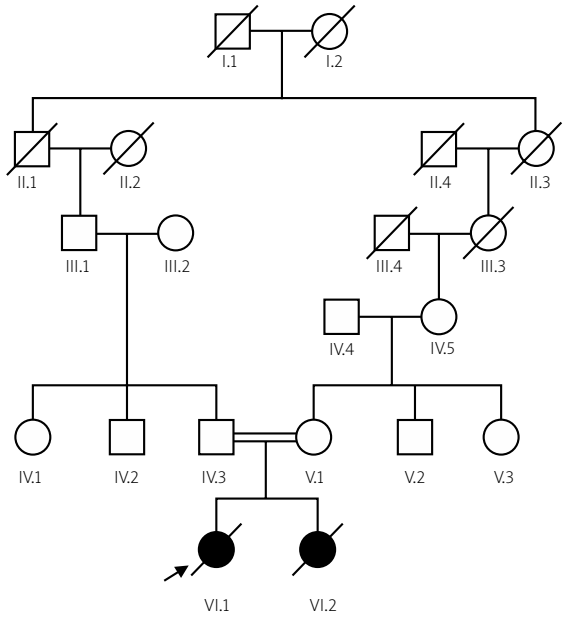

Fig. 1. Pedigree tree of the family. Note the common ancestor within the 1st generation in this consanguineous family tree. The proband is marked with an arrow. Open symbols indicate healthy males (squares) and females (circles). Filled symbols correspond to affected individuals. Deceased individuals are marked with diagonal lines. The double line indicates consanguinity in this family.

Material and methods

Case reports

The patients were two sisters who died in the second decade of life, being the only children of healthy, consanguineous (IV/V) parents (Fig. 1, Fig. 2A-C). They were born following uneventful pregnancies and deliveries. The older girl weighed 2950 g, her body length was 56 cm, OFC was 35 cm, and the Apgar score was 9 points. The younger girl weighed 2850 g, her body length was 54 cm, OFC was 32 cm, and the Apgar score was 10 points. Bilateral congenital cataract was apparent in both girls soon after birth, and a bilateral lentectomy was performed during their infancy.

The girls were severely hypotonic from early life, and their psychomotor development was delayed. At the age of a few months, infantile spasms were observed in the younger sister. Electroencephalography (EEG) showed hypsarrhythmia, so antiepileptic drugs were administered. Electroencephalography of the second sister was also severely abnormal, though no epileptic seizures were observed.

At the age of 2 years they presented with cerebellar ataxia. Limb and axial hypotonia were still observed, but a bilateral Babinski sign was also detected. Mild microcephaly was visible in both girls from early childhood.

Muscle biopsies performed at the ages of 1 and 5 years in the two sisters showed only unspecific changes in muscle fibers with mild lipid accumulation, and without any signs of myopathy or neurogenic changes (data not shown). A biochemical study of OXPHOS revealed slightly decreased activity of complex I, but blood and cerebrospinal fluid lactate were normal, both at baseline and following glucose challenge.

In the second half of the first decade of the patients' lives, mild spasticity developed, but tendon reflexes weakened progressively. Bilateral Babinski signs were still detected, and skeletal anomalies related to chronic muscle hypotonia also developed, in the form of kyphoscoliosis, thoracic deformity, and joint contractures (ulnar, iliofemoral and of the knees). Hands were dropping and planovalgus feet were to be noted. Ophthalmological examination revealed microphthalmia, microcornea and pale, atrophic optic discs. Psychomotor retardation was marked. The patients were never able to sit and walk independently. The younger girl still had epileptic seizures. The EEG of the second sister was also severely abnormal, but seizures were not observed.

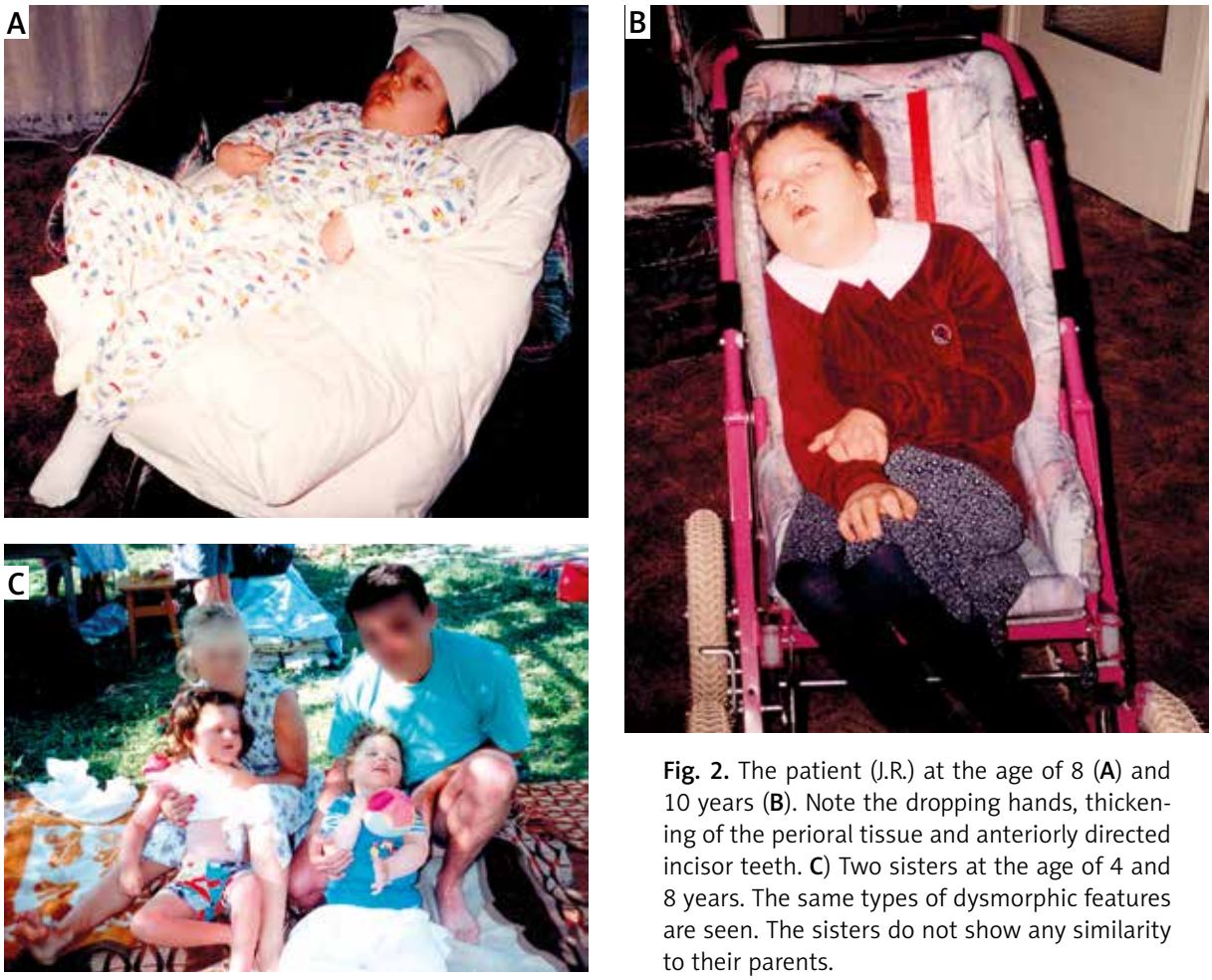

Fig. 2. The patient (J.R.) at the age of 8 (**A**) and 10 years (**B**). Note the dropping hands, thickening of the perioral tissue and anteriorly directed incisor teeth. **C**) Two sisters at the age of 4 and 8 years. The same types of dysmorphic features are seen. The sisters do not show any similarity to their parents.

Brain MRI showed slowly progressive cortico-subcortical cerebral and cerebellar atrophy as well as hypomyelination. The *corpus callosum* was thin and the brainstem was atrophic (Fig. 3). The Nerve Conduction Study (NCS) performed at 7 and 11 years, respectively, revealed progressive motor and sensory neuropathy, with demyelination and mildly expressed axonal degeneration. EMG showed neurogenic changes.

Both girls were of short stature, and deficient as regards body weight. The older girl was hypogonadal. The older girl had cardiomyopathy with left ventricular hypertrophy (148% of predicted muscle mass). Her arterial blood pressure was normal.

Molecular genetic analysis

This study was approved by the local Ethical Committee at the Cardinal Stephan Wyszynski University in Warsaw (3/2012 CSWUW). DNA isolated from the proband (VI.1) and her healthy parents (IV.3, V.1) was isolated from peripheral blood lymphocytes by the salting-out method, following receipt of the patients' informed consent.

PCR and DNA sequencing

Initially, when we suspected the diagnosis of congenital cataracts, facial dysmorphism and neuropathy (CCFDN), or Marinesco-Sjögren syndrome (MSS), we analysed the *CTDP1* and *SIL1* genes for mutations in the proband and her healthy parents. The 13 coding exons encompassing the intron-exon boundaries of the *CTDP1* gene were amplified using the polymerase chain reaction (PCR) with previously reported primers [19]. The PCR products from the proband and her parents were sequenced directly using a Dynamic terminator cycle sequencing kit (Applied Biosystems) on an ABI PRISM 3700 DNA analyzer (Applied Biosystems). To detect a common CCFDN-causing mutation, i.e. the IVS6+389C>T

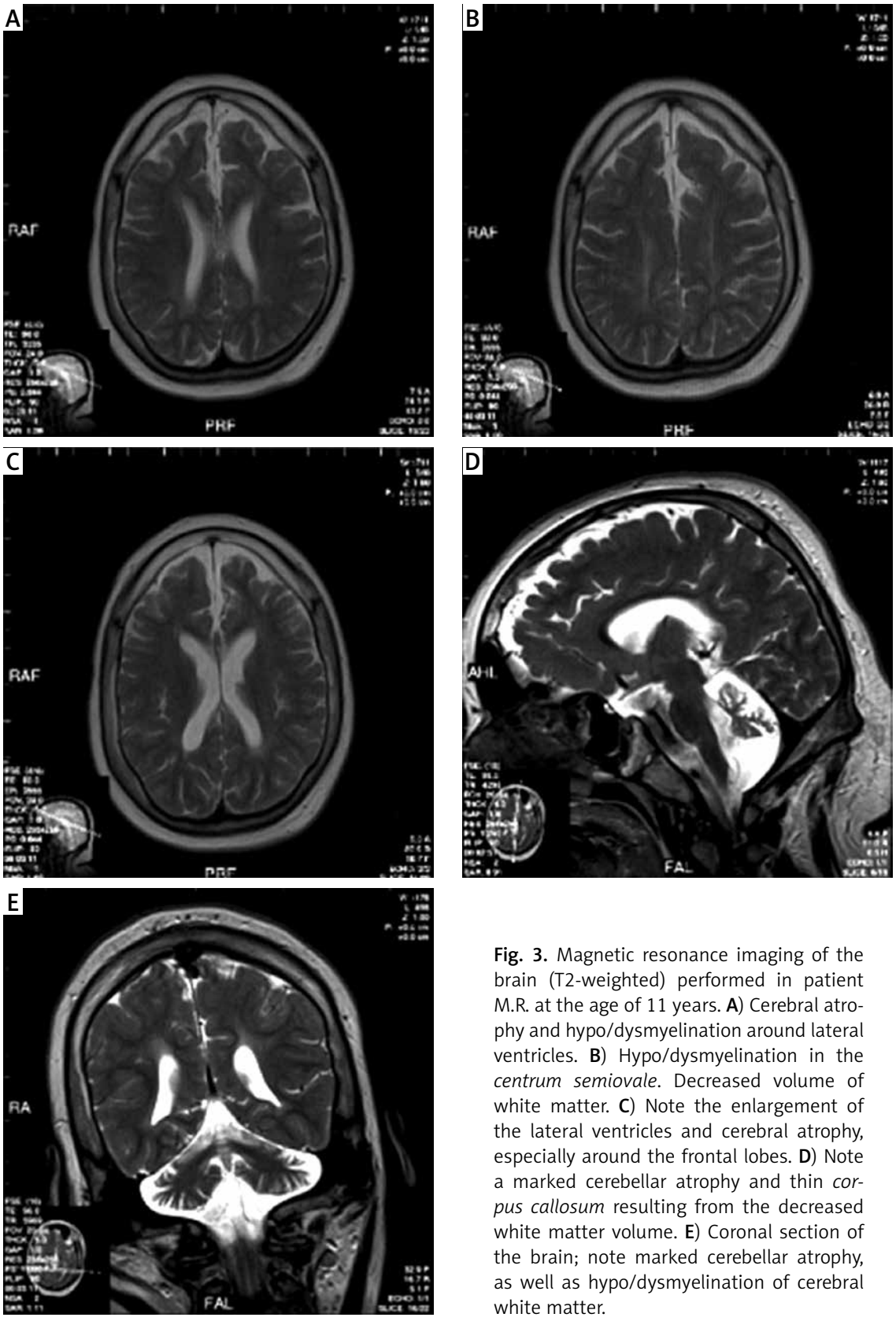

Fig. 3. Magnetic resonance imaging of the brain (T2-weighted) performed in patient M.R. at the age of 11 years. **A**) Cerebral atrophy and hypo/dysmyelination around lateral ventricles. **B**) Hypo/dysmyelination in the *centrum semiovale*. Decreased volume of white matter. **C**) Note the enlargement of the lateral ventricles and cerebral atrophy, especially around the frontal lobes. **D**) Note a marked cerebellar atrophy and thin *corpus callosum* resulting from the decreased white matter volume. **E**) Coronal section of the brain; note marked cerebellar atrophy, as well as hypo/dysmyelination of cerebral white matter.

mutation, a restriction analysis with *Nla* III endonuclease was performed using the forward primer: 5'-CACTGTGTTAGCCAGGATGG-3', and the reverse primer: 5'-GTGCCGTCTGACAGAGATGA-3'. The *SIL1* coding sequence was analysed by direct sequencing of the coding region.

Exome sequencing

Exome sequencing in the proband (VI.1) and parents (IV.3, V.1) was performed in line with the protocol from Illumina's TruSeq Exome Enrichment Guide. The qualified genomic DNA was fragmented by Covaris, ligated with adapters, purified, amplified and hybridized using a Sure Select Human All Exon 50 Mb Kit (Agilent Technologies), following the manufacturer's instructions. For enrichment, hybridized fragments were bound to the streptavidin beads, and non-hybridised fragments were washed out. The enriched library was then loaded on to a HiSeq 2000 instrument (Illumina). Exome sequencing was performed by Intelliseq sp. z o.o., Cracow.

Exome sequence data analysis

The sequence reads were analysed using the Illumina pipeline (adapter sequences were removed and low-quality reads discarded). Reads were processed by Picard, and aligned to a human reference sequence (GRCh37) using Bowtie2. BAM files were obtained for each sample using SAM tools, as well as by removing duplicate reads. SNP calling was then performed using GATK. To identify genes with recessive inheritance (the parents are consanguineous [IV/V]), we filtered data by genotype (the proband as a homozygous alternative and the parents as heterozygous, two other control probes being used as a homozygous reference), before dbSNP annotation was carried out and common variants filtered for. The SIFT tool was used in the functional and conservation prediction of SNPs.

Mutation confirmation by Sanger sequencing

The coding sequence with the intron/exon boundaries of the 7th exon of the *RAB3GAP1* gene was sequenced in family members by means of Sanger sequencing. Primers (Forward: 5'-CAGTTTGGTATTGTAAGGAGAAA-3' and Reverse: 5'-GCTAACAGACTGAACAAACAA-3') were constructed on the basis of the NG_01697.1 genomic and NM_001172435.1 RNA sequences.

Analysis of other genes

To confirm a monogenic basis of the disease in our family we analysed NGS data for all variants in three other genes involved in Warburg micro syndrome diseases, i.e. *RAB3GAP2* (NC_000001.11, NM_012414.3), *RAB18* (NC_000010.11, NM_021252.4) and *TBC1D20* (NC_000020.11, NM_144628.3).

Due to the occurrence of cardiomyopathy in one of the affected sisters, additional analysis of variants in 44 genes associated with cardiomyopathy was performed. Additionally, three genes essential in cardiac homeostasis and mitochondrial fusion (*MFN1* – mitofusin 1, *MFN2* – mitofusin 2 and *OPA1* – optic atrophy 1) were analysed.

We established a list of genes on the basis of articles by J.M. Bos *et al.* 2009, A. Huertas-Vazquea *et al.* 2013, E. Villard *et al.* 2011, K. Stark *et al.* 2010, K.N. Papanicolaou *et al.* 2012 and Y. Chen *et al.* 2011 [4,5,10,15,17,20].

Results

CTDP1 and SIL1 genes

The IVS6+389C>T mutation in the *CTDP1* gene was excluded by means of the RFLP analysis.

The sequencing of the entire *CTDP1* coding sequence (including exon-intron boundaries) revealed two sequence variants. In the proband and her mother, a heterozygous C>T transition at position 1019 resulting (by conceptual translation) in a Thr to Met amino-acid change at codon 340 (T340M) was identified in exon 7 of the *CTDP1* gene. The T340M substitution was not detected in the father of the proband. In the proband and her parents, a heterozygous transition G>A at position 2937 in the non-coding region was identified in exon 13 of the *CTDP1* gene. Analysis of the *SIL1* gene sequence did not reveal any DNA variant which could be considered a pathogenic mutation for Marinesco-Sjögren syndrome (MSS).

Whole-exome sequencing

Whole-exome analysis was performed in patient 1 as well as in her healthy mother and father. DNA from patient 2 was not available for the study. After filtering for common variants (> 1%) present in the dbSNP database, we identified 155 variants of poten-

tial importance. After filtering for nucleotide changes that potentially have a damaging effect on the protein and a high conservation ratio, we obtain only three variants among them, of which only one – c.538 G>T, p.E180X in the *RAB3GAP1* gene – is suitable for the phenotype occurring in patients. Sanger sequencing confirmed the homozygous novel mutation in the proband and a heterozygous one in their healthy parents. The remaining two homozygous variants that were found after filtering were:

– OR13C2 gene (olfactory receptor, family 13, subfamily C, member 2) rs143198170, NC_0009.12: g. 104605112_104605115delGTTA,NM_001004481.1: c. 513_516delTAAC, p. N171Kfs;
– CNOT1 (CCR4-NOT transcription complex, subunit 1): rs5817153, NC_00016.10: g. 58543412delA, NM_016284.4: c. 4434+195delT (isoform a), NM_206999.2 (isoform b): c. 4629delT, p. L1544Cfs, NM_001265612.1: c. 4419+195delT (isoform c).

Analysis of other Warburg syndrome genes

Analysis of other WARBM genes in the proband did not reveal any potentially pathogenic variants. But in the *GAP3RAB2* gene a very rare homozygous variant rs2289189 g.220157863C>G (G allele frequency 0.059), c.3275G>C with heterozygosity of 0.111 was found, this being described in the dbSNP database as a benign polymorphism.

Analysis of the genes involved in cardiomyopathy

The analysis of genes associated with cardiomyopathy revealed many SNPs, from among which only rs365990 (g.23392602A>G; c.3302T>C; p.V1101A) in the *MYH6* gene had been described previously as associated with a higher risk of cardiac conduction and rhythm disorders [4,9]. Moreover, a very rare heterozygous benign polymorphism in the *TTN* gene called rs55842557 (g.178528964G>A with allele A frequency of only 0.008, c.106787C>T, p.T35596I) was found. We identified that variant in the heterozygous state in the proband and her mother, while no mutation was identified in her father. In the *LDB3* gene, a new variant discovered was c.1318 T>C; p.S440P in VI.1, V.1 (heterozygous) and VI.3 (homozygous), but due to the homozygous variant in the healthy father this mutation probably is not associated with cardiomyopathy occurred in VI.1. A few missense benign polymorphisms, as well as numerous silent and intron mutations (not shown) were also found in other analysed genes.

Discussion

Surprisingly, even in the syndromes characterized by a peculiar morphological phenotype, molecular diagnostics may be hampered by the presence of rarely occurring symptoms. The peripheral demyelinating neuropathy and cardiomyopathy are not representative features of WARBM, however peripheral neuropathy was previously reported in two affected sisters harboring mutation within the *TBC1D20* gene [11].

Given the presence of peripheral neuropathy in our patients, we started molecular diagnostics from the syndromes in which peripheral neuropathy is a typical clinical feature.

At the beginning of our study we suspected a diagnosis of congenital cataract facial dysmorphism neuropathy (CCFDN), or Marinesco-Sjögren syndrome (MSS). In a classical phenotype-oriented gene analysis we have excluded the mutations within the *CTDP1* and *SIL1* genes that are respectively causative for CCFDN and MSS.

Due to a lack of conclusive results using the classical Sanger sequencing approach, we decided to perform whole-exome sequencing analysis (WES) in this family.

It is an open question as to whether *RAB3GAP1* mutations alone are sufficient to result in a complete phenotype of WARBM1 patients. To the best of our knowledge, the WES analysis was not widely performed to date in WARBM patients. Thus, the potential impact of other genes has not been analyzed so far. Thus, given the large family of RAB proteins and RAB modulating factors, a question arises in regard to the penetrance of the *RAB3GAP1* mutation. In fact, the patients detected by us manifested with a complete phenotype of WARBM1. In the exome analysis we found only one deleterious *RAB3GAP1* mutation; and no pathogenic mutations have been detected in other RAB and RAB-related genes. Thus, it is highly probable that a single point mutation within the *RAB3GAP1* gene is the one and only cause of WARBM1 disease. This is especially the case given that mutations in the *RAB3GAP1* gene associated with WARBM1 were described in families of very varied ethnic origin, with the number of mutations identified already exceeding 50 [14]. The clinical picture of the WARBM1 in our patients

corresponds with the phenotype described by other authors [7]. However, not typically, in our patients peripheral demyelinating motor-sensory polyneuropathy and cardiomyopathy were identified.

Interestingly, in a series of WARBM individuals with *TBC1D20* mutations, only two sisters were found to have demyelinating polyneuropathy [11]. The occurrence of peripheral demyelinating neuropathy within WARBM-affected patients is hard to estimate, since only in some have electromyographic investigations been reported.

In the whole-exome approach used in this study, we did not find any obvious deleterious mutations in the genes involved in the molecular pathogenesis of hereditary motor and sensory neuropathies (HMSN). Thus, due to a lack of mutations within known HMSN genes, we tend to consider peripheral neuropathy rather as a part of the WARBM1 phenotype than an additional clinical feature associated with a mutation of another gene (overlapping syndrome).

Cerebral and cerebellar atrophy, as well as hypoplasia of the *corpus callosum* seem to be hallmarks of WARBM [3,6,13]. In our patients too, it was possible to observe cerebral and cerebellar atrophy with hypoplasia of the *corpus callosum*. The former sign is a typical finding connected with, and resulting from, hypomyelination. The patients reported by us died in the second decade of life. The nonsense E180X mutation in the *RAB3/GAP1* mutation may be at least partially responsible for the poor clinical outcome in these patients.

In one of our patients, cardiomyopathy with left ventricular hypertrophy was detected. Since early-onset hypertrophic cardiomyopathy is often associated with the mutation of one of the sarcomere genes, we decided to screen the most often mutated genes using an exome approach. We did not detect any pathogenic mutation for the hypertrophic cardiomyopathy. It only proved possible to detect one heterozygous benign polymorphism – rs365990 in the *MYH6* gene – this being previously described as associated with a high risk of cardiac conduction and rhythm disorders [4,8,9]; as well as the heterozygous rare polymorphism rs55842557 in the *TTN* gene. Moreover, polymorphism c.1924-851A>G (rs6730157) in the *RAB3GAP1* gene has been recently described as being associated with effects on total cholesterol and high density lipoprotein cholesterol levels, and with an increased risk of sudden cardiac death [12].

For this reason, it is not definitively possible to preclude hypertrophic cardiomyopathy belonging to the WARBM syndrome phenotype.

Recently, in the consanguineous family with Kurdish-Armenian descent a large deletion encompassing exons 4-15 of the *RAB3GAP1* gene has been found in the siblings with the WARBM1 syndrome. Interestingly, in this pair of patients a mild growth hormone deficiency was detected, and X-ray examination revealed severe osteopenia, which have been not detected to date in WARBM [16]. For molecular diagnostic purposes, the lack or presence of some atypical symptoms should not preclude the molecular analysis of the *RAB* genes. Moreover, due to a limited number of reported patients, the frequency of WARBM-associated symptoms could not be estimated. Noteworthy, clinical variability of WARBM in various age-groups of patients is also unknown. Thus, a minimal set of symptoms which are necessary for WARBM diagnosis and decision concerning molecular diagnostics may be misleading.

In fact, the number of WARBM reported patients is still too low for reliable phenotype-genotype correlations to be derived.

To summarize, the patients reported by us manifest with a severe clinical form of WARBM1 associated with peripheral demyelinating neuropathy and cardiomyopathy.

Due to the unique structure of the family tree in this study (a recessive trait of inheritance and a detected common ancestor in the 6th generation), we were able to prioritize the filtering process in the interpretation of the exome data. The exome analysis identified only three probably important homozygous sequence variants in the proband. Additionally, only one sequence variant was detected in the *RAB-3GAP1* gene whose mutations had been shown previously to segregate with the phenotype of WARBM. Finally, by means of conceptual translation, the identified sequence variant within the *RAB3GAP1* gene resulted in a homozygous nonsense E180X mutation truncating the RAB3GAP1 protein. Our study confirms the usefulness of WES analysis in the pedigrees with an evident recessive trait of inheritance and evidence of the common ancestor in the family.

Because of the non-specificity of signs and symptoms of many disorders, molecular genetics is indispensable in establishing a specific diagnosis. In fact, exome analysis seems to be very serviceable in cases of unknown background. Whole-exome sequenc-

ing in our family gives a chance for the battery of biochemical, imaging and other tests to be omitted. In fact, exome analysis is a cost- and time-effective approach. Most importantly, it also reduces the discomfort of affected patients associated with a long diagnostic process.

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Disclosure

Authors report no conflict of interest.

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