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RESEARCH ARTICLE

Clinical and genetic analyses in syndromic intellectual disability with primary microcephaly reveal biallelic and de novo variants in patients with parental consanguinity

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Abstract

Background Syndromic intellectual disability (ID) with accompanying primary microcephaly is a group of rare neurodevelopmental disorders exhibiting extreme genetic and clinical heterogeneity. This layered heterogeneity can partially be resolved by unbiased genetic approaches targeting the genome with next generation sequencing (NGS) technologies, including exome sequencing (ES).

Objective This study was performed to dissect the clinical and genetic features in five distinct IDM cases.

Methods Singleton or trio ES approach followed by in-depth variant analysis using alternative inheritance models was performed.

Results We have identified biallelic loss of function variants in genes *WDR62* and *AP4M1* in three families, together with de novo missense variants in genes *SOX11* and *TRIO* in two families. ES based haplotype analysis in two cases upon identification of an identical *WDR62* variant in the homozygous state in two cases was suggestive of a small shared haplotype of 0.1 Mb. Additionally, we have shown a paternal origin for the de novo variant in *TRIO* via a polymorphic tag SNP, which enlightens the mutational mechanism for this variant.

Conclusion In populations with high parental consanguinity, an autosomal recessive inheritance pattern for data analysis is usually the most obvious choice. Therefore, heterozygous variants may be overlooked in standard NGS analyses in consanguineous families. Our findings underlie the importance of using multiple inheritance models in NGS data analysis.

Keywords Microcephaly · Intellectual disability · Exome sequencing (ES) · Parent of origin effect · *WDR62* · *AP4M1* · *TRIO* · *SOX11*

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Introduction

Neurodevelopmental disorders (NDD) is an umbrella term used for disorders affecting one or more aspects of cognition, communication, behavior and motor skills at a varying extend due to impaired brain development and function (Mullin et al. 2013). As a result, NDDs may lead to global developmental delay, intellectual disability (ID), autism spectrum disorder, attention-deficit/hyperactivity disorder and/or psychiatric conditions including schizophrenia and bipolar disorder (Morris-Rosendahl and Crocq 2020). Epilepsy is usually comorbid with NDDs (Chow et al. 2019). It has been estimated that up to 36% of all NDDs have a genetic origin (Srivastava et al. 2019). There is growing interest against monogenic origins of NDDs, where private pathogenic variations predominate as revealed via exome

sequencing (ES) (Reuter et al. 2017). Indeed, in 2019 ES was suggested as a first-tier clinical diagnostic test for individuals with NDDs (Srivastava et al. 2019).

Syndromic ID is a common subgroup of NDDs which may be accompanied by microcephaly (Ansar et al. 2019; Richard et al. 2019; Tan et al. 2020). Increasing incidence of Zika virus in recent years has drawn attention to primary microcephaly (PM) which is diagnosed during pregnancy of second trimester period or in newborns (Russell et al. 2016). PM is diagnosed with the measurement of occipitofrontal circumference (OFC) if it is less than the reference range determined by age and gender according to the world health organization. PM occurs by genetic defects; numerical chromosome aberrations, microdeletions/microduplications, monogenic and metabolic deficiencies; or by environmental causes like teratogen exposure and infections (Zaqout et al. 2017).

Herein, we aimed to investigate the clinical and genetic etiology of syndromic ID with primary microcephaly (IDpM) in 5 unrelated patients from Turkey via ES analysis. Singleton or trio ES followed by variant analysis using alternative inheritance models have led us identify biallelic loss of function variants in genes *WDR62* and *AP4M1* in three families, together with de novo missense variants in genes *SOX11* and *TRIO* in two families.

Materials and methods

Patients and clinical assessments

Comprehensive neurological examinations were performed for five cases with IDpM and their available family members at Spastic Children's Foundation of Turkey and/or at Istanbul University, Istanbul Faculty of Medicine, Department of Neurology. Review of their clinical information are presented on Table 1. Primary microcephaly, intellectual disability, hypotonia and developmental delay are core phenotypes of these cases. Informed consents were obtained for/from all family members recruited in this study in accordance with Istanbul University, Istanbul Faculty of Medicine, Clinical Ethics Committee (Reference number 2016/1231). DNA isolations were performed from whole blood using QIAamp DNA Blood Maxi kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol.

Exome sequencing and data filtering strategies

Research based singleton-ES for four cases (Case 1, Case 3, Case 4, Case 5) and trio-ES for one case (Case 2) were performed through the commercial service provided by Macrogen (Macrogen Inc.). Exonic DNA was captured with the SureSelect Human All Exon-without UTR V6Kit (Agilent Technologies, Santa Clara, CA) and sequenced on the Illumina's NovaSeq platform. Exome data analysis was performed using our in-house pipeline as previously explained (Akçakaya et al. 2019).

ES data filtering was performed using annotated variant call format (VCF) files on two levels: *Gene based*: A total of 25 (24 autosomal recessive and 1 autosomal dominant) primary microcephaly (MCPH) genes were retrieved from OMIM database considering the phenotype-genotype compatibility (Supplementary Table S1). *Variant prioritization*: Data was also analyzed for all variants using variant prioritization strategies including variant impact (truncating variants, missense and also synonymous variants) with different inheritance methods.

VCF filtered variants were first inspected manually by Integrative Genomics Viewer (IGV) and then Sanger sequenced using standard techniques if prioritized for validation and familial segregation analysis. Sanger sequence data was analyzed using CLC Main Workbench v6.5. (CLC Bio, Aarhus, Denmark). The MANE Select transcripts have been used for all variant annotations. Nucleotide and codon numbering refer to the cDNA sequence starting at the translation initiation site, with + 1 as the A of the ATG initiation codon.

Table 1 Review of clinical findings for five IDpM cases

Patients	Sex	Consan	Epilepsy	Seizure History	Hyperactivity and aggressive behavior	Spasticity	Ventricular asymmetry	Scoliosis	Strabismus	DF	Hypertelorism	Transverse palmar crease
Case 1	F	FdC	+	+	+	−	−	−	−	−	−	−
Case 2	F	SV	−	−	−	−	−	−	−	−	−	−
Case 3	F	SV	+	+	−	+	+	+	−	−	−	−
Case 4	M	FdC	−	+	−	−	−	−	+	+	+	+
Case 5	F	FdC	−	−	−	−	−	−	−	+	+	−

The core phenotypes common to all five cases, namely microcephaly, intellectual disability, developmental delay and hypotonia are not presented on the table

Consan. parental consanguinity, *DF* dysmorphic findings, *F* female, *FdC* parents are first degree cousins, *M* male, *SV* parents from same village

SNP array analysis for confirming parental status for de novo variants

DNAs of two parent offspring trios (trios for cases 4 and 5) were genotyped using Illumina Human OmniExpress-24 v1.1 BeadChip SNP array through the commercial service provided by Macrogen (Macrogen Inc.). Parent–offspring genetic data consistency for de novo variants was confirmed by whole genome trio SNP array genotyping followed by data analysis with our in-house approach (Ornek Erguzeloglu et al. 2020). Briefly, the method depends on filtering the trio data for informative SNPs (iSNPs), which are defined as SNPs that are alternatively homozygous in each parent. Accordingly, for each iSNP, the biological child of the trio should be an obligate heterozygous (Supplementary Table S2 and S3).

Genetic relatedness analysis from ES data

Relatedness analysis was performed using VCF files to ensure that the identical rare variant detected both in Case 1 and Case 2 were originated from two different individuals. The relationship between these two individuals was inferred using the 'relateness2' analysis of VCFtools utility (Manichaikul et al. 2010; Danecek et al. 2011). Two pairs of unrelated siblings (control sibling pairs A and B) and two sets of unrelated trios (control trios A and B) were added as controls to the calculation. VCF files are merged with bcftools and relatedness analysis performed with VCFtools from command-line interface. Relatedness plot is created using ggplot2 script.

Results

Autosomal recessive variants in *WDR62* and *AP4M1*

Gene based variant analysis revealed an identical homozygous and nonsense variation in two apparently unrelated cases (case 1 and 2) in gene *WDR62* (ENST00000401500.7:c.1605dup; p.Glu536Ter). Although absent from the population databases, this variant had interestingly been reported for a Turkish case again in the homozygous sate (Poulton et al. 2014). *WDR62* is associated with MCPH2 (Microcephaly 2, primary, autosomal recessive, with or without cortical malformations; MIM: 604317). The pathogenic variant has been confirmed in Case 1 and 2 along with their familial segregations via Sanger sequencing (Fig. 1a). As the variations in case 1 and 2 are exactly the same, we have performed VCF relatedness analysis to confirm that the data actually originate from two different VCF files. A *phi* score of 0.13 between case 1 and 2 supports that VCF files are unrelated (Supplementary Figure). Additionally, we have analyzed VCF files of case 1 and case2 for individual homozygosity and also haplotype sharing around the identified *WDR62* variant. The homozygosity region for case 1 was refined to chr19: 33.5–36.8 Mb (hg19), while to 36–39.2 Mb (hg19) for case 2, both encompassing gene *WDR62*. The VCF files of these two cases were separately filtered for rare variants with a global allele frequency ≤ 0.01 and identical variants around nonsense *WDR62* variant were investigated. This analysis revealed a small identical haplotype of minimum 0.1 Mb and in the homozygous state on chromosome 19 harboring this nonsense variant.

The parents of Case 3 who are from the same village have described a distant consanguinity at least 3 generations back. She was born with an eventful cesarean delivery after in vitro fertilization treatment. Variant prioritization strategy with recessive inheritance model revealed a stop codon variant in *AP4M1* (rs730882249: ENST00000359593.8:c.952C > T; p.(Arg318Ter), which had previously been associated with Spastic paraplegia 50, autosomal recessive (SPG50, MIM 612936) (Tüysüz et al. 2014). Segregation analysis was consistent with recessive inheritance of this variant in the pedigree (Fig. 1b).

De novo variants in *SOX11* and *TRIO*

Case 4 and 5 were negative both for gene based analysis and variant prioritization strategy with recessive inheritance model. We have therefore extended our analysis to possible heterozygous and de novo variants.

Filtering for rare heterozygous variants in case 4 revealed variants in *SOX11* and *DYRK1A*, which are both implicated in autosomal dominant mental retardation (MRD27: MIM615866 and MRD7: MIM614104). The *DYRK1A* gene variant rs751513164 was present in three and two gnomAD exome and genome alleles, respectively. We have therefore focused on *SOX11* variant (ENST00000322002.5:c.239C > T; p.Ser80Phe), which was not reported in GnomAD. The findings related to MRD27 (Coffin-Sirs Syndrome 9; CSS9) including delayed psychomotor development with mild hypotonia, coarse face, strabismus, short philtrum, low-set and posteriorly rotated simple ears, downs landing palpebral fissures, long eyelashes, brushy eyebrows, thin upper limp vermillion, small chin, transverse palmar crease and hypoplastic fifth toenails were also present our patient. Sanger segregation analysis was consistent with de novo heterozygosity of the variant in case 4 (Fig. 1c). iSNP analysis revealed familial consistency of 99.84% (Supplementary Table S2) confirming de novo inheritance.

A similar series of ES variant filtering approach for case 5 has identified another heterozygous and de novo variant in gene *TRIO* for Case 5 (ENST00000344204.9:c.4394A > G;

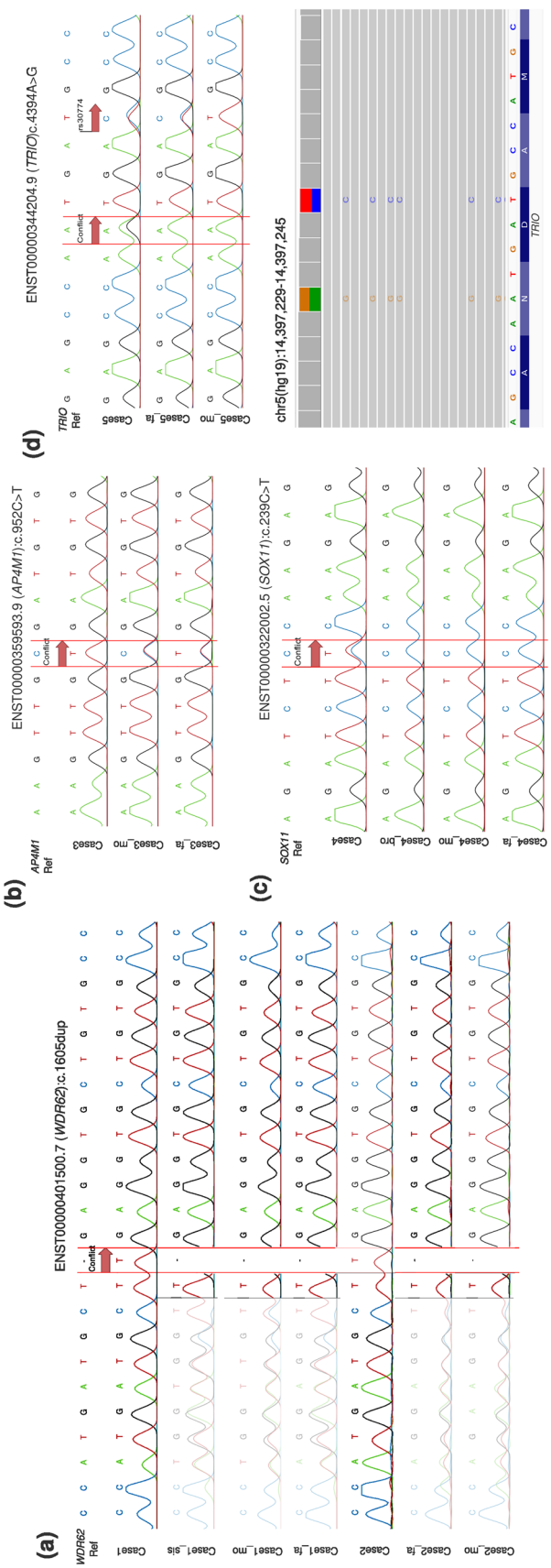


Fig. 1 Sanger sequencing based segregation analyses. **a** Segregation analyses for variants in **a** *WDR62*, **b** *AP4M1*, **c** *SOX11* and **d** *TRIO*. The IGV diagram illustrating the multiple reads neighboring two TRIO variants is also included as a lower panel in **d**. *Bro* brother, *Fa* father, *Mo* mother, *Sis* sister

Table 2 List of IDpM associated variants identified in this study

Patient ID	Gene	hg19 and hg38 positions	HGVS variant designation	RefSeq Match	Zyg	Exon	Freq	ACMG evidence	Class
Cases 1–2	*WDR62*	19:36,575,609dup 19:36,084,707dup	ENST00000401500.7:c.1605dupT; p.(Glu536Ter)	NM_001083961.2	Hom	12/32	NA	PVS1, PM2	LP
Case3	*AP4M1*	7: 99,703,604C > T 7:100,105,981C > T	ENST00000359593.9:c.952C > T; p.(Arg318Ter)	NM_004722.4	Hom	12/15	3.98E-06	PVS1, PM2, PP3, PP5	P
Case4	*SOX11*	2: 5,833,092C > T 2:5,692,960C > T	ENST00000322002.5:c.239C > T; p.(Ser80Phe)	NM_003108.4	Het DNV	1/1	NA	PM1, PM2, PP3, PS2	LP
Case5	*TRIO*	5: 14,397,234A > G 5:14,397,125A > G	ENST00000344204.9:c.4394A > G; p.(Asn1465Ser)	NM_007118.4	Het DNV	29/57	NA	PM1, PM2, PP3, PS2	LP

DNV de novo variant, *Freq* gnomAD global allele frequency, *LP* likely pathogenic, *Hom* homozygous, *Het* heterozygous, *NA* not available, *P* pathogenic, *Zyg* zygosity

p.Asn1465Ser) (Fig. 1d). *TRIO* is also associated with autosomal dominant intellectual disability (MRD44; MIM617061). This variant, which have not yet been incorporated into Ensembl database is archived in ClinVar (rs1747669042), where it has recently been implicated in MRD44 without compelling evidence. IGV inspection of the ES data neighboring this variant pinpointed a common SNP (rs30774) in the heterozygous state lying in *cis* orientation (Fig. 1d). rs30774 is evaluated as a tag SNP which suggests a paternal origin for the de novo variant. iSNP consistency for the trio was 99,83% (Supplemental Table S3).

As a result, we have detected four different variants in five families, which are all associated with their clinical findings. The variants are presented on Table 2, which are all classified according to the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG/AMP) criteria (Richards et al. 2015).

Discussion

IDs are in general characterized by limitations in intellectual and as a neurodevelopmental phenotype IDs have heterogeneous genetic and clinical backgrounds. ES is an invaluable technique with the power to detect pathogenic variants even in a single cases or in trios for NDDs (Vissers et al. 2016). Herein, via application of single or trio ES accompanied by supportive SNP array analysis has enlightened phenotype-genotype associations in five patients with syndromic ID, each having microcephaly as the striking clinical feature. Our first analysis step; gene based was also allowed to determine two recessive rare variants in *WDR62* and *AP4M1* gene. In addition, the identification of de novo variations (DNVs) in genes *SOX11* and *TRIO* should also be emphasized, which were detected in two patients both born to consanguineous parents. *DNVs* may be overlooked in consanguineous families, where homozygous variations are the usual suspects.

Our first finding was detection of identical homozygous *WDR62* variations (ENST00000401500.7:c.1605dup; p.Glu536Ter) in two apparently unrelated patients. *WDR62* is associated with autosomal recessive primary microcephaly (MCPH2; MIM604317), which can be accompanied by cortical malformations, delayed motor development, moderate to severe intellectual disability, behavioral problems, epilepsy, spasticity and ataxia (Verloes et al. 2022).

Although the *WDR62* variation in Case 1 and 2 are identical and homozygous, the patients slightly differ in their neurological features. Case 1 had drug-resistant epilepsy, hyperactivity and erratic behavior. Her brain MRI showed simplified gyral pattern and thin corpus callosum (Fig. 2a and b). Epilepsy and behavioral problems were not observed for Case 2. Relatedness analysis of Cases 1 and 2 did not

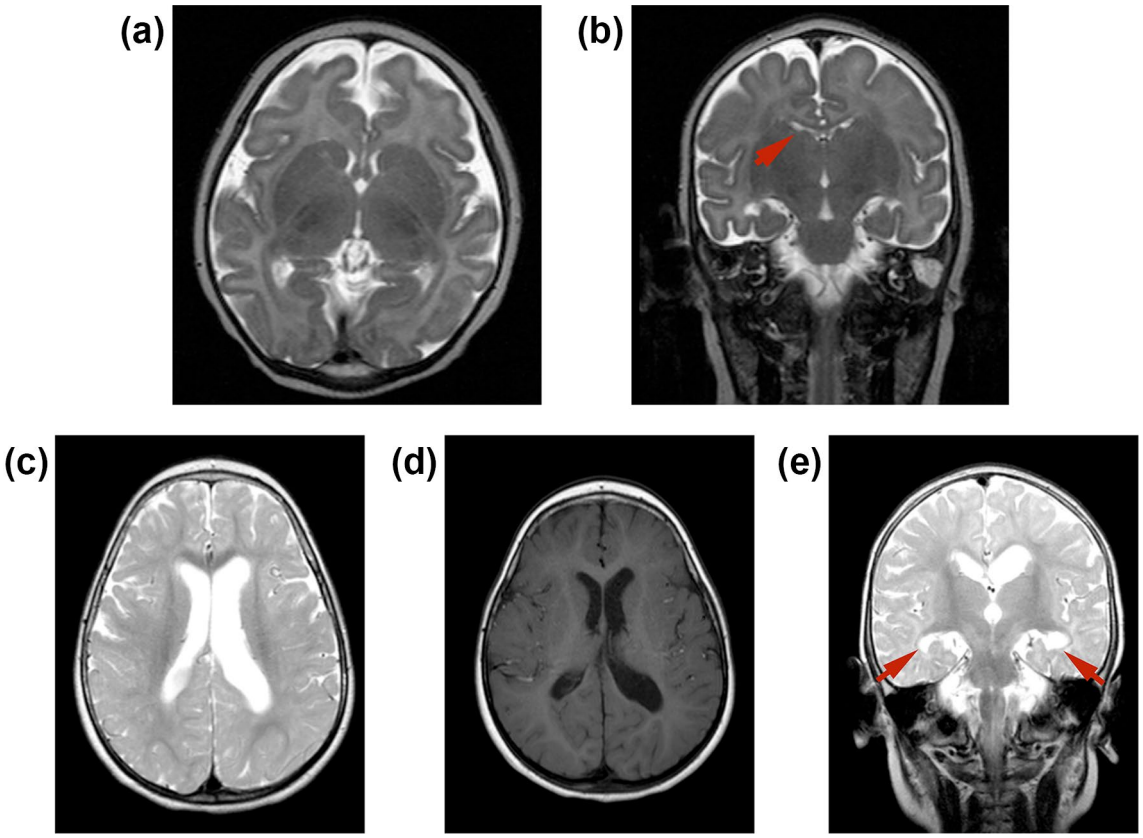


Fig. 2 MRI findings for case 1 and 3 (**a** and **b**) T2 brain MRIs of Case 1 at the age of 2 months show simplified gyral pattern and thin corpus callous (red arrow). **c**–**e** Brain MRIs of Case 3 at the age of 2 years show delayed myelination of white matter, asymmetric dilatation of the lateral ventricles, prominent dilatation at lateral ventricle temporal horns with peri-hypothalamic atrophy (red arrows)

point a close relation in-between. Additionally, haplotype analysis of VCF data in these 2 cases did not pinpoint an obvious shared haplotype around *WDR62*. Interestingly, the exact same variation in the homozygous state was also reported for another patient with a Turkish origin. She also had ID, seizures and epilepsy together with tetraparesis (Poulton et al. 2014). It may be speculated that the variant was recurrent due to a mutation hotspot given that at least case 1 and 2 are not related and do not share a common haplotype. Nevertheless, frameshift variations in *WDR62* is numerous and dispersed over this moderately large gene without any obvious hotspots as retrieved from ENSEMBL-Biomart with likely pathogenic or pathogenic consequences. Also all three cases are originally from Turkey. We therefore speculate that the variant may reside in a small haplotype block, which is in linkage disequilibrium in the relevant population for these three cases.

The *WDR62* frameshift variant results in a premature termination codon (PTC) in the 12th exon of 32 exon *WDR62* gene MANE transcript (ENST00000401500.7). Therefore, it is possible that the effect is completely null in the homozygous state due to nonsense mediated RNA decay (NMD) mechanism.

The second recessively inherited variant presented herein is *AP4M1*, which encodes a subunit of adaptor protein (AP) complex 4. APs play a crucial role in intracellular cell trafficking and particularly AP4 is ubiquitously expressed in neurons throughout the prenatal and postnatal developmental stages (Verkerk et al. 2009). Bi-allelic loss-of-function variants in four genes encoding different AP-4 subunits, including *AP4M1* have been implicated in hereditary spastic paraplegia: *AP4B1* (SPG47, MIM614066), *AP4M1* (SPG50; MIM612936), *AP4E1* (SPG51; MIM613744) and *AP4S1* (SPG52; MIM614067). The most prominent clinical features associated with *AP4S1* related SPG include microcephaly (85%), facial dysmorphism (66%), developmental delay/intellectual disability (100%), developmental regression (51%), delayed speech development (100%), neonatal hypotonia (90%), spasticity (98%) and seizures (70%) (Ebrahimi-Fakhari et al. 2020).

ENST00000359593.9(*AP4M1*):c.952C > T; p.(Arg318Ter) detected in Case 3 is mapped to the the Mu homology domain of the AP4M1 on the protein level (UniProt ID: O00189), which is a protein–protein interaction module involved in endocytosis (Prosite annotation: PRU00404). Although the PTC created by this variant

resides in relative proximity to the last exon of *AP4M1* gene MANE transcript (ENST00000359593.9; 12th of all 15 exons), it probably activates NMD mechanism, as it resides 185 bp upstream of the last exon-exon junction in the mRNA. It is generally accepted that a PTC located more than 50–55 bp upstream of the last exon–exon junction is targeted to NMD (Coban-Akdemir et al. 2018). This variant similar to the one in *WDR62* probably causes total deprivation of the gene product in the homozygous state.

The genetic finding along with the phenotype of Case 3 were compatible with *AP4M1* related SPG50. She was born with microcephaly and hypotonia and she had atonic seizures, which were under control with levetiracetam. Within few months after birth, her neonatal hypotonia was replaced by hypertonia starting from the lower extremities. Decreased myelination, white matter lesions and brisket asymmetric ventriculomegaly was observed in her brain MRI (Fig. 2c–e).

The third variant detected is missense and de novo in *SOX11*. Sry-related High Mobility Group (HMG) box (Sox) transcription factors have versatile roles in many stages of development and cell fate determination, including craniofacial, neuronal and cardiovascular development and sex determination. HMG domain is the DNA binding domain of SOX family of transcription factor (Wegner 2010). *SOX11* particularly plays an important role in brain development. It has been reported that thin brain membrane formation and brain weight decrease due to reduced proliferation and lacking normal differentiation of neuronal progenitor cells in Sox11 null mice (Wang et al. 2013). *SOX11* also has a crucial role in ocular development (Pillai-Kastoori et al. 2014).

SOX11 has been implicated in autosomal dominant Coffin-Siris Syndrome (CSS, MIM:615866). CSS9 is characterized by developmental delay and congenital malformations including microcephaly, hypoplastic nails especially of the fifth fingers in hands and/or feet and facial dysmorphology.

We have detected a novel and missense DNV in *SOX11* in Case 4 with our in depth analysis of singleton ES data. The patient exhibits the typical CSS9 phenotype and additional clinical findings including strabismus, facial dysmorphology and mild autopod findings including prominent transverse palmar crease and severely hypoplastic nail on the left hallux. The DNV on the protein level resides within the HMG domain which probably hampers the DNA binding property of SOX11 to activate downstream effector genes.

The last variant presented herein is again a missense DNV in gene *TRIO*, which encodes for a highly conserved guanine nucleotide exchange factor (GEF), namely Trio Rho GEF. The 'trio' designation assigned for this protein is related to its two GEF domains of distinct specificity and its additional serine/ threonine kinase domain (Debant et al. 1996). As expected from its functional multi-domain structure, TRIO has versatile roles in cytokinesis, cell migration, axon outgrowth and guidance, dendritic branching and synaptogenesis (reviewed in Barbosa et al. 2020).

The DNV in *TRIO* is mapped to the first GEF domain (GEFD1) on TRIO GEFD1, specifically the DH1 subdomain. GEFD1/DH1 subdomain, which is probably involves mutation hotspots, binds to the small GTPase Rac1 and regulates actin polymerization (Sadybekov et al. 2017; Barbosa et al. 2020). Variations residing in GEFD1 is associated with the microcephaly phenotype (Barbosa et al. 2020).

We have also identified a paternally inherited polymorphism (rs30774) residing four nucleotides downstream and more significantly *in cis* with the *TRIO* DNV via IGV visual inspection (Fig. 1d). The father was about 31 years old at the age of conception fitting him into the young to moderate age interval as suggested by Goldmann et al. (2016). At this age interval, fathers inherit about 30 DNVs to the offspring. Both *SOX11* and *TRIO* DNVs result in base transitions, which make up almost 70% of all phased DNVs. In short read NGS platforms, it is not always possible to detect parent of origin effect as in *SOX11*-DNV, due to lack of long and uninterrupted runs of reads with multiple enclosed variants. Nevertheless, the 'tag' SNP, namely rs30774 is close enough to the *TRIO*-DNV for effective parental phasing.

In conclusion, we have utilized singleton or trio ES approaches to identify underlying variations associated with IDpM. Our in depth analysis, including parental validation for DNVs, vcf analyses for relatedness and haplotype comparisons and finally parent-of-origin detection have led us delineate 4 variations in genes *WDR62*, *SOX11*, *AP4M1* and *TRIO* in five pedigrees. *SOX11* and *TRIO* DNVs are novel in the sense that their clinical and genetic associations had never been presented before. Although all of the affected children were born to consanguineous parents, we have found DNVs for 2 of them underlying the necessity of evaluating dominant inheritance in ES data. Determining whether the genetic variant responsible for the condition is inherited or de novo is extremely important for families, especially in terms of calculating the risk of recurrence in the field of genetic counseling. For the DNVs presented herein, we did not find any evidence of mosaicism in any parent, at least in the blood derived DNA. However, especially possible germline or gonosomal mosaicism of parents would increase the recurrence risk for so called DNVs (Shu et al. 2021).

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Author contributions All authors contributed to the study conception and design. All wet laboratory applications and variant filtering steps were performed by SM. NHA and UO took part in material collection together with clinical and genetic analyses. ZY was responsible for clinical analyses. Bioinformatic analyses were carried out by BS. The first draft of the manuscript was written by SAUI and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data availability The data that support the findings of this study are available from the corresponding author, upon reasonable request.

Declarations

Conflict of interest Sevcan Mercan, Nihan Hande Akcakaya, Baris Salman, Zuhal Yapici, Ugur Ozbek and Sibel Aylin Ugur Iseri declare that they have no conflict of interest.

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