







ORIGINAL ARTICLE

ATP1A3 mosaicism in families with alternating hemiplegia of childhood

Xiaoling Yang¹  | Xiaoxu Yang²  | Jiaoyang Chen¹ | Shupin Li¹ | Qi Zeng¹  | August Y. Huang²  | Adam Y. Ye^{2,3,4}  | Zhe Yu^{3,4} | Sheng Wang^{6,7} | Yuwu Jiang¹ | Xiru Wu¹ | Qixi Wu^{4,5}  | Liping Wei² | Yuehua Zhang¹ 

¹Department of Pediatrics, Peking University First Hospital, Beijing, China

²Center for Bioinformatics, State Key Laboratory of Protein and Plant Gene Research, School of Life Sciences, Peking University, Beijing, China

³Academy for Advanced Interdisciplinary Studies, Peking University, Beijing, China

⁴Peking-Tsinghua Center for Life Sciences, Peking University, Beijing, China

⁵Human Genetic Resources Core Facility, School of Life Sciences, Peking University, Beijing, China

⁶Dr Liping Wei's lab, National Institute of Biological Sciences, Beijing, China

⁷College of Biological Sciences, China Agricultural University, Beijing, China

Correspondence

Prof Yuehua Zhang, Department of Pediatrics, Peking University First Hospital, No.1 of Xian Men Street, Xicheng District, Beijing 100034, China. Email: zhangyhdr@126.com

Prof Liping Wei, Center for Bioinformatics, Peking University, Room 307, Wang Ke-Zhen Building, 5th Yiheyuan Road, Beijing 100871, China. Email: weilp@mail.cbi.pku.edu.cn

Dr Qixi Wu, Human Genetic Resources Core Facility, School of Life Sciences, Peking University, Room 342, Wang Ke-Zhen Building, 5th Yiheyuan Road, Beijing 100871, China. Email: wuqx@pku.edu.cn

Funding information

National Natural Science Foundation of China, Grant/Award Number: 81571265 and 31530092; National Natural Science Foundation for Young Scientists of China, Grant/Award Number: 31801043; Peking University Clinical Cooperation "985 Project", Grant/Award Number: PKU-2014-1-1

Alternating hemiplegia of childhood (AHC) is a rare and severe neurodevelopmental disorder characterized by recurrent hemiplegic episodes. Most AHC cases are sporadic and caused by *de novo* ATP1A3 pathogenic variants. In this study, the aim was to identify the origin of ATP1A3 pathogenic variants in a Chinese cohort. In 105 probands including 101 sporadic and 4 familial cases, 98 patients with ATP1A3 pathogenic variants were identified, and 96.8% were confirmed as *de novo*. Micro-droplet digital polymerase chain reaction was applied for detecting ATP1A3 mosaicism in 80 available families. In blood samples, four asymptomatic parents, including two paternal and two maternal, and one proband with a milder phenotype were identified as mosaicism. Six (7.5%) parental mosaicisms were identified in multiple tissues, including four previously identified in blood and two additional cases identified from paternal sperms. Mosaicism was identified in multiple tissues with varied mutant allele fractions (MAFs, 0.03%-33.03%). The results suggested that MAF of mosaicism may be related to phenotype severity. This is the first systematic report of ATP1A3 mosaicism in AHC and showed mosaicism as an unrecognized source of previously considered "*de novo*" AHC. Identifying ATP1A3 mosaicism provides more evidence for estimating recurrence risk and has implications in genetic counseling of AHC.

KEYWORDS

alternating hemiplegia of childhood, ATP1A3, *de novo*, micro-droplet digital PCR, mosaicism

Xiaoling Yang and Xiaoxu Yang contributed equally to this study.

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

© 2019 The Authors. Clinical Genetics published by John Wiley & Sons A/S. Published by John Wiley & Sons Ltd.

1 | INTRODUCTION

Alternating hemiplegia of childhood (AHC, MIM: 614820) is a rare and predominantly sporadic neurological disorder characterized by attacks of hemiplegia and other paroxysmal manifestations such as abnormal eye movement and dystonia.¹ Additional symptoms may appear later in the disease process.² Several study groups have identified *de novo* pathogenic variants in the *ATP1A3* gene as the cause of AHC in 93.3% to 100% of patients.^{3–5}

De novo variants have been discovered to be a more prominent disease-causing mutation type than inherited variants in numerous neurological genetic disorders, such as Dravet syndrome and autism spectrum disorders.^{6,7} *De novo* variant was an alteration in a gene that is present for the first time in one family member as a result of a mutation in a germ cell (egg or sperm) of one of the parents or in the fertilized egg itself. *De novo* variants are detectable in the disease-affected probands but undetectable in either of their parents in genomic DNA from peripheral blood sample. The consistency of gene-associated phenotypes and patients' manifestations were confirmed clinically.⁸ Many variants were considered *de novo* by either direct Sanger sequencing or next-generation sequencing (NGS) results validated by Sanger sequencing.^{3–5} *De novo* variants are considered to be either mainly prezygotic variants in the germ cells of parents, or postzygotic variants in the offspring.^{9,10} They have been reported to have strong paternal origin bias in large-scale family data.^{11–13} Some postzygotic mosaicisms in the parents may occur at the early stage and affect both the germ cells and somatic cells, known as gonosomal mosaicism.¹⁴ Recent publications have further reported that some “*de novo*” pathogenic variants are actually inherited from underestimated parental mosaicism.^{15,16}

In AHC, *de novo* single nucleotide pathogenic variants are more common than inherited variants.^{3–5} However, few families with autosomal dominant inheritance have been reported. A family with two AHC half-sisters born to an asymptomatic mother and different fathers, suggesting maternal mosaicism.¹⁷ Hully *et al* reported two unrelated families with two full siblings with *ATP1A3* variants presenting epilepsy and ataxia, and the authors suggested the potential occurrence of parental *ATP1A3* mosaicism.¹⁸

More sensitive genetic testing approaches are required for the detection and validation of mosaicism in AHC-affected families with *ATP1A3* variants, especially in parents with multiple AHC-affected children. NGS-based deep sequencing methods, such as personal genome machine amplicon sequencing for mosaicism (PASM) and molecular inversion probes, can be used to detect mutant allele fraction (MAF) as low as 1%, and duplex sequencing can detect even lower percentages.¹⁹ However, deep sequencing methods targeting the whole exome or genome require significantly increased sequencing depth to achieve higher detection limits. Next-generation micro-droplet digital polymerase chain reaction (mDDPCR) can be used to detect MAF as low as 0.01%, as is suitable for hotspot variants.²⁰

Here, we first report parental *ATP1A3* mosaicism in AHC families, regarded as “*de novo*” via Sanger sequencing, by mDDPCR and PASM using blood, sperm, and other tissues. We also identified *ATP1A3* mosaicism in one proband with milder AHC.

2 | MATERIALS AND METHODS

2.1 | Patient recruiting and inclusion criteria

AHC patients were recruited from Peking University First Hospital from August 2005 to May 2016. This study and the methodologies involved were in accordance with the relevant guidelines and regulations of the Institutional Review Board at Peking University (IRBPU) under the approval IRB00001052-11087. Written informed consent was provided by the patients or their statutory guardians. We labeled each family with Axxx according the enrolling time. In each family, each person has separate IDs. For example, A052 was the family ID. In this family, each family member was assigned a separate ID as: A05203: proband; A05201: father and A05202: mother.

All typical patients fulfilled the published clinical diagnostic criteria for AHC^{1,2}: (a) onset of first symptoms before 18 months of age; (b) repeated episodes of hemiplegia involving alternating body sides; (c) episodes of bilateral hemiplegia or quadriplegia, starting bilaterally as a separate attack or as a generalization of a hemiplegic episode; (d) disappearance of all symptoms upon sleeping, and symptoms possibly resuming later after waking; (e) other paroxysmal disturbances, including dystonic spells, abnormal oculomotor symptoms, or autonomic phenomena, occurring during hemiplegic spells or independently; and (f) evidence of developmental delay or neurologic abnormalities, including choreoathetosis, dystonia, or ataxia. Atypical cases with onset age later than 18 months but who fulfilled all other diagnostic criteria for AHC were also included.^{21,22} A Chinese AHC cohort consisting of 105 probands, 101 sporadic and 4 familial, were recruited (Figure 1A). Fifty-one patients had been described in our previous publication,⁵ and the remaining patients were not reported. The four multiplex families included one dominant family with two affected family members, two sets of monozygotic twins who were both affected with AHC, and one family with sibship of an affected brother and sister.

2.2 | DNA isolation, *ATP1A3* variant screening, and functional prediction of variants

Genomic DNA from peripheral blood was extracted using a salting-out procedure. Genomic DNA from buccal swab, saliva, hair follicles and urine was extracted using a QIAamp DNA Micro Kit (#56304, Qiagen, Germany). Skin biopsies were collected from a 5-mm-diameter skin punch, and DNA was extracted using an All Prep DNA/RNA Mini Kit (#80204; Qiagen). Sperm samples were purified with a PureSperm 40/80 assay (Nidacon, Sweden). Genomic DNA of purified sperm cells and unpurified semen were extracted using a phenol-chloroform extraction method.¹⁵

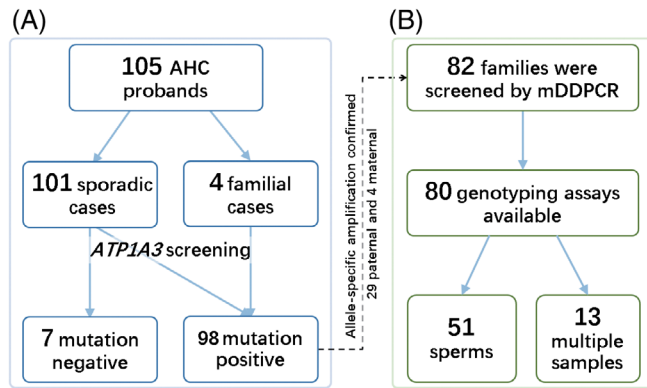


FIGURE 1 Schematic flowchart of patient recruiting and screening procedures. A, Schematic flowchart for patient recruiting and variant screening procedures. B, TaqMan MGB genotyping assays could be designed to perform variant allele discrimination in available families [Colour figure can be viewed at wileyonlinelibrary.com]

ATP1A3 variants were screened by Sanger sequencing or panel NGS. Before 2015, 74 patients were screened by Sanger sequencing. After 2015, the other 31 patients were sequenced by NGS panel sequencing. A total of 153 genes including *ATP1A3* were included in the panel and listed in Supporting Information, Table S1. All 23 coding exons and their flanking regions were sequenced using primers as previously described.⁴ Variants described in this article were based on reference cDNA NM_152296.4, reference amino acid sequence NP_689509.1, and reference genome assembly hg19. Deleterious variants detected in the probands were then sequenced in the blood samples of their parents or other available family members. Hundred unrelated healthy individuals were sequenced as controls. Functional predictions of the variants were carried out using conventional

prediction software as well as the classifiers we developed for AHC or *ATP1A3*.^{5,23} The variant and phenotype information have been deposited in <https://databases.lovd.nl/shared/genes/ATP1A3>.

2.3 | Detection of mutant allele origin and paternity

Allele-specific PCR (ASPCR) was carried out to determine the origins of the mutant alleles in the probands. Primers for amplifying the SNP fragments are listed in Table S2. For ASPCR, one or two pairs of allele-specific primers were designed based on the informative SNPs. The patients' two alleles were analyzed separately using paternal or maternal-specific primers containing different bases of the target variants. The primers used for ASPCR are listed in Table S3.

Short tandem repeat (STR) analysis was carried out for all informative families to confirm paternity and gender using 11 highly polymorphic microsatellite markers. The forward primer of each pair was labeled with FAM fluorophores. Primers for STR analysis are provided in Table S4. Labeled amplicons were detected on an ABI 3730 automated sequencer (Applied Biosystems by Thermo Fisher) and analyzed using genotype software (Gene Marker 1.5; SoftGenetics).

2.4 | mDDPCR screening and PASM for mosaicism

Raindrop mDDPCR was used for the detection of low-fraction mosaicism in 80 available families (Figure 1B). Genotyping assays were first tested using an endpoint-genotyping experiment. Ultraviolet treatments were carried out after each reaction to reduce contamination. After droplet emulsion generation and PCR reactions, post-PCR emulsions were subjected to a droplet detector, and data were analyzed

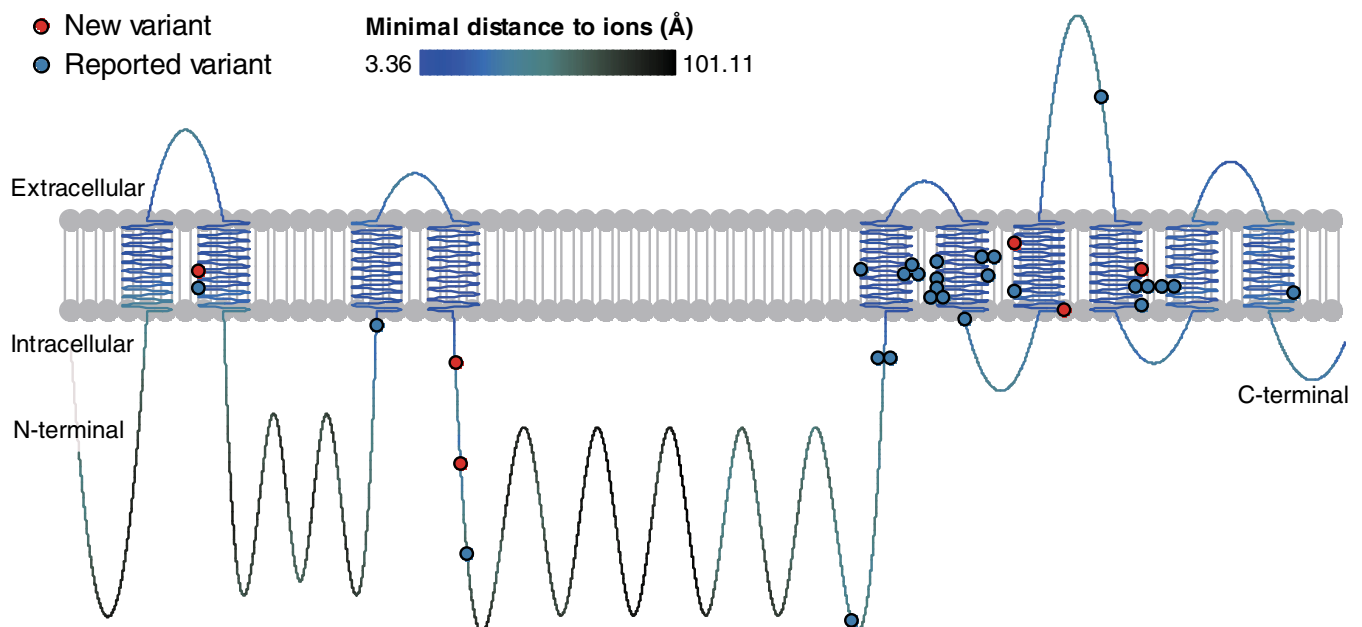


FIGURE 2 Locations of *ATP1A3* variants shown on protein domains. Thirty-three variants were identified. Six newly identified *ATP1A3* variants (red dots) and variants reported previously (blue dots) are shown [Colour figure can be viewed at wileyonlinelibrary.com]

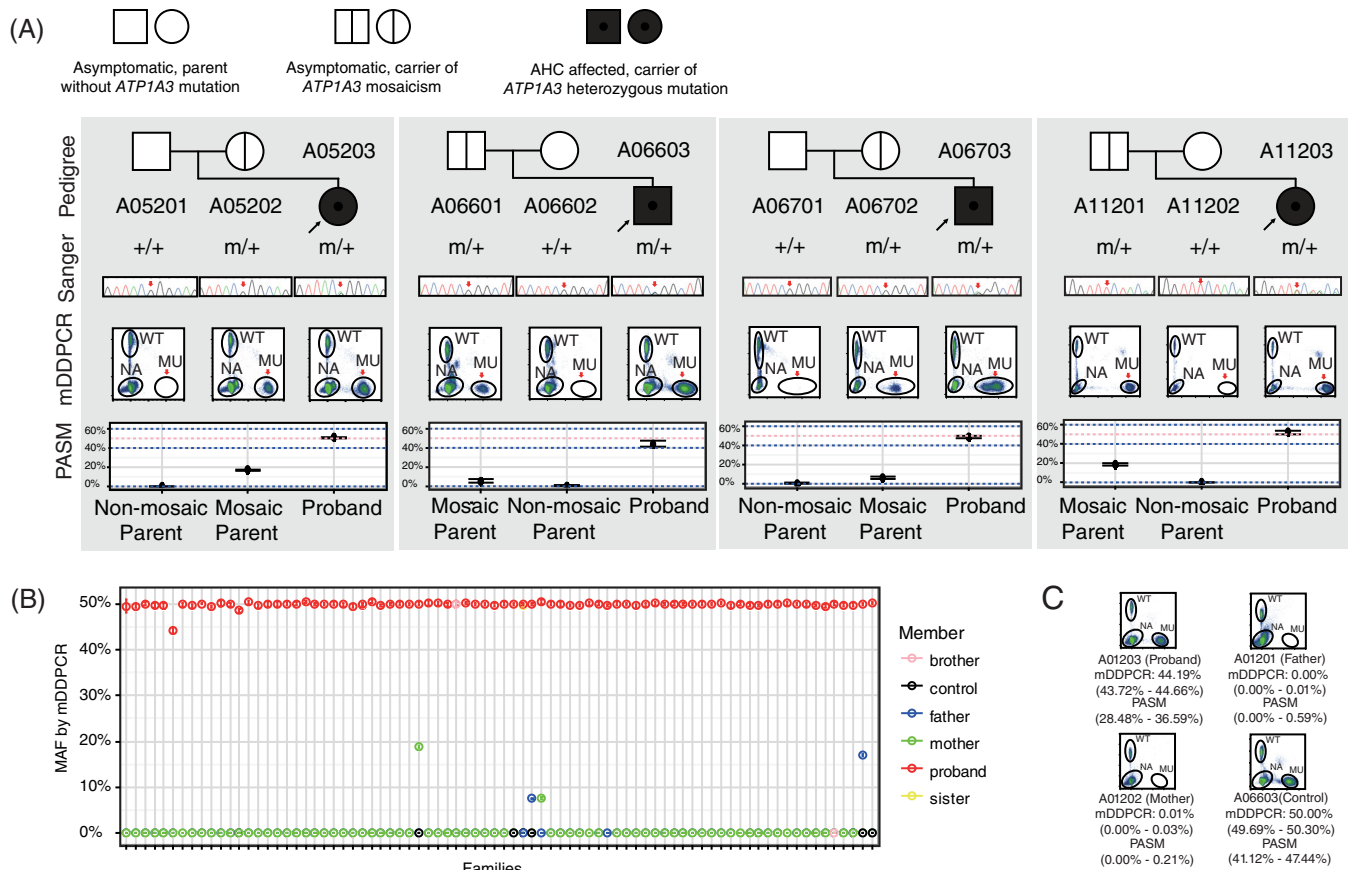


FIGURE 3 mDDPCR and PASM detected *ATP1A3* variants from blood samples of 80 AHC families. A, Four parental mosaic variants were detected by mDDPCR, and their pedigree charts are shown. Mosaic variants are clearly shown on the flow cytometry scatter plots of mDDPCR under the cluster name of “MU” at the bottom right corner. Peripheral blood from the proband was used as a positive control; the performance of the negative controls is not shown. B, mDDPCR considered AHC-affected families with “*de novo*” *ATP1A3* variants. Probands were all detected with a mutant allelic fraction (MAF) of 50%. The lower bounds of the 95% binomial CIs of the measured MAFs were under 0.01% in the non-mosaic parents and negative controls. C, The MAF identified by mDDPCR and PASM confirmed that A01203 carried a pathogenic variant on c.2839G>C/p.(Gly947Arg) with the MAF and the 95% confidence intervals that met the detection criteria for a mosaic variant. The proband A06603, who was detected with a heterozygous variant at the same base c.2839G>C/p.(Gly947Arg), was chosen as a positive control [Colour figure can be viewed at wileyonlinelibrary.com]

using RainDrop Analyst V3 software (RainDance Technologies, Billerica, Massachusetts). A binomial distribution was fitted, and the 95% confidence intervals (CIs) of MAFs were calculated. If the lower bound of the 95% CI of the MAF detected by mDDPCR was >0.01% and the upper bound was <50.00%, it was considered to be a positive detection according to our previous benchmark test.¹⁵ MAFs in detectable *ATP1A3* mosaic families were further quantified using PASM. The primers of PASM detection were provided in Table S5.

2.5 | Prenatal diagnosis

Prenatal diagnosis was performed upon further pregnancy of one proband's mother at 22 gestational weeks. A 20-mL amniotic fluid sample was collected under ultrasound guidance and cultured before DNA extraction. Fetal DNA was extracted using a Wizard Genomic DNA Purification Kit (Promega) according to the manufacturer's instructions. Before DNA sequencing, maternal cell contamination was excluded by STR analysis, as described above. The sex of the fetus

was determined by karyotype analysis and PCR amplification of the sex-determining region Y (NM_003140).

3 | RESULTS

3.1 | Sanger screening of *ATP1A3* pathogenic variants in probands and parents

Thirty-three deleterious variants were identified in the blood samples from 98 (93.3%, 98/105) probands (Table S6 and Figure 2). Of the 33 variants, 6 were novel. None of the 33 variants were common SNPs in dbSNP 137, and they were all absent in 1000 Genomes, the Personal Genome Project, ESP6500, gnomAD or ExAC. Their functional effects were predicted by Polyphen2, iFish and our logistic classifier (Table S6). The predicted functional effects for variants from this study and reported causal variants from the literature are similar, and these were different from the benign missense variant in the *ATP1A3* coding region in ExAC (Figures S1 and S2). According to the ACMG

TABLE 1 Positive mosaic families with blood samples of different origins (MAF and 95% CI in %)

Variant info			Father blood		Mother blood		Control blood		Proband blood						
Family Origin	Chr	Position ^a	Ref	Alt	Var (cDNA) ^b	Var (protein) ^c	mDDPCR	PASM	mDDPCR	PASM	mDDPCR	PASM	mDDPCR	PASM	
A012	Proband	19	42471896	C	G	c.2839G>C	p.(Gly947Arg)	0.00 (0.00-0.01)	0.0 (0.0-0.6)	0.01 (0.00-0.03)	0.0 (0.0-0.2)	0.0 (0.00-0.02)	0.0 (0.0-0.2)	44.19 (43.72-44.66)	32.5 (28.5-36.6)
A052	Maternal	19	42473598	C	T	c.2677G>A	p.(Gly893Arg)	0.00 (0.00-0.01)	0.0 (0.0-0.0)	18.82 (18.51-19.14)	17.1 (16.7-17.6)	0.00 (0.00-0.02)	0.0 (0.0-0.0)	49.98 (49.60-50.35)	50.9 (49.9-51.9)
A066	Paternal	19	42471896	C	G	c.2839G>C	p.(Gly947Arg)	7.53 (7.33-7.74)	4.5 (2.8-6.7)	0.01 (0.00-0.02)	0.0 (0.0-0.4)	0.00 (0.00-0.02)	0.0 (0.0-0.2)	50.00 (49.69-50.30)	44.3 (41.1-47.4)
A067	Maternal	19	42471896	C	T	c.2839G>A	p.(Gly947Arg)	0.00 (0.00-0.01)	0.2 (0.0-1.3)	7.65 (7.40-7.91)	6.4 (5.7-7.2)	0.00 (0.00-0.01)	0.0 (0.0-0.2)	50.41 (49.88-50.95)	49.2 (48.0-50.5)
A112	Paternal	19	42486187	GAA	c.1063	c.1063	p.(Glu355del)	17.05 (16.82-17.28)	18.8 (17.7-19.9)	0.00 (0.00-0.01)	0.0 (0.0-0.1)	0.0 (0.00-0.01)	NA	49.93 (49.64-50.22)	52.6 (50.7-54.6)

Abbreviations: CI, confidence interval; MAF, mutant allele fraction; mDDPCR, micro-droplet digital polymerase chain reaction; NA, not available.

^aGenomic position based on hg19.

^bAll variants based on cDNA NM_152296.4.

^cReference amino acid sequence NP_689509.1.

standard and guidelines,²⁴ all variants were classified as likely pathogenic.

For 93 out of 98 probands with *ATP1A3* variants, Sanger sequencing was performed in the blood DNA of their parents. We identified 96.8% (90/93) as *de novo* pathogenic variants and 3.2% (3/93) as inherited. For the three patients with inherited variants, one patient inherited the heterozygous variant from her AHC-affected mother,⁵ and the other two patients inherited the variant from the asymptomatic mother (A05202) or father (A11201), suggesting the mosaicism due to the lower signal (Figure 3A). ASPCR amplification were performed in 33 families with available SNPs. Twenty-nine (87.9%) were paternal and four (12.1%) were maternal, indicating significant parent-of-origin sex bias. One index case of paternal origin is shown in Figure S3, and the variants and parental origins are listed in Table S7.

In the family with two affected siblings (Family A065, Figure S4), the brother and sister carried the same pathogenic variant c.2401G>A/p.(Asp801Asn), and no variant signals were detected from the parents' blood DNA by Sanger sequencing. ASPCR amplification confirmed that both the proband and his sister inherited the variant from their father (Figure S5).

3.2 | Mosaic variants were detected by mDDPCR in the blood of four parents and one atypical proband

Among AHC families that were considered to carry “*de novo*” or “parental mosaic” *ATP1A3* pathogenic variants by Sanger sequencing, 82 families agreed to participate to further genetic tests of mosaicism screening, including Family A052, A112 and A065. Twenty-four genotyping assays were available for 80 families according to an endpoint genotyping qPCR analysis (Figure S6 and S7).

Four of 80 (5%) parental mosaic families were detected from the peripheral blood samples by mDDPCR. The MAFs were quantified and validated as 18.82% in the mother of Family A052, 17.05% in the father of Family A112, 7.65% in the mother of Family A067 and 7.53% in the father of Family A066. The MAFs of PASM were similar to those obtained by mDDPCR (Figure 3A and Table 1).

Mosaicism was also confirmed by mDDPCR and PASM in an atypical AHC proband (A01203) carrying the hotspot variant c.2839G>C/p.(Gly947Arg). MAF measured using the blood sample of proband A01203 was 44.19% by mDDPCR and 32.5% by PASM, which was significantly lower than all the other probands (Figure 3B, C). The onset age and the age of first hemiplegia of A01203 were distinctly later. The clinical phenotype of the A01203 was milder, compared with the four probands carrying the same heterozygous variant (Table 2).

3.3 | Two germline mosaic variants were only detected in paternal sperm samples

Given the high frequency of the paternal origin of the mutant alleles, we speculated that a collection of paternal sperm and multiple parental peripheral tissues might provide further evidence for parental mosaicism and important information for genetic counseling. Sperm

samples from 51 fathers were collected (Table S8), including parental mosaic cases, and healthy sperm was purified and subjected to mDDPCR. Apart from the mosaicisms that were detectable in paternal blood, mosaicisms were detected in sperms of two additional cases, A01501 (MAF of 0.03%) and A06501 (MAF of 12.42%; Figure 4A,B). Taken together, six (7.5%) parental mosaic cases were identified from the *ATP1A3* mutated AHC affected families.

3.4 | Mosaic variants were detected in multiple peripheral samples

Multiple samples from parents, including buccal swab, saliva, hair follicles, skin punch and urine, were collected from 13 individuals from 11 AHC families. Among the 13 individuals, 6 parents were from five mosaic families (A052, A065, A066, A067, and A112) and the other 7 parents were from six non-mosaic families (A012, A023, A050, A061, A082 and A111). Mosaic variants were also detected in multiple samples from blood-positive mosaic parents (Figure 4B and Table 3), but with different MAF values. Heterogeneity of the MAFs was observed among the different tissues, and the shared mosaicism suggested that the variants might have occurred at the early stages of embryonic development. High correlation was observed between MAFs measured

by PASM and mDDPCR ($R^2 = 0.9722$; Figure 4C), showing that the differences of MAF we observed are reliable.

To analyze the MAF differences among multiple samples, we performed hierarchical clustering for square-root-transformed MAFs detected by mDDPCR from the parental samples (Figure 4D). Euclidean distances were used for comparisons. From the clustering result, the probands showed the lowest similarity to the other samples. Samples from the blood of normal controls and non-mosaic parents had higher similarities. Most samples from mosaic parents showed a similar pattern. MAFs in blood and saliva were the most similar because of their mesodermal origin. Buccal swabs containing ectoderm-derived oral epithelium as well as mesoderm-derived white blood cells showed a similar MAF to the skin punch. These findings showed that the cell type might be an important factor influencing MAF. Additional cell divisions from the common progenitor cells might also contribute to this phenomenon. The mDDPCR raw flow cytometry scatter plots are presented in Figure S8, and MAF differences are compared in Figure S9.

3.5 | Prenatal diagnosis in families with parental *ATP1A3* mosaicism

Prenatal tests were then carried out for the mother from Family A067 c.2839G>A/p.(Gly947Arg) who was pregnant and expressed worries

TABLE 2 Phenotype of probands carrying the p.(Gly947Arg) pathogenic variant

	Patients ID				
	A01203	A02903	A05503	A06603	A08203
Sex	M	F	M	M	M
Father	–	–	–	–	–
Mother	–	+	–	–	–
Age of onset (months)	30	5	7	5	3
Abnormal eye movement	–	–	–	+	–
Dystonia	–	+	+	–	+
Hemiplegia	+	–	+	–	–
Quadriplegia	–	–	–	–	–
Seizure	–	–	–	–	–
Hemiplegia start (months)	30	11	7	8	3
Hemiplegia duration (days)	0.01~4	2~7	0.01~3	1~2	2~3
Hemiplegia frequency (per month)	3~15	1~2	2~3	2	2~3
Quadriplegia	Uncertain	+	+	–	–
Abnormal eye movement	+	+	–	+	–
Dystonia	+	+	+	+	+
Epilepsy	–	–	–	–	–
Automatic dysfunction	–	–	–	–	–
Dysphagia	+	–	–	–	–
Dysarthria	+	–	–	–	–
Respiratory disturbance	–	–	–	–	–
Ataxia	–	Uncertain	+	+	–
Developmental delay	+	+	+	+	Uncertain

Abbreviations: F, female; M, male

of AHC recurrence in the fetus. The mother was a mosaic carrier of the same *ATP1A3* pathogenic variants as her first child. The MAFs measured 7.65% from blood, 8.08% from saliva, 4.87% from buccal swab, 5.9% from urine and 0.05% from hair follicle (Table 3), suggesting a higher risk of recurrence. DNA of the fetus was extracted from the amniotic tissues, and Sanger sequencing was carried out to detect the same *ATP1A3* pathogenic variants of the proband. No detectable mutant signals were obtained in the fetus (Figure S10).

4 | DISCUSSION

In our Chinese cohort of 101 sporadic cases and 4 familial cases, 33 *ATP1A3* pathogenic variants were identified in 93.3% of the AHC patients. Of these, 96.8% were identified as *de novo* variants. We identified six (7.5% of 80 families, 4/80 in peripheral blood and 2/51 in sperm) mosaicism in the asymptomatic parents and one mosaic variant in the proband with milder phenotype by mDDPCR and PASM.

Sanger sequencing could only detect mosaicism with an MAF higher than 10%. For A05202 and A11201, the variant signal highly suggested the mosaicism and MAF of 18.82% and 17.05% was validated with mDDPCR. However, the low-level MAF could not be detected using conventional Sanger sequencing or low-coverage NGS. For A06601 and A06702, the Sanger sequencing result failed to tell the mosaicism and MAF of 7.53% and 7.65% were detected by

mDDPCR. More importantly, even for whole-exome data with an average depth of 200x, it was relatively difficult to determine mosaicism with MAF ~1%. For the majority of mosaic variants with a lower MAF, only more sensitive detection techniques could identify and quantify the mosaicism. From our benchmark tests and multiple validation of ultra-low fraction mosaicism, PASM (with an average sequencing depth of ~10 000x) could detect MAF as low as 0.5%,^{15,25,26} and mDDPCR could detect MAF as low as 0.01%.^{15,25}

The phenomenon of mosaicism was prevalent in neurodevelopmental diseases, such as Dravet syndrome and *PCDH19*-related epilepsy.^{15,26,27} In the family of A065, two siblings were both diagnosed with AHC carrying the same pathogenic variant c.2401G>A/p.(Asp801Asn), however, this variant was not detected in father's blood. By mDDPCR, MAF of 12.42% was detected in his sperm. When two siblings were affected in one family, parental mosaicism should be considered. Genetic counseling and appropriate prenatal diagnosis are strongly recommended. Mosaicism might have originated after the progenitor cells of the germline tissue diverged from somatic tissues. These results alerted us that using samples from the parental germline might provide more important information compared with peripheral samples in clinical genetic testing. Testing for mosaicism in all available tissues could enable us to determine the stage of occurrence of the deleterious variants. For “*de novo*” pathogenic variants in genetic diseases, more sensitive techniques may be performed to detect unrecognized low levels of parental mosaic

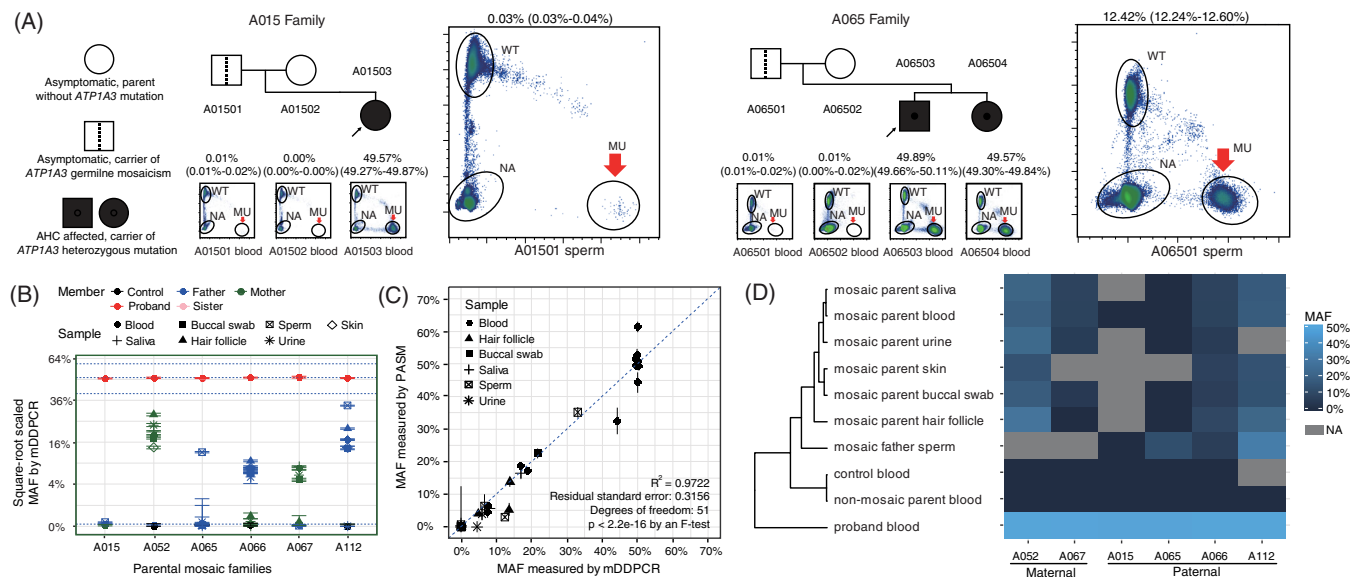


FIGURE 4 Screen of multiple parental peripheral samples and paternal sperm samples identified two additional mosaic cases. A, Mutant alleles were identified from the sperm samples of the A015 and A065 fathers (A01501 and A06501, sperm samples are enlarged); no signal that could pass the threshold was identified from their blood samples. MAFs and 95% binomial confidence intervals (CIs) are labeled for each sample. B, Mosaic variants are detectable from multiple parental peripheral samples. Error bars show the 95% CIs from binomial estimations for MAFs. Shapes are used to distinguish tissues, and colors distinguish family members. MAFs of A01501 sperm were difficult to distinguish from 0. C, The multiple parental samples were also examined by PASM, and the correlation of the PASM and mDDPCR-estimated MAFs is shown. D, Hierarchical clustering for square-root-transformed MAFs measured by mDDPCR from multiple parental peripheral samples is shown using Euclidean distance measurement. Mosaic parental urine samples clustered closer to the branch of non-mosaic control samples than other mosaic parental samples. Blood and saliva MAFs clustered closer than other parental samples. Skin punch and hair follicle samples showed similar MAFs. Details for the mDDPCR analysis for parental tissues are shown in Figures S8 and S9 [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 3 Positive mosaic families with multiple tissue samples of different origins (MAF and 95% CI in %)

Variant info				Mosaic parents' multiple peripheral samples										
Family	Origin	Chr	Position ^a	Ref	Alt	Var (cDNA) ^b	Var (protein) ^c	Father sperm	Blood	Buccal swab	Saliva	Hair follicle	Urine	Skin punch
A015	Paternal	19	42474436	C	T	c.2443G>A	p.(Glu815Lys)	0.03 (0.03-0.04)	0.01 (0.01-0.02)	NA	NA	NA	NA	NA
A052	Maternal	19	42473598	C	T	c.2677G>A	p.(Gly893Arg)	NA	18.82 (18.51-19.14)	17.60 (17.19-18.02)	20.51 (20.27-20.75)	28.25 (27.58-28.93)	23.24 (22.48-24.01)	14.13 (13.64-14.62)
A065	Paternal	19	42474557	C	T	c.2401G>A	p.(Asp801Asn)	12.42 (12.24-12.60)	0.01 (0.01-0.02)	0.01 (0.00-0.04)	0.01 (0.00-0.08)	0.00 (0.00-0.01)	0.00 (0.00-0.98)	NA
A066	Paternal	19	42471896	C	G	c.2839G>C	p.(Gly947Arg)	6.41 (6.26-6.56)	7.53 (7.33-7.74)	6.48 (6.34-6.62)	8.05 (7.82-8.29)	9.64 (9.39-9.90)	5.49 (4.17-7.09)	7.70 (7.48-7.93)
A067	Maternal	19	42471896	C	T	c.2839G>A	p.(Gly947Arg)	0.00 (0.00-0.00)	7.65 (7.40-7.91)	4.87 (4.52-5.23)	8.08 (7.90-8.27)	0.05 (0.00-0.25)	5.90 (4.80-7.16)	NA
A112	Paternal	19	42486187 _42486189	GAA	c.1063	p.(Glu355del) _1065del		33.03 (32.78-33.27)	17.05 (16.82-17.28)	13.88 (13.30-14.49)	16.92 (16.65-17.18)	21.66 (21.37-21.95)	0.00 (0.00-0.00)	14.03 (13.59-14.49)

Abbreviation: CI, confidence interval; MAF, mutant allele fraction; NA, not available.

^aGenomic position based on hg19.

^bAll variants based on cDNA NM_152296.4.

^cReference amino acid sequence NP_689509.1.

variant that may be delivered to the offspring. We also believe that the use of mDDPCR and PASM could help to validate potential mosaicism in families with AHC and other genetic disorders with seemingly recurrent *de novo* variants.¹⁷

Mosaicism in the AHC proband is also reported for the first time in this study. The patient (A01203) was clinically diagnosed as an atypical AHC case, because his age of onset was later than 18 months. His pathogenic variant was identified as *de novo* after validation in his parents. The variant c.2839G>C/p.(Gly947Arg) was reported in other studies³ and was confirmed to be related to the AHC phenotype. In other studies, Panagiotakaki et al reported p.Gly947Arg appeared to correlate with the most favorable prognosis, compared to the other two frequent variants (p.Asp801Asn and p.Glu815Lys).²⁸ Our observations were consistent with other studies. In this study, five probands, including A01203, carried the same variant p.G947R. The onset age of A01203 was later, the frequency and duration of hemiplegia were less severe, and his developmental delay was milder, compared with the other four probands. MAF measured from the blood sample in A01203 was 44.19%, which was significantly lower than all the other probands (Figure 3B). In this proband, mosaicism was only tested in the DNA from peripheral blood. We would like to look at levels of mosaicism in other tissue types if the patient consents to participate in the testing. It is reasonable to presume that MAF in the proband's brain might be even lower, contributing to the phenotypic differences. The observation of attenuated phenotype in the mosaic probands is in accordance with our previous report in Dravet syndrome,²⁶ however, we still need more patients to study the mosaicism in AHC probands. Mosaicism in the proband has been reported in other genes such as *PCDH19* (8.3%, 6/73), *SCN1A* (1.3%, 4/320), *SCN2A* (6.4%, 7/110), and *CDKL5* (8.8%, 8/91), according to a recent large-scale study of epilepsy-related neurodevelopmental disorders.²⁹

Based on our observations^{15,16,30} and others' reports,³¹⁻³³ detectable mosaic variants are derived either from early embryonic development or from clonal expansion during cell proliferation. Of the six validated parental mosaic cases, four were paternal and two were maternal. More paternal mosaicism than maternal mosaicism was observed. Regarding the four maternal pathogenic variants detected by ASPCR, mDDPCR could not detect mosaicism from the blood of carriers of the maternal pathogenic variants. As female germ cells were not collected and screened for mosaicism, variants that could only be observed in female germline and not in other tissues were likely to be missed in our study, and thus the overall proportion of maternal mosaicism cases might have been underestimated.

We observed that the differences in MAF between tissues showed similarities between mesoderm and ectoderm-derived samples, similar to our previous observations for samples from Dravet syndrome families.¹⁵ We also found that mosaic variants that were detectable in paternal blood could be detected in their sperm, although the sample size was small. However, MAFs in the sperm of AHC fathers are not always higher than those in their blood. The Pur-eSperm assay we used to purify male germline cells with reproductive activity could guarantee the vitality of the sperm. Thus, MAFs measured in the sperm samples directly reflected the proportion of sperm

carrying deleterious alleles at the time of donation. Our results emphasize the urgency of including sperm detection as well as multiple parental samples for genetic testing and counseling for parents who have already had a child with AHC.

Based on our published results in Dravet syndrome cohorts, probands carrying mosaicism with a higher MAF suggest a disease-related phenotype.¹⁵ Six parents with lower level MAF of *ATP1A3* mosaicism (<18.82%) were asymptomatic. One mosaic proband with higher level MAF of *ATP1A3* mosaicism (44.19%) had milder phenotype compared with other heterozygous *ATP1A3* AHC patients. We speculated that MAF of mosaicism was related to the severity of clinical manifestations. Parents carrying lower MAF mosaicism who were missed by panel NGS sequencing, whole-exome sequencing, or Sanger sequencing tended to be asymptomatic.^{15,26} For the seven probands without *ATP1A3* variant, the more likely hypothesis is that additional pathogenic genes are responsible for AHC. We are planning to carry out whole-exome sequencing to identify new pathogenic genes.

In conclusion, this is the first report to identify parental mosaicism of *ATP1A3* in AHC families. In this study, we showed that 7.5% (6/80) of presumed “*de novo*” *ATP1A3* variants with AHC were discovered to be mosaic, and MAF of mosaicism was speculated to be related to severity of clinical manifestations. These findings have significant implications in genetic counseling for AHC patients.

ACKNOWLEDGEMENTS

This study was supported by National Natural Science Foundation of China (Grant No. 81571265 and 31530092), National Natural Science Foundation for Young Scientists of China (No. 31801043), and Peking University Clinical Cooperation “985 Project” (PKU-January 1, 2014). We thank the patients and their families for their participation and support. We thank Rui Wei, Jiayin Li and Shun Huang for the sample collection. We thank Dr. Xiaodong Wang from CipherGeneLtd for her valuable suggestions.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY

We have deposited all the variation and phenotype data in the LOVD database (Patient ID 00155754-00155762, 00163007-00163023, 00163692-00163741, 00163984, 00163999-00164008; variant ID 359660-359685, 367799-367848, 368415-368457, 368462, 368479-368488). Raw data containing mDDPCR flow cytometry signals (FCS files) are available at <https://pan.baidu.com/s/1i5vi5Op> under access code “wgt6”.

Author contributions

X.Y.1, X.Y.2, Q.W., S.L., Q.Z., and J.C. collected the data. Y.Z., X.Y.1, S.L., Q.Z., and J.C. performed the clinical diagnoses and recruited the patients. Q.W., X.Y.2, and X.Y.1 designed the experiments. X.Y.2, A.Y.H., and A.Y.Y. carried out the bioinformatics and statistical analyses. X.Y.1, X.Y.2, Y.Z., Q.W., L.W., A.Y.H., and A.Y.Y. wrote and revised the manuscript. Y.Z., L.W., Q.W., X.W., and Y.J. conceived the project.

ORCID

Xiaoling Yang  <https://orcid.org/0000-0002-7953-5678>

Xiaoxu Yang  <https://orcid.org/0000-0003-0219-0023>

Qi Zeng  <https://orcid.org/0000-0003-2856-9808>

August Y. Huang  <https://orcid.org/0000-0002-0416-2854>

Adam Y. Ye  <https://orcid.org/0000-0002-1542-0740>

Qixi Wu  <https://orcid.org/0000-0002-9959-2629>

Yuehua Zhang  <https://orcid.org/0000-0003-1432-5423>

REFERENCES

- Bourgeois M, Aicardi J, Goutieres F. Alternating hemiplegia of childhood. *J Pediatr*. 1993;122:673-679.
- Panagiotakaki E, Gobbi G, Neville B, et al. Evidence of a non-progressive course of alternating hemiplegia of childhood: study of a large cohort of children and adults. *Brain*. 2010;133:3598-3610.
- Heinzen EL, Swoboda KJ, Hitomi Y, et al. De novo mutations in *ATP1A3* cause alternating hemiplegia of childhood. *Nat Genet*. 2012;44:1030-1034.
- Rosewich H, Thiele H, Ohlenbusch A, et al. Heterozygous de-novo mutations in *ATP1A3* in patients with alternating hemiplegia of childhood: a whole-exome sequencing gene-identification study. *Lancet Neurol*. 2012;11:764-773.
- Yang X, Gao H, Zhang J, et al. *ATP1A3* mutations and genotype-phenotype correlation of alternating hemiplegia of childhood in Chinese patients. *PLoS One*. 2014;9:e97274.
- Claes L, Del-Favero J, Ceulemans B, et al. De novo mutations in the sodium-channel gene *SCN1A* cause severe myoclonic epilepsy of infancy. *Am J Hum Genet*. 2001;68:1327-1332.
- Neale BM, Kou Y, Liu L, et al. Patterns and rates of exonic de novo mutations in autism spectrum disorders. *Nature*. 2012;485:242-245.
- Hoischen A, van Bon BW, Gilissen C, et al. De novo mutations of *SETBP1* cause Schinzel-Giedion syndrome. *Nat Genet*. 2010;42:483-485.
- Ku CS, Tan EK, Cooper DN. From the periphery to centre stage: de novo single nucleotide variants play a key role in human genetic disease. *J Med Genet*. 2013;50:203-211.
- Ronemus M, Iossifov I, Levy D, Wigler M. The role of de novo mutations in the genetics of autism spectrum disorders. *Nat Rev Genet*. 2014;15:133-141.
- Goldmann JM, Wong WS, Pinelli M, et al. Parent-of-origin-specific signatures of de novo mutations. *Nat Genet*. 2016;48:935-939.
- Turner TN, Coe BP, Dickel DE, et al. Genomic patterns of de novo mutation in simplex autism. *Cell*. 2017;171:710-722.e712.
- Jonsson H, Sulem P, Kehr B, et al. Parental influence on human germline de novo mutations in 1,548 trios from Iceland. *Nature*. 2017;549:519-522.
- Biesecker LG, Spinner NB. A genomic view of mosaicism and human disease. *Nat Rev Genet*. 2013;14:307-320.

15. Yang X, Liu A, Xu X, et al. Genomic mosaicism in paternal sperm and multiple parental tissues in a Dravet syndrome cohort. *Sci Rep*. 2017;7:15677.
16. Dou Y, Yang X, Li Z, et al. Postzygotic single-nucleotide mosaicisms contribute to the etiology of autism spectrum disorder and autistic traits and the origin of mutations. *Hum Mutat*. 2017;38:1002-1013.
17. Kramer U, Nevo Y, Margalit D, Shorer Z, Harel S. Alternating hemiplegia of childhood in half-sisters. *J Child Neurol*. 2000;15:128-130.
18. Hully M, Ropars J, Hubert L, et al. Mosaicism in ATP1A3-related disorders: not just a theoretical risk. *Neurogenetics*. 2017;18:23-28.
19. Schmitt MW, Kennedy SR, Salk JJ, Fox EJ, Hiatt JB, Loeb LA. Detection of ultra-rare mutations by next-generation sequencing. *Proc Natl Acad Sci USA*. 2012;109:14508-14513.
20. Watanabe M, Kawaguchi T, Isa S, et al. Ultra-sensitive detection of the pretreatment EGFR T790M mutation in non-small cell lung cancer patients with an EGFR-activating mutation using droplet digital PCR. *Clin Cancer Res*. 2015;21:3552-3560.
21. Mikati MA, Kramer U, Zupanc ML, Shanahan RJ. Alternating hemiplegia of childhood: clinical manifestations and long-term outcome. *Pediatr Neurol*. 2000;23:134-141.
22. Roubergue A, Roze E, Vuillaumier-Barrot S, et al. The multiple faces of the ATP1A3-related dystonic movement disorder. *Mov Disord*. 2013;28:1457-1459.
23. Wang M, Wei L. iFish: predicting the pathogenicity of human non-synonymous variants using gene-specific/family-specific attributes and classifiers. *Sci Rep*. 2016;6:31321.
24. Richards S, Aziz N, Bale S, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med*. 2015;17:405-424.
25. Wang K, Lai S, Yang X, et al. Ultrasensitive and high-efficiency screen of de novo low-frequency mutations by o2n-seq. *Nat Commun*. 2017;8:15335.
26. Xu X, Yang X, Wu Q, et al. Amplicon resequencing identified parental mosaicism for approximately 10% of "de novo" SCN1A mutations in children with Dravet syndrome. *Hum Mutat*. 2015;36:861-872.
27. Liu A, Yang X, Yang X, et al. Mosaicism and incomplete penetrance of PCDH19 mutations. *J Med Genet*. 2019;56:81-88.
28. Panagiotakaki E, De Grandis E, Stagnaro M, et al. Clinical profile of patients with ATP1A3 mutations in alternating hemiplegia of childhood—a study of 155 patients. *Orphanet J Rare Dis*. 2015;10:123.
29. Stosser MB, Lindy AS, Butler E, et al. High frequency of mosaic pathogenic variants in genes causing epilepsy-related neurodevelopmental disorders. *Genet Med*. 2018;20:403-410.
30. Huang AY, Yang X, Wang S, et al. Distinctive types of postzygotic single-nucleotide mosaicisms in healthy individuals revealed by genome-wide profiling of multiple organs. *PLoS Genet*. 2018;14:e1007395.
31. Abyzov A, Tomasini L, Zhou B, et al. One thousand somatic SNVs per skin fibroblast cell set baseline of mosaic mutational load with patterns that suggest proliferative origin. *Genome Res*. 2017;27:512-523.
32. Lodato MA, Rodin RE, Bohrsen CL, et al. Aging and neurodegeneration are associated with increased mutations in single human neurons. *Science*. 2018;359:555-559.
33. Myers CT, Hollingsworth G, Muir AM, et al. Parental mosaicism in "De novo" epileptic encephalopathies. *N Engl J Med*. 2018;378:1646-1648.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Yang X, Yang X, Chen J, et al. ATP1A3 mosaicism in families with alternating hemiplegia of childhood. *Clin Genet*. 2019;96:43–52. <https://doi.org/10.1111/cge.13539>