**ARTICLE OPEN**

Biallelic truncation variants in ATP9A are associated with a novel autosomal recessive neurodevelopmental disorder

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**INTRODUCTION**

Intellectual disability (ID) is a highly heterogeneous disorder with hundreds of associated genes. Despite progress in the identification of the genetic causes of ID following the introduction of high-throughput sequencing, about half of affected individuals still remain without a molecular diagnosis. Consanguineous families with affected individuals provide a unique opportunity to identify novel recessive causative genes. In this report, we describe a novel autosomal recessive neurodevelopmental disorder. We identified two consanguineous families with homozygous variants predicted to alter the splicing of ATP9A which encodes a transmembrane lipid flipase of the class II P4-ATPases. The three individuals homozygous for these putatively truncating variants presented with severe ID, motor and speech impairment, and behavioral anomalies. Consistent with a causative role of ATP9A in these patients, a previously described Atp9a+/− mouse model showed behavioral changes.

**RESULTS**

Clinical report

We identified three affected individuals from two unrelated consanguineous families of Pakistani and Iranian origin. The main clinical features of the affected individuals are reported in Table 1 and in Fig. 1.

Family 1 is from the Khyber Pakhtunkhwa region of Pakistan. As indicated in the pedigree, the unaffected parents (III:3 and III:4), who are first cousins, have six children. The oldest and youngest siblings (IV:1 and IV:7) exhibited similar clinical features that include delayed childhood milestones, severe ID, mild hypotonia, attention deficit hyperactivity disorder (ADHD), aggressive behavior, bilateral eye squints, and impaired vision. The oldest affected daughter (IV:1) presented with microcephaly (<1st percentile, −3.12 SD), however the head circumference of the second affected sibling, the youngest daughter, (IV:2) is in the normal range (39th percentile). We could not perform brain magnetic resonance imaging (MRI) because the family lives in a very remote area and did not agree to travel due to COVID19 outbreak and the high rate of infections in the region. While the other siblings (IV:2, IV:3, IV:4, and IV:5) were unaffected, we note that pregnancy IV:6 was not carried to term (Fig. 1).

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The proband (IV:1) of the Iranian family 2 is the only child born from a couple of first cousins (Fig. 1). Childbirth was unremarkable. The parents noticed a delay in the development of both language and walking (18 months). The proband started epileptic episodes at 3 years of age and seizures were controlled with sodium valproate. An abnormal EEG with epileptiform activity was reported. Brain MRI was normal. At the time of the last visit, the child did not present motor paralysis or coordination deficit, but he had an abnormal gait. At 11 years of age, height, weight, and head circumference were in the normal range with 140 cm, 45 kg, and 53 cm, respectively. Eye contact was impaired and there was complete language dysfunction. He is presenting with severe ID, prominent stereotypic movement disorder, and autistic features. The proband has arched eyebrows with round, downturned eyes, thin lips, bulbous nose, and a short philtrum. The proband’s cousin was also reported to be affected by a neurodevelopmental disorder. He is presenting with moderate ID, autistic features, and epilepsy. However, he does not have any motor or coordination problem. The different severity of ID, growth parameters, and the absence of motor impairment are possibly indicative of a different genetic etiology.

**Exome analysis**

In family 1, whole-exome sequencing (WES) was performed in the proband (IV:1) to exclude variants in genes previously reported to cause ID or developmental delay. Subsequently, SNP-array was performed in both affected individuals (IV:1 and IV:7), parents (III:3 and III:4) and an unaffected sibling (IV:3). Homozygosity mapping revealed a 2.5 Mb region of homozygosity (chr20:49010965-51638043) common in both patients (IV:1 and IV:7) but not in the parents (III:3 and III:4) and an unaffected sibling (IV:3). In total, six homozygous variants from the WES data of the proband (IV:1) were present in the segregating ROH (chr20:49010965-51638043) (as mentioned in the Supplementary Table 1), but the splicing variant (NM_006045.3:c.799+1G>T) in ATP9A was the only mutation with the MAF < 1% (in any of the population in the gnomAD database). (Fig. 1). The variant was not present in gnomAD17, Bravo (https://bravo.sph.umich.edu/freeze5/hg38/) or our local database of >500 Pakistani controls. Its segregation in the family was confirmed by Sanger sequencing, in particular, the youngest sister and second affected sibling is homozygous for this variant (Fig.1). The change at the conserved first nucleotide of the donor splice site was predicted to cause abnormal splicing by SpliceAI17 (score DS_DL = 0.99), MaxEntScan18 (MaxEntScan_diff = 8.504), and NNsplice19. RNA samples from affected individuals were not available to assess RNA splicing.

Our search for more cases led to the identification of a second family. The WES of proband IV:1 from family 2 also revealed the presence of a homozygous splicing variant in ATP9A, a base pair substitution in intron 3 of ATP9A (NM_006045.3:c.799+1G>T) in ATP9A was the only mutation with the MAF < 1% (in any of the population in the gnomAD database). The change at the conserved first nucleotide of the donor splice site was predicted to cause abnormal splicing by SpliceAI17 (score DS_DL = 0.99), MaxEntScan18 (MaxEntScan_diff = 8.504), and NNsplice19. RNA samples from affected individuals were not available to assess RNA splicing.

**Table 1. Clinical features of patients with homozygous ATP9A splicing variants.**

| Family | 1 | 1 | 2 |
|--------|---|---|---|
| Individual | IV:1 | IV:7 | IV:1 |
| Sex | F | F | M |
| Origin | Pakistani | Pakistani | Iranian |
| Consanguineous parents | Yes | Yes | Yes |
| Age at last evaluation (years) | 28 | 21 | 11 |
| ATP9A variant (gDNA) | Chr20:50305602 C > A | Chr20:50305602 C > A | Chr20:50342357 C > A |
| General characteristic | | | |
| Head circumference (cm) | 51 | 54 | 53 |
| Height (cm) | 149 | 169 | 140 |
| Weight (kg) | 64 | 67 | 45 |
| Microcephaly | + | – | – |
| Strabismus | + | + | – |
| Facial dysmorphism | + | + | + |
| Neurodevelopment | | | |
| Severe Intellectual disability | + | + | + |
| Motor delay | + | + | + |
| Speech delay/ dysfunction | + | + | + |
| Fine motor impairment | + | + | + |
| Epilepsy | – | – | + |
| Brain MRI anomalies | n.d. | n.d. | n.d. |
| Behavioral anomalies | | | |
| ADHD | + | + | n.d. |
| Stereotypic movement | n.d. | n.d. | + |
| Autistic features | – | – | + |
| Aggressiveness | + | + | n.d. |

*n.d. not determined, ADHD attention deficit hyperactivity disorder.*
Testing of the aberrant RNA splicing was not possible due to the unavailability of the patient's RNA or cells. Sanger sequencing confirmed the segregation of the potentially causative variant (Fig. 1), i.e., the variant is heterozygous in the proband’s parents (III:2 and III:3), his aunt (III:4) and absent in his uncle (III:1). Homozygosity mapping of proband 1 revealed that the ATP9A variant is embedded in a putative 6.83 Mb region of homozygosity (ROH) (chr20[GRCh37]: 45358223-52192534). While we did not find any likely pathogenic variants in known ID genes in proband IV:1 of family 2 (based on the Panelapp gene list for ID20; Supplementary Table 2), we cannot exclude those variants besides the ATP9A one might play a role in the patient’s phenotype. In particular, we identified homozygous variants in CCDC88C (NM_001080414.4: c.1126 C > T, p.Arg376Trp) and ZNF407 (NM_017757.3: c.5497 > T, p.Pro1833Ser), two genes previously implicated in neurodevelopmental disorders but associated with phenotypes different than the one found in our proband. Bi-allelic variants in CCDC88C were associated with a form of congenital hydrocephalus21–23, while variants in ZNF407 have been recently implicated in an AR form of ID with microcephaly, short stature, hypotonia, and ocular anomalies24,25.

**DISCUSSION**

Autosomal recessive ID is characterized by extensive genetic heterogeneity. Still, many patients do not receive a molecular diagnosis, suggesting that a considerable number of causative genes have not yet been identified4,26. We described three individuals from two consanguineous families with different homozygous splicing variants in canonical splice sites of the ATP9A gene. All three patients present with severe ID, motor delay, speech and fine motor impairment, and behavioral anomalies. Both affected sisters (IV:1 and IV:7) of family 1 had an attention deficit hyperactivity disorder-like phenotype combined with aggressiveness, whereas proband IV:1 from family 2 presented with autistic features, including prominent stereotypic movements, and lack of eye contact.

ATP9A is under constraint (intolerance to missense variants z-score = 4.15; pLI = 1; LOEUF = 0.2) according to gnomAD27. Its yeast homolog, NEO1, was shown to be an essential gene28, while the absence of the C. elegans orthologous TAT-5 resulted in disrupted cell adhesion and morphogenesis in worms’ embryos29. Whereas ablation of the mouse orthologous Atp9a did not diminish survival, the Atp9a−/− mice engineered and phenotyped by the International Mouse Phenotyping Consortium were hyperactive and showed a significant increased exploration in new environment reminiscent of the behavioral symptoms of our patients30,31. Depletion of ATP9A were lethal in human hepatoma HepG2 cells but not in other cell lines including HeLa, HEK293T, MCF-7, and THP-1, suggesting that the absence of ATP9A could be tolerated in certain tissues but not in others12,15. ATP8A2, another P4-ATPase highly expressed in the brain, has been implicated in a
recessive disorder characterized by cerebellar ataxia, ID, and disequilibrium syndrome (CAMRQ, MIM 615268), or severe hypotonia, ID, and optic atrophy with or without encephalopathy. A de novo balanced translocation leading to haploinsufficiency of this gene has been also proposed as the cause of moderate ID and hypotonia.

Downregulation of ATP9A has been associated with a significant increase of extracellular vesicles release, in particular the endosome, altering its recycling. Depletion of ATP9A reduces the plasma membrane expression of the glucose transporter GLUT1 and increases its level in the endosome, altering its recycling. Deficiency of GLUT1 has been associated with a neurological disorder with a variable phenotype including epilepsy, movement disorders, mild to severe ID, and acquired microcephaly in some cases. Similarly, alteration in the recycling endosomal processes by mutations in the sodium exchanger have been associated with Christianson syndrome (MIM 300243), a neurodevelopmental disorder characterized by ID, speech impairment, epilepsy, postnatal microcephaly, truncal ataxia, and hyperactivity.

Since the original submission of this paper and the deposit of our data in medRxiv, a study describing additional ATP9A cases was published. This latter study reports three affected individuals from two consanguineous families with homozgyous loss of function variants, p.(Arg290*) and c.542 + 1 G > A; p.(Ser184Profs*16) in ATP9A, and phenotypic manifestations similar to our study. Patients are all presenting with mild or severe ID, motor and speech delay. Behavioral anomalies, including attention deficit, were also reported in all affected individuals. All patients were noted to have microcephaly, a feature observed only in individual IV:1 of family 1 but not in her sister, IV:7. They were all reported to have short stature and failure to thrive, which are not observed in our patients. In the other cohort, strabismus was reported for only another affected individual but not in his brother, while here it is observed in both affected Pakistani sisters. Combined with ours, these results strengthen the hypothesis of the causative role of ATP9A biallelic truncation variants in a novel neurodevelopmental syndrome.

In conclusion, we describe a novel AR neurodevelopmental disorder. In two unrelated consanguineous families, we identified variants predicted to affect the splicing of ATP9A. The three individuals homozygous for these putatively truncating variants presented with severe ID, motor and speech impairment, and behavioral anomalies. Consistent with a causative role of ATP9A in the patients’ phenotypes, Atpp9a−/− mouse model showed behavioral changes.

METHODS

Recruitment

The current study was approved by the IRBs of the Khyber Medical University, Peshawar, Pakistan, and the University Hospitals of Geneva, Switzerland (Protocol number: CER 11-036). Informed consent forms were obtained from guardians of all affected individuals who participated in this study. Informed consent was obtained for the publication of photos from the guardians of the affected individuals of family 1.

Exome sequencing

The proband IV:1 of family 1 was subjected to exome sequencing (ES). DNA was enriched using SureSelect Human All Exon v6 capture kit (Agilent Technologies, Santa Clara, CA, USA) and sequenced on an Illumina HiSeq 4000 platform, with an average coverage of 120x at each nucleotide position. ES data were analyzed with an in-house customized pipeline that is based on published algorithms including BWA, SAMtools, PICARD (http://broadinstitute.github.io/picard/) and (GATK) . Initial screening for known or novel pathogenic mutations in the reported ID genes was performed. The 720 K SNP array was performed in parents (III:3 and III:4), affected (IV:1 and IV:7) and unaffected individuals (IV:3 and IV:5) of family 1 to identify Runs of Homozygosity (ROH) using PLINK as described previously. ROH and exome sequencing data were analyzed with CATCH to determine variants that were present in ROHs of patients (IV:1 and IV:7) but not in normal individuals of family 1. Subsequently, the variants were filtered manually by using the criteria described in published studies.

The exome of IV:1 from family 2 was captured using the xGen Exome Research Panel v2 (Integrated DNA Technologies) and sequenced using the illumina HSseq 4000 platform according to the manufacturer’s protocols. The overall mean-depth base coverage was 153-fold and 97% of the targeted region was covered at least 20-fold. Read mapping and variant calling were performed as described using the Varapp software. Homozygous and hemizygous variants with a MAF <1% in the general population (1000genome, EVS, gnomAD) were retained and screened for variants in reported ID genes (Supplementary Table 1). Homozygosity mapping was performed with AutoMap, which uses Variant Call Format (VCF) files from WES.

Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon request. The identified variants have been submitted to ClinVar under accession numbers SCV001911505-506.

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