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Exome sequencing revealed a compound heterozygous mutations in *RTTN* gene causing developmental delay and primary microcephaly

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ABSTRACT

RTTN (Rotatin) (OMIM 614833) is a large centrosomal protein coding gene. *RTTN* mutations are responsible for syndromic forms of malformation of brain development, leading to polymicrogyria, microcephaly, primordial dwarfism, seizure along with many other malformations. In this study we have identified a compound heterozygous mutation in *RTTN* gene having NM_173630 c.5225A > G p. His1742Arg in exon 39 and NM_173630 c.6038G > T p.Cys2013Phe in exon 45 of a consanguineous Saudi family leading to brain malformation, seizure, developmental delay, dysmorphic feature and microcephaly. Whole exome sequencing (WES) techniques was used to identify the causative mutation in the affected members of the family. WES data analysis was done and obtained data were further confirmed by using Sanger sequencing analysis. Moreover, the mutation was ruled out in 100 healthy control from normal population. To the best of our knowledge the novel compound heterozygous mutation observed in this study is the first report from Saudi Arabia. The identified compound heterozygous mutation will further explain the role of *RTTN* gene in development of microcephaly and neurodevelopmental disorders.

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1. Introduction

Primary Microcephaly (MCPH, OMIM 251200) is stated as the congenital genetic neurodevelopmental disorder demonstrating as small brain size along with seizure and intellectual disability. The head circumference (HC) is reduced from -4 SD (standard deviation) to -2 SD at the time of birth below the age and sex of the individual (Nicholas et al., 2010; Bilgüvar et al., 2010; Kaindl et al., 2010). In the primary microcephaly most of the patients have normal weight, height, physical appearance as well as chromoso-

mal banding pattern and normal brain scan result. In some of the cases it is also observe that patients showing abnormal chromosomal analysis and short stature with mild to severe intellectual disability (Woods et al., 2005; Nicholas et al., 2010). Studies on brain growth also revealed that primary microcephaly is due to the abnormal developmental process and not because of the degeneration or regression of the neuronal tissues (Bundey, 1997; Faheem et al., 2015). The occurrences of primary microcephaly is much more in the populations ranging between 1.3 and 150 per 100,000 births where rate of consanguineous marriages is high such as Middle East and Asia and much lower in Caucasians where consanguinity is lower (Woods and Parker, 2013). In the OMIM genes database 25 genes associated with primary microcephaly has been reported so far.

RTTN gene in human with (OMIM 610436) chromosomal location 18q22.2 translates a centrosomal protein called Rotatin mainly related to ciliary functions. Kheradmand et al., for the first time in 2012 reported a homozygous mutation in *RTTN* gene in the three members were the product of a consanguineous family from Turkey having short stature, epilepsy, microcephaly, and polymicrogyria. Later on, Shamseldin et al. (2015) reported a homozygous

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mutation in intron 23 of the *RTTN* (c.2885 + 8A-G) gene resulting in a premature stop codon (S963X) in a family having primary microcephaly and severe intellectual disability along with another homozygous mutation in Saudi male where c.3190A > C and protein change p.lys1064gln with intrauterine growth retardation, short stature, primary microcephaly along intellectual disability. Further, a Canadian family was also reported having mutation where c.1732G > C p. ala578-to-pro in exon 13 of *RTTN* gene, and a compound heterozygous mutation c.5750A > G resulting growth retardation and primary microcephaly in the affected members of the family (Shamseldin et al., 2015). Recently, a homozygous c.2953A > G transition (chr18.6,780,1710 T-C, GRCh37) at the penultimate base of exon 23 of the *RTTN* gene in Moroccan family, resulting in an p.Arg985Gly (R985G) substitution was reported by Grandone et al. 2016 and similarly a homozygous mutation was also reported (Cavallin et al., 2018). Moreover, a compound heterozygous mutation was also reported where c.4186delC p. E1397Kfs*7 and c.2594A > G and protein change p.H865R in *RTTN* gene. Later on *RTTN* gene mutations was linked with the brain and other organs deformities along with primary microcephaly and primordial dwarfism (Grandone et al., 2016; Shamseldin et al., 2015). The exact reason of the brain and other organs deformities along with primary microcephaly and primordial dwarfism is still unknown as recently, we have reported many novel mutations such as *PGAP2* (Naseer et al., 2016a, 2016b), *SATMBP* (Naseer et al., 2016a, 2016b) *NT5C2* (Naseer et al., 2020a, 2020b), *PUS7* and *AASS* genes related to microcephaly but the still lot of studies need to be done to identify the cause of the disease (Naseer et al., 2020a, 2020b).

Although few of *RTTN* gene mutations causing primary microcephaly have been reported so far. In the present study we identified a novel compound heterozygous mutation in *RTTN* gene that will be helpful for adding information in the disease data base and also helpful for the geneticists and clinicians to establishing a reliable diagnostic strategies for primary microcephaly families in Saudi population.

2. Materials and methods

2.1. Sample collection

We recruited a Saudi family with clear phenotype of primary microcephaly. Pedigree was carefully made by interviewing the family. Peripheral blood samples of all members of the family and from 100 healthy unrelated control were collected in EDTA tubes. Genomic DNA was extracted through QIAamp genomic DNA extraction or similar kits according to the manufacturers' protocols (<https://www.qiagen.com/pk/products/top-sellers/qiaamp-dna-minikit/#orderinginformation>). Genomic DNA quantification was performed with Nanodrop spectrophotometer (<https://www.thermofisher.com/order/catalog/product/ND-LITE-PR>) and visualized with CYBR Safe (ThermoFisher, USA) dye via running on 1% agarose horizontal gel electrophoresis apparatus. Informed consent was taken prior to the study from all participants. This study was permitted by the local ethical committee of King Abdulaziz University and followed all the guidelines according to the Helsinki's declaration 2013. The detailed family pedigree was drawn as shown in Fig. 1.

2.2. Magnetic resonance imaging (MRI)

MRI examination of the brain plain reveals, technique: T1WI axial, coronal and sagittal T2WI axial finding straight is prominent measuring in the superoanterior part. 6 mm. Sulci and extra cerebral CSF spaces are slightly prominent. Myelination pattern is nor-

mal for the age of the patient. Normal ventricular system. No midline shift. No tonsillar herniation noted.

2.3. Karyotype analysis

Peripheral blood samples was used for karyotype analysis. Cells culture were subjected to multiple steps including harvesting, preparation of slide, staining and microscopic analysis, before obtaining the complete image of the chromosomes. Two lymphocyte cultures per patient were initiated in sterile T-25 flasks by adding 0.5–0.7 ml blood and 0.2 ml phytohemagglutinin solution to 10 ml of complete media consisting of RPMI 1640 50 ml, and fetal bovine serum (FBS) 10 ml along with 0.5 ml penicillin/streptomycin solution and 0.5 ml L-glutamine and the cultures were incubated at 37 °C for 72 h. After incubation for 48 h, 0.1 ml 5-Fluoro-2-deoxyuridine (FUdR) solution was added in thymidine culture flask and the contents were mixed gently. Following the incubation period 0.2 ml of thymidine was also added and the contents were mixed and re-incubated at 37 °C. Three hours before cell harvest, 0.1 ml of ethidium bromide solution was added in thymidine culture flask. Then, 0.65 ml of colcemid (10 µg/ml) was added to regular flask and thymidine flask 10 min and 15 min before cell harvest respectively. Following 25 min, the cultures were terminated and the contents of the two culture flasks were transferred into centrifuge tube for centrifugation for 10 min at 1500 rpm. Cells were fixed by adding 1 ml freshly made fixative. The fixed cell suspension was centrifuged at 1500 rpm for 10 min, the supernatant removed, leaving approximately 1 ml, depending on the size of pellet. The cells were resuspended, using a narrow glass pipette. One to two drops of the cell suspension were dropped onto each marked slide from a height over vapour of hot water. Slides were left to dry at room temperature overnight. The spreads quality and mitotic index of the slide was checked using an Olympus phase contrast microscope (Model No. BX51TF). For G-Banding and staining four coplin jars were prepared and each containing the following solution. Coplin jar one (48 ml of isotonic buffer + 2 ml of trypsin solution). Coplin jar two (50 ml isotonic buffer). Coplin jar three (50 ml Gurr Buffer pH 6.8) and coplin jar four (15 ml of re-filtered Leishman stain +45 ml Gurr Buffer solution pH 6.8). As for chromosome trypsin treatment, the slides were dipped in coplin jar one for 1 min. Then the slides were soaked for 3 s in coplin jar two and jar three respectively. The slides were stained with fresh Leishman stain for 2–3 min in coplin jar four. Immediately the slides were rinsed in running water and air dried and observed using the Nikon microscope for the presence of optimal chromosome bands. The position of the metaphase spreads upon finding was captured by using the Cytovision tools, the chromosomes of captured metaphases were separated, arranged in pairs, compared pairs band by band and checked for any abnormality.

2.4. Whole exome sequencing

To find out the pathogenic mutation that may be the cause of primary microcephaly WES was performed for the affected members of the family. The samples for sequencing were processed according to the guide from Agilent SureSelect Target Enrichment Kit (SureSelect_v6 Agilent USA) while whole exome sequencing was done using the Illumina HiSeq 2000/2500. Further, the libraries were sequenced using sequencer from Illumina. The variants were filtered using frequency, protein effect, quality, pathogenicity, genomic position and relations with the disease. The variants or single nucleotide polymorphisms and short indels candidates are detected at nucleotide resolution at this stage. SNPs identified are compared to 1000 genome (<https://www.interna->

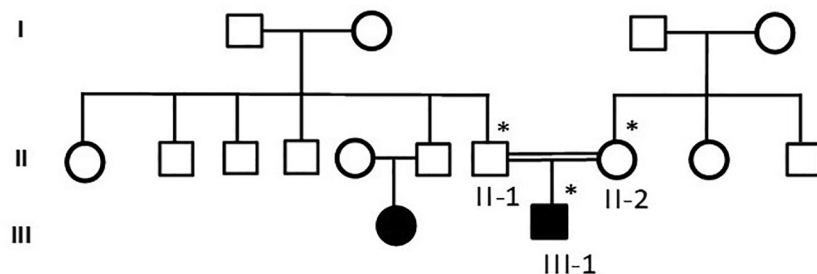


Fig. 1. A detailed pedigree of a consanguineous Saudi family. The samples available for the study were marked with satiric sign.

tionalgenome.org/), SnpEff (<http://snpeff.sourceforge.net/SnpEff.html>) and genomAD databases (<https://gnomad.broadinstitute.org/>). We utilized various bioinformatics tools to find the causative variant for primary microcephaly. Laser gene Genomic Suite v. 12 (DNASTAR, Madison, WI, USA). Further, we used ArrayStar v. 12 (Rockville, MD, USA) to tag variant alleles based on dbSNP142. The sequences obtained in FASTQ were further mapped to human reference genome which is termed as “alignment”, this alignment is performed through Borrow-Wheel arrangement tool (<http://bio-bwa.sourceforge.net/bwa.shtml>) and this alignment was done with reference genome hg19 for human (<http://hgdownload.cse.ucsc.edu/goldenPath>). FASTQ raw data files are here converted to BAM files format. The BAM files obtained in the previous step were annotated with Toolkit for Genome Analysis (<http://www.broadinstitute.org/gatk>). It is an excellent and successful tool for variant discovery in next generation sequencing data.

2.5. Sanger sequencing (SS)

Selected variants were confirmed through SS so that in exome they have not been picked up as false positive. In SS first primers were selected through Primers3 plus online software, then the target DNA will be PCR amplified, purified through PCR purification kits and then Sanger sequenced through ABI genetic analyzers with help of BigDye Terminator V3.1 Cycle Sequencing kit. These technologies provide sequence reads of 400–700 nucleotides and thus the target variants are easily confirmed through electropherograms obtained from ABI genetic analyzers.

2.6. RTTN protein docking and alignment of the protein

SWISS-MODEL docking was used for the modeling of both wild-type and mutated protein structures for the position of p.His1742Arg and for the position of p.Cys2013Phe. Sequencing alignment of different species was also done and highlighted the strong conservation of the two variants at p.His1742Arg and p.Cys2013Phe by down loading the data of different proteins from Ensembl gene browser (<https://m.ensembl.org/index.html>) and alignment of the sequencing was done using the BioEdit software.

3. Results

3.1. Clinical reports of the patient

Proband III-1 a boy was of 2 years old at time of examination. He was the first baby of the consanguineous Saudi family as shown in Fig. 1a. This was the second case in the family history as another boy of four year old first cousin from father side have the same phenotypes but blood samples was not available for analysis. He has microcephaly of -4 SD measured as occipitofrontal head cir-

cumference along with mild to modest intellectual disability, dysmorphic features, developmental delay and epileptic seizure as well. Furthermore, typical feature of primary microcephaly with reduce head circumference was present. He had normal chromosomes, no metabolic as well as no neurological problem was observed. Karyotype study based on 15 metaphases analysis resulted as 46XY a normal male without any chromosomal changes. Brain MRI resulted in microcephaly with an abnormal triangular appearance of the frontal skull bones. Sulci and extra cerebral CSF spaces are slightly prominent. Myelination pattern is normal for the age of the patient. Normal ventricular system. No midline shift. No tonsillar herniation noted. The MRI report showed microcephaly with an abnormal triangular appearance of the frontal skull bones mostly related to premature closure of the metopic suture. There was no acute brain insult, mass-effect, hydrocephalus or space occupying lesion. He was intellectually disabled. He has similar feature of primary microcephaly like sloppy forehead large ears.

3.2. Whole exome sequencing

WES for the proband III-1 was done and the resulted FASTQ files were converted to BAM and then BAM files were converted to variant call format (vcf) file having 113,251 variants were obtained. These variants were utilized for the identification of mutation that may leads to the disease based on novel/rare (MAF + 0.01%) frequency, functional (predicted damaging by Polyphen/SIFT), homozygous/heterozygous state, genomic position, pathogenicity, protein effect, and earlier associations with the disease related phenotype. We applied different filters and bioinformatics tools and we did not find any homozygous mutation than we applied more filters to find out the heterozygous mutations and we identified a compound heterozygous mutation NM_173630 c.5225A > G p.His1742Arg in exon 39 and NM_173630 c.6038G > T p.Cys2013Phe in *RTTN* gene. The variant was not found in the large reference population cohort of the Genome Aggregation Database (gnomeAD, n >120,000 exomes and >15,000 genomes). To best of our knowledge these variants have been not been reported in the any medical literature or in the disease related variation database like Human Gene Mutation Database (HGMD) or ClinVar. The total number of mutations reported for *RTTN* gene so far collected from the literature has been mentioned in the Table 1.

3.3. Sanger sequencing

The compound heterozygous mutation identified after WES analysis were further validated by using Sanger sequencing analysis in all other available family members by using the primers for the observed mutations. The Sanger sequencing results confirm the compound heterozygous mutation NM_173630 c.5225A > G p.His1742Arg in exon 39 and NM_173630 c.6038G > T p.

Table 1
Mutation spectrum in *RTTN* gene.

S. No	Mutation	Consequence	State	Origin	Disease	References
1	c.2796A > T	p.Leu932Phe	homozygous	Turkish	Polymicrogyria	Kheradmand et al., 2012
2	c.80A > G	p.Cys271Tyr	homozygous	Turkish	Polymicrogyria	Kheradmand et al., 2012
3	c.2885 + 8A > G	p.Ser963*	homozygous	Yemen	Primary microcephaly & primordial dwarfism	Shamseldin et al., 2015
4	c.3190A > C	p.Lys1064Gln	homozygous	Saudi Arabia	Primary microcephaly & primordial dwarfism	Shamseldin et al., 2015
5	c.1732G > C	p.Ala578Pro	homozygous	Canadian	Primary microcephaly & primordial dwarfism	Shamseldin et al., 2015
6	c.5750A > G	p.Asp1917Gly	homozygous	–	Primary microcephaly & primordial dwarfism	Shamseldin et al., 2015
7	c.2953A > G	p.Arg985Gly	homozygous	Moroccan	microcephaly-related craniofacial dysmorphism, axial hypotonia, and limb spasticity	Grandone et al., 2016 and Cavallin et al., 2018
8	c.190G > T	p.Val64Phe	homozygous	Moroccan	microcephaly-related craniofacial dysmorphism, axial hypotonia, and limb spasticity	Wambach et al., 2018
9	c.32-3C > T	–	homozygous	Moroccan	microcephaly-related craniofacial dysmorphism, axial hypotonia, and limb spasticity	Wambach et al., 2018
10	c.4186delC / c.2594A > G	p.E1397Kfs*7/ p.H865R	Compound heterozygous	Netherlands	Polymicrogyria with seizures	
11	c.5225A > G/ c.6038G > T	p.His1742Arg/p.Cys2013Phe	Compound heterozygous	Saudi Arabia	Microcephaly related craniofacial dysmorphism, axial hypotonia, and limb spasticity	Present study

Cys2013Phe in *RTTN* gene as one parent was normal at one position and was heterozygous at other position and similarly the other parent was heterozygous at the position where the other parent was normal while both heterozygous mutations were present in the affected boy as shown in Fig. 2. Finally, to find it out that the identified variants are pathogenic for the family and to rule out this possibility the Sanger sequencing for 100 unrelated control people were also done.

3.4. *RTTN* protein docking and alignment of the protein

Swiss docking modeling was done for both wildtype and mutated showed wildtype and mutated structure of Rotatin protein prediction for the position of p.His1742Arg and for the position of p.Cys2013Phe. The automated protein structure

homology-modelling model was done using online SWISS-MODEL database as shown in Fig. 3AB. Sequence alignment of different species was also done and highlighted the strong conservation of the two variants at p.His1742Arg and p.Cys2013Phe as shown in Fig. 3C.

4. Discussion

The *RTTN* gene is very important gene that is responsible for centrosomal protein translation. The exact function of this gene is still not very clear, but the studies showed that human mutant fibroblasts showed anomalous growth of cilia. Further, Real-Time Quantitative Reverse Transcription qRT-PCR showed the involvement and dysregulations of numerous genes in the signaling pathway for brain development of the Sonic Hedgehog (Kheradmand

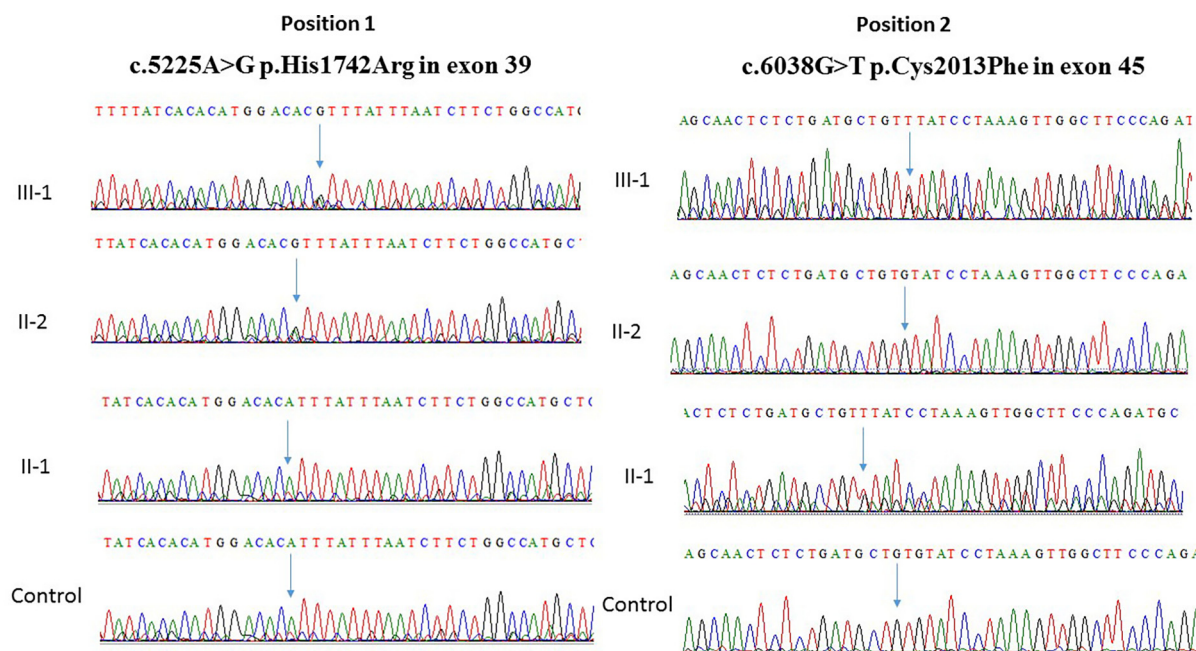


Fig. 2. Sanger sequencing of the individuals participated in the study. A novel compound heterozygous mutation was identified in the affected member III-3 where c.5225A > G p.His1742Arg in exon 39 and c.6038G > T p.Cys2013Phe in exon 45 of a consanguineous Saudi family. The father II-1 was homozygous having “A” allele at the position and the mother II-2 was heterozygous at c.5225A > G at this position while the father was heterozygous at the position c.6038G > T and mother II-2 was homozygous having “C” allele. The affected member was heterozygous at both positions c.5225A > G p.His1742Arg in exon 39 and c.6038G > T p.Cys2013Phe in exon 45 clearly representing mutation pattern of compound heterozygous manner.

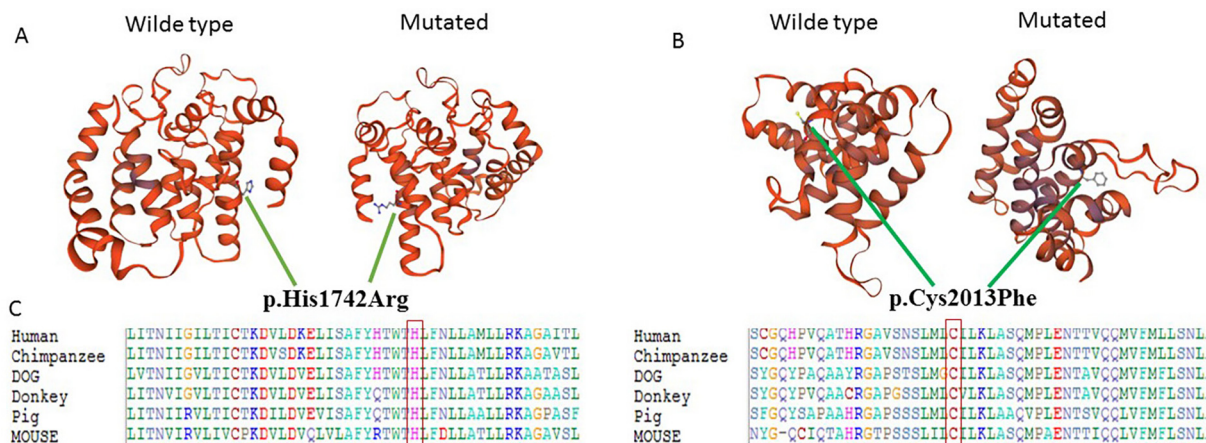


Fig. 3. A. Representation of wildtype and mutated structure of Rotatin protein prediction for the position of p.His1742Arg. B. Representation of wildtype and mutated structure of Rotatin protein prediction for the position of p.Cys2013Phe. The automated protein structure homology-modelling model was done using online SWISS-MODEL database. C. Representation of different species alignment was done and highlighted the strong conservation of the two variants at p.His1742Arg and p.Cys2013Phe.

et al., 2012). *Rtt*n expression in mesoderm and telencephalon in mice embryos was also observed. If the gene is knockout mortality of embryo occurs at the age of E.11.5. Moreover, many other major developmental defects include an abnormal distinction of the somites, notochord degeneration, the neural tube, randomization of heart looping and axial rotation failure was also studied (Faisst et al., 2002).

The *RTTN* gene has been added in list of gene mutations that are linked with the cortical malformations. Two different consanguineous families reported with mutations in *RTTN* gene in four patients having intellectual disability, seizures and polymicrogyria in brain MRI (Kheradmand et al., 2012). Polymicrogyria is a condition of abnormal cortical malformation considered by small undulating gyri with combination of the different molecular layer in two sulci. This condition also linked with a normal or irregular gyration that might be the result of brain damage with hypoxic ischemic or may be due to any developmental disorders with different environmental conditions (Squier and Jansen, 2014).

RTTN gene knockdown study in *Drosophila* ortholog also suggested that the mechanism of *RTTN*-related with asymmetry that is due to abnormal cilia development along with shortening of the cilia size (Kheradmand et al., 2012; Stevens et al., 2009). The normal development of cilia play very important and critical role in the formation of the nodal flow, that is linked with a primary cue that cause the interruptions of the left and right symmetry that is a standard condition of primary ciliary dyskinesia (Nonaka et al., 1998; Okada et al., 2005).

In the Murine embryos any genetically abolished in *RTTN* gene resulted in expression demonstrate delayed neural tube closure, randomized heart looping along with left–right sidedness abnormalities (Faisst et al., 2000; Chatterjee et al., 2007). Different studies suggest species specific changes in the role of *RTTN* such as fetal mortality linked with genetic dismissal for *RTTN* encoded functions during cardiac development *RTTN*-mediated cardiac abnormalities in humans, while in murine model showed different phenotypic conditions in the null alleles along with biallelic splice variants or missense mutations was also observed in the newborns and children. Genetic disorder of *RTTN* gene and its homolog *Ana3* in *Drosophila* related to the expression in fly models murine further explain the role and the importance of this important gene for the development of brain (Stevens et al., 2009).

Wambach et al. (2018) reported that patients as similar neurologic conditions such as reduced white matter, corpus callosum agenesis, microcephaly, cortical dysplasia, simplified gyral pattern

and nervous system (bilateral microphthalmus, renal pyelocaliectasis and cryptorchidism) conditions as reported by different patients with biallelic *RTTN* variants (Shamseldin et al., 2015; Kheradmand et al., 2012; Grandone et al., 2016). Whereas some ophthalmologic findings such as optic nerve hypoplasia, rudimentary retinal vasculature, pale optic disks and misshapen orbital globes along with skeletal findings such as thin ribs, hypoplastic mandible, gracile appearing bones, syndactyly and augmented density of the temporal bones were not reported in previous studies (Wambach et al., 2018).

RTTN gene encodes a large 2,226 amino acid protein called Rotatin and the exact function is still unknown as having two domains Armadillo-like that is highly conserved among species. These Armadillo domains control the interaction between proteins by controlling the transducing indications of cell adhesion molecules to the cytoskeleton. (Shamseldin et al., 2015; Kheradmand et al., 2012).

Previously, studies showed that mice with *Rtt*n knockout having the missing the murine ortholog of *RTTN*, showed many defects such as notochord degeneration, with lacking axial rotation, embryonic lethality, irregular differentiation of the neural tube, severe hydrocephalus along with loss of the left and right requirement of the heart (Faisst et al., 2002). These abnormalities leading to the disorders disturbing primary and motile cilia Goetz and Anderson, 2010; Badano et al., 2006; Gerdes et al., 2009). Furthermore *Rtt*n mouse mutant also showed has anteroposterior and dorsoventral modeling defects as showed in the *Kif3a* and *Kif3b* mutant mice, which control the motor subunits of kinesin-II for ciliary transport (Chatterjee et al., 2007; Melloy et al., 1998; Marszalek et al., 1999).

5. Conclusions

Finally, in this study we reported a novel compound heterozygous mutation in *RTTN* gene, this will further help in population screening of primary microcephaly families that is required for better understanding and management of this disease for efficient and accurate genetic testing. These type of reports are helpful for patient management and to reduce of the disease risk through genetic counselling in the population. Moreover, our study enlarges the mutation data of *RTTN* gene related to MCPH and offers new insight into the types and frequencies of the mutations in Saudi population, which will further help to identify innovative strategies to tackle primary microcephaly.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author's contribution

MIN, AGC designed the study. AAA and MIN conducted the experiments. OYM, MIN, AAA analyzed the data. MIN, and AGC wrote the paper. All authors contributed to the editing of the paper and the scientific discussions.

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Further reading

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