The alkylglycerol monooxygenase (*AGMO*) gene previously involved in autism also causes a novel syndromic form of primary microcephaly in aconsanguineous Saudi family

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Introduction

Autosomal recessive primary microcephaly (MCPH) is a clinically and genetically heterogeneous group of neurodevelopmental disorders. Patients are characterised by intellectual disability, developmental delay and a characteristic sloping forehead. The abnormal brain development leads to a marked reduction in occipitofrontal head circumference (−b 3SD) [1,2]. Microcephaly and intellectual disability have also been reported with skeletal deformities or primordial dwarfism[3] including phenotypes of Seckel syndrome, Silver-Russell syndrome, microcephalic osteodysplastic primordial dwarfism types I/III and II, and Meier-Gorlin syndrome.

To date, 18 genes have been reported for MCPH and overlapping phenotypes. Homozygosity mapping followed by candidate gene sequencing has previously been used in an attempt to determine the underlying genetic cause of MCPH [4–7]. However, whole exome sequencing (WES) when combined with homozygosity mapping works more efficiently for the molecular diagnosis of rare conditions [8,9].

In Saudi Arabia, social or ethnic isolation into conserved tribal structures and large family sizes leads to a high incidence of consanguinity (56%) and therefore this population has a high risk of autosomal recessive disorders [10]. Whilst investigating autosomal recessive neurological disorders of Saudi population, we have found WES as a successful molecular diagnostic tool by complementing it with genome-wide homozygosity mapping [11,12].

In this study, we aimed to identify the genetic cause of MCPH in a consanguineous family from Saudi Arabia, using genome-wide homozygosity mapping in combination with WES technologies.

Materials and methods

Ethical approval and sample enrolment

Prior to the commencement of this study, informed written consent was signed by each patient or by the legal guardians of participants, including agreement on publishing the research outcomes. The study was approved, according to the Declaration of Helsinki, by the Institutional Review Board (IRB) of the Princess Al-Jawhara Albrahim Center of Excellence in Research of Hereditary Disorders and the Unit of Biomedical Ethics Research Committee (ref. # 24-14), Faculty of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia.

The four-generation family tree, shown in Fig. 1, was drawn after interviewing the family elders who originated from a remote area in the South-Western region of Saudi Arabia. Peripheral blood samples from five family members, including both parents, were collected in EDTA tubes and the genomic DNA was extracted using standard methods.

Microarray and exome analyses

To investigate the possible involvement of chromosomal aberration, and for the purpose of homozygosity mapping, we performed 300 K SNP microarray analysis (Illumina, San Diego, CA, USA) on two affected patients (IV-1 and IV-2), the parents (III-1 and III-2) and an unaffected (IV-3) family member. The data were analysed as described previously [13]. Whole exome paired-end sequencing analysis with 100× coverage was performed on the two affected patients by preparing 51 Mb SureSelect V4 libraries (Agilent Technologies, USA), and the sequence reads were run on a HiSeq2000 platform (Illumina, San Diego, CA, USA). Bioinformatic analyses for causative variant identification and Sanger validation of the candidates was performed as described previously [11,14].

Candidate gene prioritisation criteria

All of the affected individuals in the family were males, hence there was a possibility of X-linked inheritance. For this reason, we used both X-linked and autosomal recessive models for candidate gene identification. Non-pathogenic or variants of unknown significance were filtered out using the following criteria: X chromosome hemizygous variants with a minor allele frequency higher than 0.05; for the autosomal recessive model variants with a minor allele frequency higher than 0.05 in both affected and those not present within the regions of homozygosity or those found in in-house exomes ($n = 16$). In silico prediction tools (MutationTaster2 [15] and Provean [16]) were used to predict likely pathogenicity of each variant. The selected variants were genotyped using Sanger sequencing in all

available family members to validate the WES data and to determine whether or not the variant in question co-segregated with disease status. Ethnically matched controls were screened to exclude the possible population-specific common polymorphisms.

Results

Clinical features

The two affected individuals (IV-1 and IV-2) had microcephaly and global developmental delay. Patient IV-1, an 8-year-old boy, was born at full term to unaffected parents. His parents were first degree cousins and were of 30 (father) and 25 (mother) years of ages at the time of his birth. His measurements of occipitofrontal head circumference (OFC) 42.2 cm, height 88 cm and weight 10.5 kg were less than 5th centile of the normal growth ranges and were thus suggestive of failure to thrive. Patient IV-2, a 6-year-old boy, had similar clinical features to his elder brother (IV-1). He had 40 cm OFC, 74 cm height and 7.9 kg weight. Fragile X syndrome DNA tests were negative in both. They were unable to speak a single word, had difficulty in standing and could not walk without a support. They relied on mashed food due to difficulty in swallowing and were dependent for their toileting needs. They had a flat occiput, bilateral 5th finger clinodactyly, and short hand and fingers (Fig. 2, a–d). They had hypogonadism in the form of a micropenis less than 10th centile of the normal growth ranges. They self-mutilated and frequently hit their heads or bit their hands. They also had subclinical hypothyroidism. Brain magnetic resonance imaging (MRI) of patient IV-1 showed delayed myelination and thinning of the corpus callosum (Fig. 2, e–f).

Genetic analysis

Regions of homozygosity

Genome-wide SNP microarray genotyping revealed six regions of homozygosity shared by the two patients on three different chromo-somes containing 822 genes in total (Table 1).

Whole exome sequencing and Sanger sequencing validation

Whole exome sequencing revealed that the two affected patients had 30,774 homozygous variants on autosomes and 866 hemizygous variants on X chromosome. The stated prioritisation criteria for both autosomal recessive and X-linked models generated a list of just six putative causal variants (Table 2). Sanger sequencing demonstrated that five of these six variants (those in *CXorf59*, *GPR112*, *SHROOM4*, *RUFY3* and *MEOX2*) either did not co-segregate with the disease in this family, or were present in in-house healthy controls. However, Sanger sequencing across the single base deletion variant in *AGMO* (NM_001004320: c.967delA; p.Glu324Lysfs12*) confirmed its co-segregation with the disease phenotype in a recessive manner, i.e. the affected individuals were homozygous for this deletion whilst both parents in the family were heterozygous (Fig. 3). The possibility of this deletion being a population-specific polymorphism was excluded by screening 178 chromosomes of ethnically matched unaffected or healthy controls; however, this variant was not found outside the family. In addition, this alteration was also not listed in the Exome Aggregation Consortium database containing 60,706 individuals' exome data worldwide. The c.967delA deletion results in the substitution of a glutamate residue at position 324 with a lysine residue and further affects the normal reading frame, adding 12 amino acids and ultimately a premature termination codon (p.Glu324Lysfs12*) as revealed by analysis with BioEdit Sequence Alignment Editor software version 6 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) (Supp. Fig. S1).

Discussion

To date, 18 genes have been assigned to MCPH and overlapping phenotypes worldwide [8], and half of these genes have been discovered through WES analyses. Here with this study, we have added another gene to this list via

investigating a consanguineous family from Saudi Arabia.

Clinical features of our patients including intellectual disability, developmental delay, reduced height and growth retardation were resembling more closely MCPH in comparison with Seckel and Silver-Russell's syndromes. In fact, Seckel syndrome patients present severe short stature [1] which was not observed in our patients. Some features of Silver-Russell's syndrome including delayed psychomotor development, clinodactyly and short stature [17] were although comparable to those observed in our patients. However, microcephaly observed in our cases, is not a feature of Silver-Russell's syndrome [18]. Furthermore, clinodactyly was a minor finding in our patients and other radiological surveys for middle or distal phalangeal hypoplasia or syndactyly of the 2nd and 3rd toes did not reveal any associated skeletal abnormalities in our patients, as observed in previous studies of Silver Russell's syndrome [18].

The MCPH proteins have been divided into six classes based on their involvement in cell cycle dynamics, centrosome functioning, ciliogenesis and neuronal cell migration [8]. Keeping in view the functional role of the shortlisted candidate variants in Table 2, more than one gene could potentially be causative for the phenotype in our patients. For example, chromosome X open reading frame 59 (*CXorf59*), also known as cilia and flagella associated protein 47 (*CFAP47*), could be of interest based on the observation that it be- longs to the class of MCPH genes involved in ciliogenesis [8]. The shroom family member 4 (*SHROOM4*) gene encodes an actin-associated protein implicating the cytoskeletal organisation [19], and its mutations are associated with Stocco dos Santos X-linked mental retardation syndrome [20]. G protein-coupled receptor 112 (*GPR112*) is a marker for enterochromaffin cells and gastrointestinal neuroendocrine carcinomas [21]. However, these three X-linked variants did not segregate correctly with the disease phenotype and their presence in healthy males excluded the possibility of them being causative in our patients. In mice, the *Rufy3* protein is specifically expressed in neurons and it interacts with actin-bundling protein to control the molecular regulation of axonal outgrowth and cell polarisation in neurons [22]. However, the variant of *RUFY3* identified in our study was also found to be homozygous in in-house healthy controls and was predicted to be a neutral polymorphism (Table 2). Therefore, it seems unlikely that *RUFY3* is the causal variant in our patients. Genome-wide rare copy number variant association studies have established that heterozygous alterations in *MEOX2* affect amyloid-β peptide metabolism or signalling in Alzheimer's disease in humans [23]. Meox-2 knockouts are also susceptible to cleft palate in mice [24] and limb muscle differentiation anomalies in chicken [25]. Interestingly, the *MEOX2* variant identified in our patients co-segregated with the disease phenotype in an autosomal recessive fashion, and was also predicted to be a disease causing. However, its homozygous presence in ethnically matched healthy controls excluded any possible pathogenic involvement in our patients. Co-segregation of the *MEOX2* variant with the disease phenotype is not surprising because it resides within the same locus as the *AGMO* and therefore may be in linkage disequilibrium with this gene. Furthermore, variants co-segregating with the disease phenotype and predicted to be disease causing in in-silico analysis cannot be declared pathogenic unless they are ruled out in ethnically matched healthy controls. Very recently, a similar example was observed in WES data analysis of a large Pakistani autosomal recessive congenital ichthyosis family, where more variants co-segregated with the disease phenotype but the causative mutation was only confirmed in *PNPLA1* gene [26].

The alkylglycerol monooxygenase (*AGMO*, *OMIM 613738*) gene, also known as *TMEM195*, is located on chromosome 7p21.2 and has 13 coding exons producing a protein of 445 amino acids (Ensembl Genome Browser ID: ENSG00000187546). Within the cell, AGMO is located in the lipid bilayer of the endoplasmic reticulum [27]. The AGMO enzyme has a fatty acid hydroxylase motif with eight conserved histidine residues [28], which are required for binding to its iron cation cofactor during its enzymatic activity [27]. AGMO is a highly hydrophobic mixed-function oxidase that cleaves the ether bond of saturated alkylglycerol in the presence of tetrahydrobiopterin (*BH4*), which helps in hydroxylation of alkylglycerol, and the product rapidly decays into an aldehyde and a glycerol derivative [27].

Studies have revealed the importance of AGMO in lipid biology both in human and animal models. It is the only enzyme known to cleave the O-alkyl bond of ether lipids, which are essential components of cell membranes in the brain, protect eyes from cataracts, interfere with or mediate signalling processes, and are required for spermatogenesis [29]. Genome-wide association data from various studies have considered AGMO as a potential candidate for IGF/insulin-like signalling [30] and congenital heart disease [31] in humans. In cancer cells, decreased or lack of O-alkyl monooxygenase enzyme activity may lead to cell death due to the accumulation of lipid ethers in these tissues [32]. Increased AGMO activity has been observed, together with a fatty aldehyde dehydrogenase (*ALDH3A2*, *OMIM 609523*), in Chinese hamster ovary cells during toxic fatty acid homeostasis in various tissues [27]. Recent

studies in Caenorhabditis elegans have revealed that AGMO, in combination with BH4, has a profound impact on a wide range of lipid biology, cuticle integrity, murine lipidome and signalling events [33,34].

Mutations in other genes encoding proteins participating in lipid biosynthesis and other members of the pathways followed by AGMO are also known to be involved in syndromic forms of neurological disorders in human. For example, alterations in intracellular phospholipase A1 encoded by *DDHD2* gene, which has a role in lipid biochemistry and membrane trafficking, have been reported in hereditary spastic paraplegia with intellectual disability [12,35–37]. Deficiency of BH4 [38] leads to a group of rare inherited neurological disorders affecting either organs including the central nervous system or the peripheral hepatic system [39]. Large homozygous deletions and point mutations in the *ALDH3A2* gene have been reported in Sjögren-Larsson syndrome, a neurocutaneous disorder with intellectual disability and skin barrier abnormalities [13,40].

Previously, AGMO was known as putative sterol desaturase (*FLJ16237*) and an association with autism in humans had been reported [41]. The biallelic single base deletion (*AGMO*, *c.967delA*) identified in our patients is suggestive of complete loss of function of AGMO due to premature protein truncation or mRNA decay [42]. Loss of function of AGMO, which is one of the key enzymes in lipid biology, may disrupt the signalling pathways leading to the pathologic effects, as observed in our patients, in the same fashion as its cofactor BH4 and adherent *ALDH3A2* enzyme are previously reported in other neurological disorders [38, 40]. Taking together the biological function of AGMO in lipid biosynthesis and its potential role in a previously reported neurological disorder, we infer that the variant identified here leads to the disease phenotype in our family.

Conclusion

We present the first evidence of a mutation in AGMO being associated with an autosomal recessive microcephaly in humans. This new disease gene broadens the spectrum of genetic heterogeneity in MCPH phenotypes and may also be implicated further in other patients with MCPH from Saudi Arabia and other populations. Although next generation sequencing alone has made a greater contribution to disease gene identification in the recent past, the complementation of homozygosity mapping with WES analysis may be considered more useful for the genetic analysis of MCPH phenotypes in highly inbred populations.

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Figures and Tables

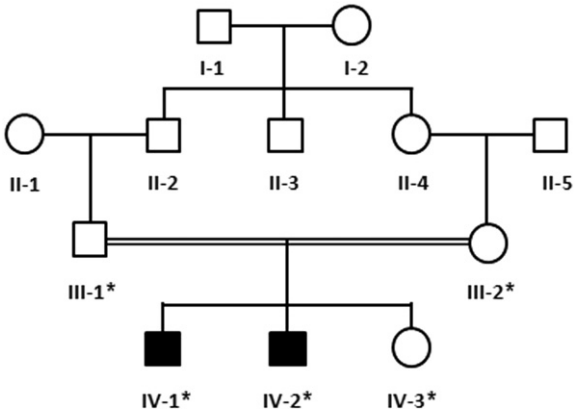

Fig. 1. A consanguineous family from Saudi Arabia showing the disease phenotype segregating in an autosomal recessive manner. The samples available for genetic testing are marked with asterisks.

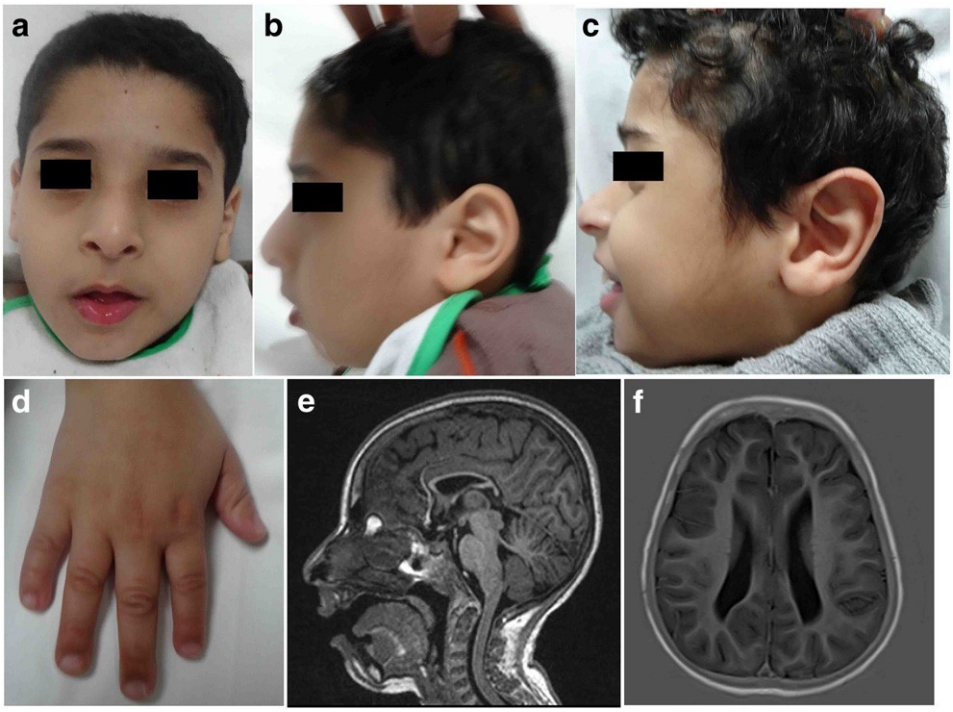

Fig. 2. Clinical presentation of the affected individuals: microcephaly in patients IV-1 (a–b) and IV-2 (c), clinodactyly in patient IV-1 (d), hypoplastic corpus callosum, mildly dilated ventricles and prominent sulci in patient IV-1 (e–f).

Table 1

List of regions of homozygosity shared by the affected individuals in the family.

No	Chromosome	Physical position	Size in Mb	Flanking SNP(s)	Genes in the region
1	4q13.3-4q21.21	71,509,431–77,660,731	6.15	rs3796704 and rs344141	76
2	4q23-4q28.2	102,751,076–129,867,280	27.11	rs10516487 and rs10028124	242
3	7p21.3-7p21.2	11,022,230–15,725,967	4.7	rs218966 at and rs144533382	23 including *AGMO*
4	7p11.2-7q11.23	53,103,554–77,247,821	24.14	rs11238247 and rs9640663	397
5	7q31.1-7q31.31	113,518,502–117,199,533	3.68	rs2974938 and rs213950	31
6	12q24.33-12q24.33	131,306,314–133,768,553	2.46	rs6486602 and rs61960670	53

Table 2

List of candidate variants revealed by whole-exome sequencing.

Chr	Gene	Exon	RefSeq accession number	c.DNA position	Amino acid change	Frequency in ExAc	In silico protein prediction

						database	MutationTaster2	Provean
X	*CXorf59*	3	NM_173695	c.A155G	p.N52S	0	U	N
X	*GPR112*	17	NM_153834	c.7969_7971del	p.2657_2657del	0	P	N
X	*SHROOM4*	6	NM_020717	c.3365_3366insAGCAGCAACAGC	p.K1122delinsKQQQQ	0	P	U
4	*RUFY3*	1	NM_001130709	c.C155A	p.A52D	0.005	P	N
7	*AGMO*	10	NM_001004320	c.967delA	p.E324Kfs12*	0	D	U
7	*MEOX2*	1	NM_005924	c.C61A	p.P21T	0.001	D	N

Chr, chromosome; RefSeq, reference sequence; ExAc, Exome Aggregation Consortium database; P, polymorphism; D, damaging, deleterious or disease causing; N, neutral or tolerated.; U, unknown.

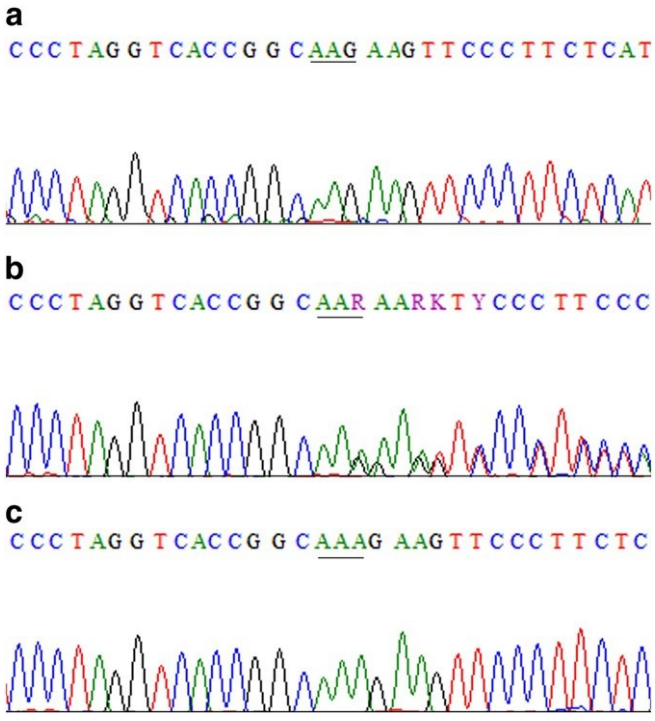

Fig. 3. Sanger sequence analysis: a homozygous deletion mutation (c.967delA) in exon 10 of the *AGMO* gene in affected (a), obligate carriers (b) and wild type or ethnically matched control individuals (c).