ORIGINAL ARTICLE

Mosaicism and incomplete penetrance of *PCDH19* mutations

Aijie Liu,[1,2] Xiaoxu Yang,[3] Xiaoling Yang,[1] Qixi Wu,[4,5] Jing Zhang,[1] Dan Sun,[6] Zhixian Yang,[1] Yuwu Jiang,[1] Xiru Wu,[1] Liping Wei,[3] Yuehua Zhang[1]

[1]Department of Pediatrics, Peking University First Hospital, Beijing, China
[2]Department of Pediatric Neurology, Capital Institute of Pediatrics, Beijing, China
[3]Center for Bioinformatics, State Key Laboratory of Protein and Plant Gene Research, School of Life Sciences, Peking University, Beijing, China
[4]School of Life Sciences, Peking University, Beijing, China
[5]Peking-Tsinghua Center for Life Sciences, Peking University, Beijing, China
[6]Department of Neurology, Wuhan Children's Hospital, Wuhan, China

Correspondence to
Dr Liping Wei, Center for Bioinformatics, State Key Laboratory of Protein and Plant Gene Research, School of Life Sciences, Peking University, Beijing 100871, China; weilp@mail.cbi.pku.edu.cn and Professor Yuehua Zhang, Department of Pediatrics, Peking University First Hospital, Beijing 100034, China; zhangyhdr@126.com

AL, XxY and XIY contributed equally.

Check for updates

ABSTRACT

Background Mutations in the *PCDH19* gene have mainly been reported in female patients with epilepsy. To date, *PCDH19* mutations have been reported in hundreds of females and only in 10 mosaic male epileptic patients with mosaicism.

Objective We aimed to investigate the occurrence of mosaic *PCDH19* mutations in 42 families comprising at least one patient with *PCDH19*-related epilepsy.

Methods Two male patients with mosaic *PCDH19* variants were identified using targeted next-generation sequencing. Forty female patients with *PCDH19* variants were identified by Sanger sequencing and Multiple Ligation Probe Amplification (MLPA). Microdroplet digital PCR was used to quantify the mutant allelic fractions (MAFs) in 20 families with *PCDH19* variants.

Results Five mosaic individuals, four males and one female, were identified in total. Mosaic variant was confirmed in multiple somatic tissues from one male patient and in blood from the other male patient. Among 22 female patients harbouring a newly occurred *PCDH19* variant identified by Sanger sequencing and MLPA, Sanger sequencing revealed two mosaic fathers (9%, 2/22), one with two affected daughters and the other with an affected child. Two asymptomatic mosaic fathers were confirmed as gonosomal mosaicism, with MAFs ranging from 4.16% to 37.38% and from 1.27% to 19.13%, respectively. In 11 families with apparent de novo variants, 1 female patient was identified as a mosaic with a blood MAF of 26.72%.

Conclusion Our study provides new insights into phenotype-genotype correlations in *PCDH19* related epilepsy and the finding of high-frequency mosaicism has important implications for genetic counselling.

INTRODUCTION

Point mutations or rearrangements involving the *PCDH19* gene (MIM# 300460) located on Xq22.1 cause epilepsy and mental retardation mainly in females.[1–3] The main clinical manifestations of *PCDH19*-related epilepsy include early seizure onset, generalised or focal seizures highly sensitive to fever and brief seizures occurring in clusters.[4] The disorder shows an unusual X-linked inheritance affecting heterozygous females and sparing hemizygous males.[1] However, males with mosaic mutations can also be affected.[5] This mode of inheritance might be explained by 'cellular interference'.[5 6] According to this hypothesis, the coexistence of wild-type and mutant cell populations may scramble cell-cell communication.

Recently, next-generation sequencing (NGS) technologies have enabled the identification of mosaicism for *PCDH19* point mutations in male patients with epilepsy.[7 8] To date, *PCDH19* mutations have been reported in hundreds of females and only in 10 mosiac male patients with epilepsy.[5–9] In addition, mosaic *PCDH19* mutations have been reported in mildly affected or even unaffected mothers.[10 11] Interestingly, we identified an asymptomatic mosaic father with two affected daughters.[12] Our previous study revealed parental mosaicism for approximately 10% of apparent de novo *SCN1A* mutations in children with Dravet syndrome.[13] Since de novo *PCDH19* mutations occurred in more than half of the sporadic cases,[14] we suspected that parental mosaic mutations of *PCDH19* might be underestimated.

In this study, we investigated 42 Chinese families comprising at least one patient with *PCDH19*-related epilepsy (online supplementary figure 1). Our objectives were to estimate the frequency of mosaicism in a Chinese cohort of *PCDH19*-related epileptic families and to determine the role of mosaicism in phenotypic variations, thereby providing better informed genetic counselling.

METHODS

Subjects

We recruited 42 *PCDH19*-related epileptic families from the Pediatric Clinic of Peking University First Hospital and Wuhan Children's Hospital between October 2007 and July 2017 (online supplementary figure 2). Written informed consent was provided by participants or their statutory guardians before enrolment. In the 42 families, 21 families were previously reported[12] and 21 were families newly recruited. The clinical features and the results of the mutation analysis in these new families are summarised in table 1.

Genetic analysis

Peripheral blood leucocytes obtained from the 42 families, as well as samples obtained from other available tissues, were used for this study. Genomic DNA from peripheral blood was isolated using a simple salting-out procedure.[15] Genomic DNA from saliva, hair follicles, buccal swab, urine and finger nails of four individuals with suspected mosaicism was collected using a QIAamp DNA micro kit (Qiagen) or a TIANamp micro DNA kit (Tiangen).[13 16] A PureSperm 40/80 assay (Nidacon) was used for the purification of vital sperm from

Table 1 The clinical manifestations of 21 newly assessed patients with epilepsy with *PCDH19* pathogenic variants

Proband no. (Sex)	Sz onset (months)	Sz types	Fever sensitivity	Seizure clusters	Status epileptic	Intellectual disability	Psychiatric problem	AEDs	Age (years) and seizure frequency at last follow-up	PCDH19 variants	Transmission	Reported/novel
22 (F)	41	Focal	–	+	–	+	–	VPA	4.5/seizure free for 1 year	c.1987delT p.S663PfsX13	Father (1 sib affected)	Novel
23 (F)	27	GTCS, Focal, MS	+	+	–	+	Aggression	OXC, TPM, LTG, VPA	8/seizure free for 2 years and 4 months	c.1240G>A p.E414K	Asymptomatic Mother	Reported
24 (F)	13	GTCS, Focal	+	+	–	–	–	VPA	3/8 months between clusters	Whole *PCDH19* deletion	De novo	Reported
25 (M)	5	Focal	–	+	–	+	Aggression	OXC, LEV, VPA, TPM	3/1–2 months between clusters	c.317T>A p.M106R (Mosaic)	De novo	Novel
26 (M)	9	GTCS, AbS, MS	+	+	–	+	Aggression, ASD	VPA. TPM	9/seizure free for 5 years	c.158dupT p.D54GfsX35 (Mosaic)	De novo	Novel
27 (F)	4	GTCS, Focal, AbS	–	+	–	+	Aggression, ASD	OXC, LEV, VPA, TPM	4/monthly clusters	c.262G>T p.D88Y	De novo	Reported
28 (F)	6	GTCS, Focal	–	+	–	+	Aggression, ASD	VPA, OXC, LEV	4/4 months between clusters	c.497_498insA p.Y166X	Familial (3-generation affected)	Reported
29 (F)	12	GTCS	+	+	–	–	–	LEV	2/6 months between clusters	c.2341delA p.I781SfsX19 (Mosaic)	De novo	Reported
30 (F)	11	GTCS, Focal	+	+	–	+	–	VPA, LEV	2/3 months between clusters	c.471C>G p.D157E	De novo	Novel
31 (F)	24	GTCS, Focal	+	+	–	–	–	OXC	1/4 months between clusters	c.2113C>T p.R705X	Father	Novel
32 (F)	18	GTCS	+	+	–	+	–	VPA, LEV, TPM	7/seizure free for 1 year and 6 months	c.134_135delAC p.D45GfsX43	Father	Reported
33 (F)	10	GTCS	+	+	–	–/–	Aggression	PB, VPA, LEV	2/6–8 months between clusters	c.64delC p.L22fsX8	De novo	Novel
34 (F)	21	GTCS, Focal	+	+	–	–	Aggression	VPA, LEV, CZP	4/seizure free for 1 year and 2 months	c.183_184insT p.R62SfsX27	De novo	Novel
35 (F)	9	GTCS	+	+	–	+	Aggression	TPM, LEV	6.5/5 months between clusters	c.1019A>G p.N340S	Asymptomatic Mother	Reported
36 (F)	42	GTCS	+	+	–	+	Aggression	VPA, LEV, TPM	7/seizure free for 2.5 years	c.339_340insC p.V114RfsX112	Familial (3-generation affected)	Novel
37 (F)	15	GTCS, Focal	+	+	–	+	Aggression	LEV, CZP	4.5/5 months between clusters	c.1178C>T p.P393L; c.1191G>C p.Q397H	Father	Reported; Novel
38 (F)	16	GTCS, Focal	+	+	–	+	Aggression	VPA, LEV, CBZ, LTG	11.5/seizure free for 1 year and 9 months	c.1133C>G p.S378X	De novo	Novel
39 (F)	14	GTCS	+	+	–	+	Aggression	TPM	2/5 months between clusters	c.1681C>T p.P561S	De novo	Reported
40 (F)	17	GTCS, Focal	-	+	+	+	Aggression	OXC, VPA	2/2–7 months between clusters	c.2849-1delG	Asymptomatic Mother	Novel

Continued

Table 1 Continued

Proband no. (Sex)	Sz onset (months)	Sz types	Fever sensitivity	Seizure clusters	Status epileptic	Intellectual disability	Psychiatric problem	AEDs	Age (years) and seizure frequency at last follow-up	PCDH19 variants	Transmission	Reported/novel
41 (F)	8	GTCS, Focal	+	+	+	+	Aggression, ASD	LEV, VPA, LTG	2.5/monthly clusters	c.1804C>T;p.R602X	De novo	Reported
42 (F)	10	Focal	+	+	–	+	–	OXC, VPA, CZP, LEV	3/1–10 months between clusters	Whole PCDH19 deletion	De novo	Reported

Abs, absence seizure; AED, antiepileptic drugs; ASD, autism spectrum disorder; CBZ, carbamazepine; CZP, clonazepam; GTCS, generalized tonic clonic seizure; LEV, levetiracetam; LTG, lamotrigine; MS, myoclonic seizures; OXC, oxcarbazepine; PB, phenobarbital; Sz, seizure; TPM, topiramate; VPA, sodium valproate.

paternal semen sample, and DNA was extracted from purified sperm using a phenol-chloroform extraction method.

Two male patients with mosaic *PCDH19* variants were ascertained by clinical testing, both with epilepsy and intellectual/development disabilities. For male patient 25, a custom-designed panel for capturing the coding exons of 470 genes associated with epilepsy,[17] including *SCN1A* and *PCDH19* (NM_0011848801.1), based on the Agilent SureSelect Target Enrichment technique (Zhongguancun Huakang Gene Institute, China) was used. For male patient 26, another custom-designed panel capturing the coding exons of 153 genes associated with epilepsy (online supplementary table S1), including *SCN1A* and *PCDH19,* were synthesised by Agilent Technologies on a chip (MyGenostics, Baltimore, Maryland, USA). Targeted gene capture, massively parallel sequencing and sequence alignment were performed for the two epileptic and intellectual disability patients as described previously.[17 18] All sequencing data were based on the reference human genome build hg19.

PCDH19 point variants were detected by PCR and Sanger sequencing as previously described.[12] SALSA Multiple Ligation Probe Amplification (MLPA) probe mix P330-A2 was applied to confirm *PCDH19* rearrangements. Segregation analysis was performed for all available members of a pedigree. If neither parent had the variant, then parent-offspring trios were tested by microsatellite markers analysis at the X chromosome to ensure that the variant occurred de novo.

Quantification of mutant versus wild-type allele

Customised TaqMan SNP Genotyping Assays (Life Technologies, Grand Island, New York, USA), consisting of mutation-specific primers and fluorescent-labelled allele discrimination probes, were used to quantify the mutant allelic fraction (MAF) of each site measured using the RainDance Raindrop microdroplet digital PCR platform (mDDPCR, RainDance, Billerica, Massachusetts, USA). mDDPCR was performed in 20 families with *PCDH19* variants (including four families with a male mosaicism, 11 parent-offspring trios with apparent de novo heterozygous mutations in probands by Sanger sequencing, 3 families with mild affected or asymptomatic mothers and 2 families with hemizygous fathers), as previously described.[13]

Mosaic individuals were normalised to the normal control identified in the same family. In families with a mosaic or hemizygous father, we also controlled for PCR efficiency by assuming that 100% of the affected offspring's cells harboured a mutation.

RESULTS
PCDH19 mutations in 42 families with epilepsy

By the end of July 2017, a total of 42 probands with epilepsy were identified to carry *PCDH19* pathogenic variants, including 40 females and 2 mosaic males. Forty of the probands exhibited point variants (21 previously reported) and two (2/42, 5%) probands demonstrated whole-gene deletion of *PCDH19*. We found 37 different pathogenic variants, including 15 missense variants, 12 small frameshift insertions and deletions (indels), 7 nonsense variants, 1 small in-frame insertion, 1 splicing variant and 1 whole gene deletion. In 21 newly described probands, we identified 12 novel and 9 reported variants (table 1). All four novel missense variants were on exon 1 and were predicted as pathogenic using the in silico prediction tools Mutation Taster Server, Polyphen-2 and SIFT. None of the novel mutations was detected in 100 Chinese healthy controls, the dbSNP or ExAC databases.

Among the 40 female patients, 19 patients were identified with inherited variants and 21 patients demonstrated apparent de novo variants by Sanger sequencing and MLPA. In our 40 female proband families, 60 females were confirmed to carry *PCDH19* variants and 54 of had an epileptic seizure. Thus, the penetrance of females with the *PCDH19* variants was estimated as 90% (54/60) in our study. Twelve hemizygous fathers and the previously reported mosaic father (patient 13's father) were asymptomatic.[12]

mDDPCR analysis was performed in 20 families with *PCDH19* pathogenic variants, involving 65 participants, of which 23 were males and 42 were females (including 4 families with a male mosaicism, 11 parent-offspring trios of which the probands were detected with seemingly de novo variants, 3 families with mild or asymptomatic mothers and 2 families with hemizygous fathers). The data are shown in online supplementary table S2. To evaluate the accuracy of the MAFs quantified by mDDPCR, we screened two fathers with hemizygous *PCDH19* variants as controls. The MAF was measured as 99.69% (95% binomial CIs 99.58% to 99.77%) and 99.95% (95% binomial CIs 99.88% to 99.99%). In addition, the 95% CI of MAFs measured in eight females with an inherited variant ranged from 44.53% to 55.17%.

Mosaicism in male patients

Two male mosaic patients (Probands 25 and 26) with epilepsy demonstrated intellectual disability. Proband 26 was also diagnosed with autism spectrum disorder. They were identified by targeted NGS. A total of 189 males with epilepsy have been screened using gene-panel testing from 2014 to 2017, only two (2/189, 1%) were identified with *PCDH19* variants. In patient 25, the c.317T>A (p.Met106Arg) variant in *PCDH19* was preliminary detected as a hemizygous mutation. Manual inspection revealed 85% of the mutant allele in an 86X coverage region (MAF 85.0%). Sanger sequencing confirmed the existence of *PCDH19* mosaic variants in his blood, buccal epithelium samples. It also suggested the existence of *PCDH19* mosaic variant in his saliva and urine samples. However, the variant was present in almost 100% of the hair follicles because it appeared in a hemizygous status (figure 1A). In patient 26, the c.158dupT (p.Asp54GlyfsX35) variant was detected as a mosaic mutation by NGS. It revealed 33% of the mutant allele in a 124X coverage region (MAF 33.1%). We sequenced every mosaic variant bidirectionally, for at least twice, in technical replicates starting from independent PCR reactions. Segregation analysis demonstrated that these two variants occurred de novo. MLPA analysis did not detect any *PCDH19* deletion/duplication in the two male patients.

We confirmed and quantified the MAFs in the two male mosaic cases by mDDPCR. The MAFs of male patient 25's multiple somatic tissues (blood, hair follicles, buccal swab, finger nails, saliva and urine) were 67.43%–98.46% (figure 2). The MAF of male patient 26's blood DNA was 37.08%.

Patient 25 was 3 years old at last follow-up. He was born full term to a G2P2 34-year-old mother. His birth weight was 3.25 kg. Both the pregnancy and neonatal course were unremarkable. Familial and personal neurological antecedents were negative. However, his mother was diagnosed with a teratoma on a physical examination at the age of 29 years old between her two pregnancies. The patient developed seizures at 5 months of age lasting 20–50 s. A cluster of attacks occurred every 1–2 months, with each cluster lasting for 1–15 days. He has predominantly afebrile focal tonic seizures. Antiepileptic drugs were arbitrarily
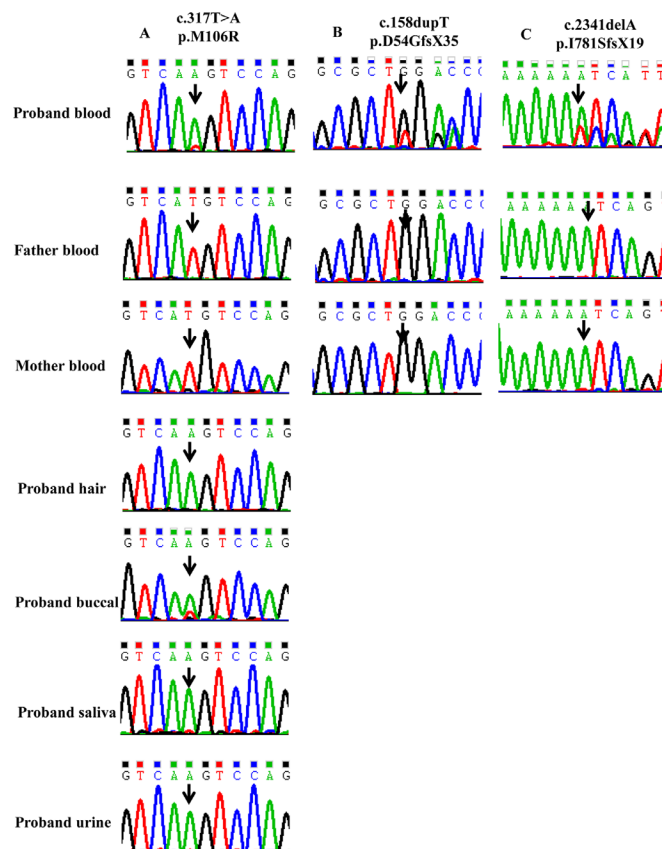

Figure 1 Sanger sequencing chromatograms of three mosaic probands families. (A) Sanger sequencing shows that the proband is mosaic for the *PCDH19* variant (c.317T>A) in blood, buccal epithelium, saliva and urine. The variant appears to be hemizygous in his hair follicles. The variant is absent in his parents' blood. (B) Sanger sequencing shows that the proband is mosaic for the *PCDH19* variant (c.158dupT) in blood. The variant is absent in his parents' blood. (C) Sanger sequencing shows that the proband is mosaic for the *PCDH19* variant (c.2341delA) in blood. The variant is absent in her parents' blood.

stopped after he had been treated with oxcarbazepine, levetiracetam and valproate without effect. He tended to display excessive motor activity and was described as restless. His cranial MRI was normal. He has no other significant medical concerns. His psychomotor development, prior to the onset of epilepsy, was reported as normal. He manifested severe cognitive impairment after seizure onset. An interictal EEG (while medicated with oxcarbazepine and levetiracetam) at 9 months was normal. An EEG (2 days after seizures cluster) at 1 year was abnormal, demonstrating epileptic discharges in the bilateral occipital and temporal posterior regions. An EEG (during the time of the seizure cluster) at 13 months recorded dozens of focal seizures from the anterior region.

Patient 26 was 9 years old at last follow-up. He was born 37^{+4} to a G1P2 mother. His birth weight was 2.3 kg. Both the pregnancy and neonatal course were unremarkable. Familial and personal neurological antecedents were negative. At the age of 9 months, he presented the first cluster of febrile seizures. Multiple seizure types occurred including febrile and afebrile generalised tonic-clonic seizures, myoclonic seizures and absence seizures. Seizures were highly sensitive to fever. He had been treated with phenobarbital and subsequently with valproate and topiramate. His seizures were controlled by valproate and topiramate since he was 4 years
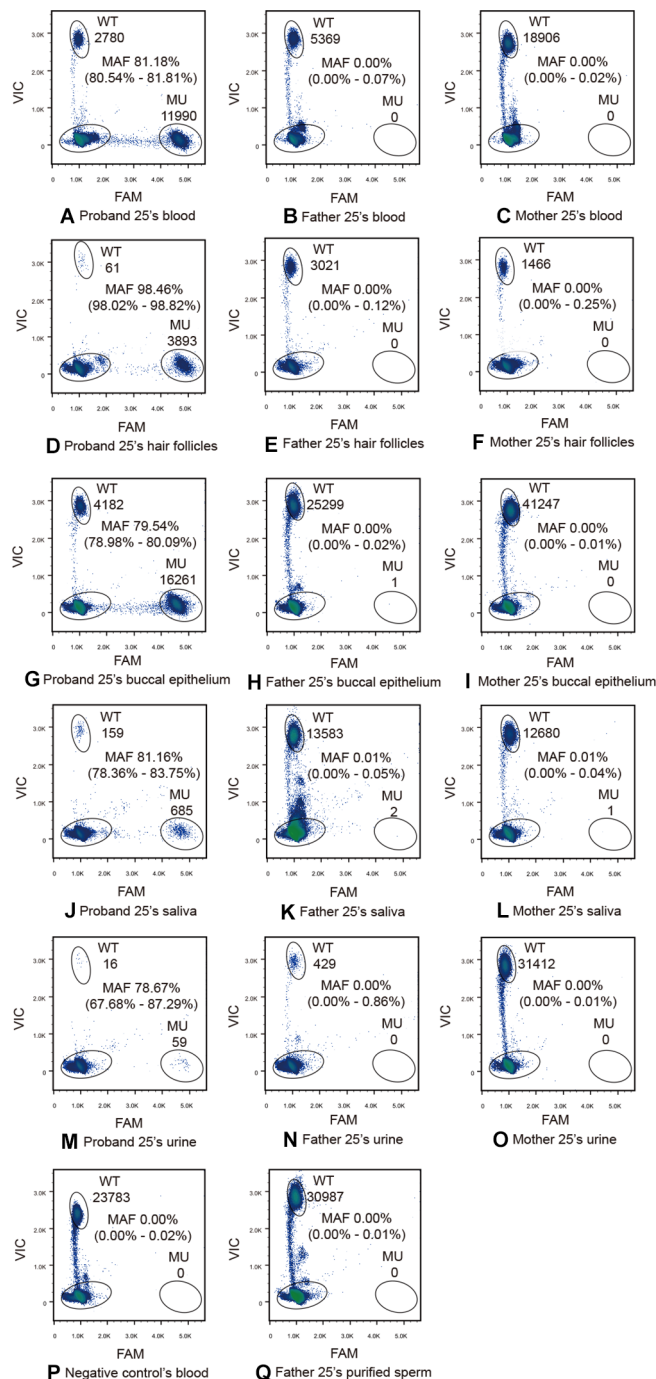
Figure 2 The mDDPCR results of Family 25. Detection of *PCDH19* c.317T>A mutation in Proband 25's multiple tissues (blood, buccal epithelium, saliva and urine). The wild-type and mutant population are circled in the top panels and bottom panels, respectively, with the % MUT indicated in the top right corner (MUT drops/total of WT+MUT droplets). MAF, mutant allelic fraction; mDDPCR, microdroplet digital PCR; WT, wild type.

old. He had autistic traits, excessive motor activity and was described as restless. His cranial MRI was normal. He had no other significant medical concerns. An EEG (while medicated with valproate and topiramate) at 2 years and 10 months was abnormal, showing diffuse slowing of background activity, epileptic discharges in the bilateral, frontal and central regions, evident during drowsiness.

Mosaicism in the female patient

In 11 families in which the probands with apparent de novo variants by Sanger sequencing, 1 female patient (female patient 29) was identified as a mosaic with a blood MAF of 26.72% (figure 1C). No parental mosaicism was identified in these 11 families.

Patient 29 was 2 years at the last follow-up. She was born full term. Both the pregnancy and neonatal course were unremarkable. She had a history of febrile seizure at the age of 12 months. At the age of 15 months, she presented the first cluster of febrile seizures. She has predominantly generalised tonic-clonic seizures and focal seizures. Her seizures were highly sensitive to fever. Her cranial MRI was normal. She had no other significant medical concerns. Her EEG at 15 months captured six seizures, which manifestated staring blankly, head deviation to the back and tonic in four limbs. The EEG was abnormal, showing diffuse slowing of background activity and epileptic discharges in the bilateral regions, predominantly in the left hemisphere. She had been treated with levetiracetam since 15 months. She had two more clusters of seizures during the next year. She had normal development.

Parental mosaicism

Since technical limitations and manual limitations are the frequent reasons for missed *SCN1A* mutations (including heterozygous and mosaic mutations),[19] we carefully reconfirmed the Sanger sequencing results in the 19 families, which were initially assumed as de novo *PCDH19* point variants. Consequently, another asymptomatic mosaic father (patient 1's father) was identified, as shown in figure 3A and B. Sanger sequencing confirmed the *PCDH19* mosaic variants in Father 1's blood, hair follicles, buccal swab, finger nails, saliva, urine and purified sperm. Sanger sequencing confirmed *PCDH19* mosaic variants in Father 13's blood, buccal swab, finger nails, saliva and sperm. However, the variant was absent in his urine. Thus, the two fathers were confirmed as gonosomal mosaicism. In addition, the two mosaic fathers identified by Sanger sequencing were confirmed as a somatic (blood, hair follicles, buccal swab, finger nails, saliva and urine) and gonadal mosaicism by mDDPCR, with MAFs of 4.16%–37.38% and 1.27%–19.13%, respectively, (figure 4 and figure 5).

The clinical details of Family 1 and Family 13 have been reported. Proband 1 is a sporadic case (patient 1 in Liu *et al* 2017).[12] Proband 13 has an affected sibling (patient 13 in Liu *et al* 2017).[12]

Incomplete penetrance in heterozygous females is unrelated to mosaicism

We further investigated whether mosaicism plays a role in females with a mild phenotype or those who are asymptomatic. The mother of patient 4 had febrile and afebrile seizures before 13 years of age and exhibited normal intelligence. The mother of patient 6 had only febrile seizures in her childhood. The MAFs from multiple somatic tissues (blood, hair follicles, buccal swab, saliva and urine) in patient 14's mother ranged from 47.20%–49.64%. On the basis of these data, these patients were determined not to exhibit somatic mosaicism.

DISCUSSION

Mosaicism is a common biological phenomenon. It describes an individual who has developed from a single zygote and has two or more populations of cells with distinct genotypes.[20] Mosaicism can occur at any time during development after the formation
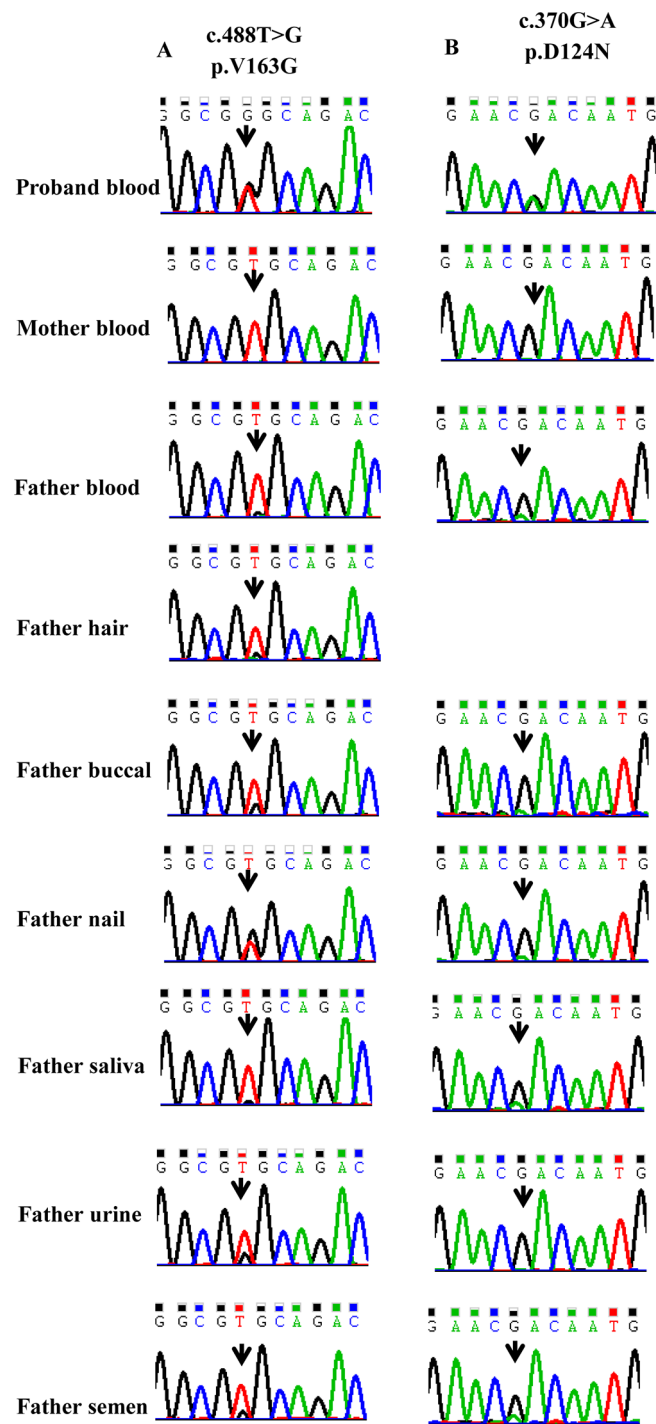
Figure 3 Sanger sequencing chromatograms of two parental mosaic families. (A) Sanger sequencing shows that the proband is heterozygous for the *PCDH19* variant (c.488T>G) and her father is mosaic for the same variant in blood, hair, buccal epithelium, finger nails, saliva, urine and purified sperm. The variant is absent in her mother's blood. (B) Sanger sequencing shows that the proband is heterozygous for the *PCDH19* variant (c.370G>A) and her father is mosaic for the same variant in blood, buccal epithelium, finger nails, saliva, urine and purified sperms. The variant is absent in her mother's blood.
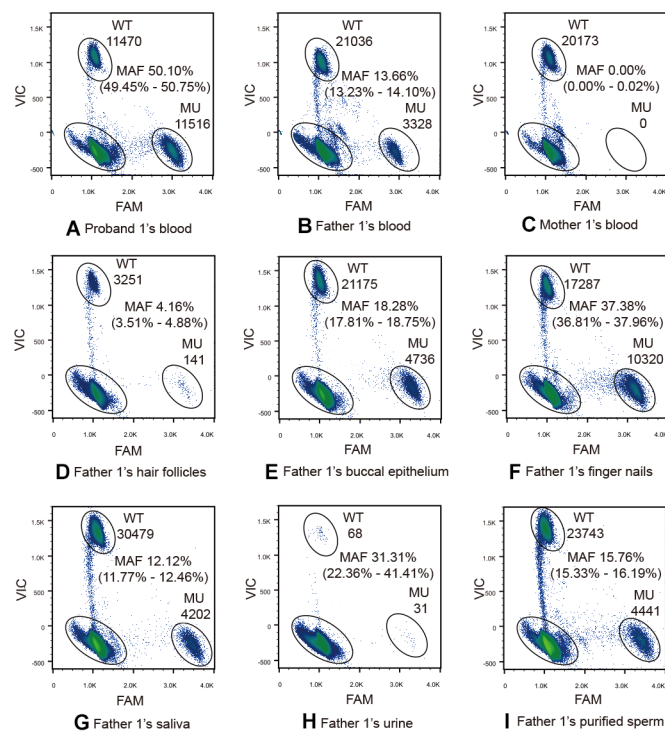

Figure 4 The mDDPCR results of Family 1. Detection of *PCDH19* c.488T>G mutation in Proband 1's blood and her father's multiple tissues (blood, hair follicles, buccal epithelium, finger nails, saliva, urine and purified sperms). The wild-type ('WT') and mutant ('MUT' or 'MU') population are circled in the top panels and bottom panels, respectively, with the % MUT indicated in the top right corner (MUT drops/total of WT+MUT droplets). MAF, mutant allelic fraction; mDDPCR, microdroplet digital PCR; WT, wild type.

of the fertilised egg.[21] It is known that somatic mutations have important roles in cancer[22 23] and monogenic diseases.[24–30] In monogenic diseases, if mutations occur in the early embryo stage, the individual may be composed of mixed gonadal and somatic mosaicism.[31] Somatic and gonadal mosaicism in the parents can cause recurrent transmission of human genetic disease.[31 32] This phenomenon has been reported in more than 100 genes, such as *DMD*, *SCN1A*, *ACTA1* and so on.[24 26 33] Somatic mosaicism can also contribute to variable phenotypic expressivity.[33 34] For the purpose of this study, we focused on the somatic mosaicism in *PCDH19*.

Until recently, 10 male mosaic epilepsy patients with *PCDH19* mutations have been reported.[5–9] In this study, we identified two new male mosaic epilepsy patients with *PCDH19* mutations by NGS. Previously, male epilepsy patients with mosaic *PCDH19* mutations were explained by the 'cellular interference' mechanism because the coexistence of wild-type and mutant cell populations may scramble cell-cell communication.[5] However, we identified an asymptomatic mosaic father who had two affected daughters.[12] Apparent de novo mutations may actually be inherited from mosaic parents.[13] However, mutations in mosaic parents could be undetectable due to technical limitations. We reanalysed the Sanger sequencing results of 19 families in which the probands were initially considered as de novo *PCDH19* point mutations. The second asymptomatic mosaic father, who was initially overlooked, was found in families which had one child with apparent sporadic *PCDH19*-related epilepsy. Our identification of the two asymptomatic mosaic fathers implies that the frequency of paternal mosaicism might be underestimated. The identification of asymptomatic mosaic fathers is inconsistent with previous reports, in which mosaic males have clinical manifestations.[5–9] We hypothesised that the ratio of mutant versus wild-type protocadherin 19 protein in the brain determines the phenotypes in mosaic males. To test
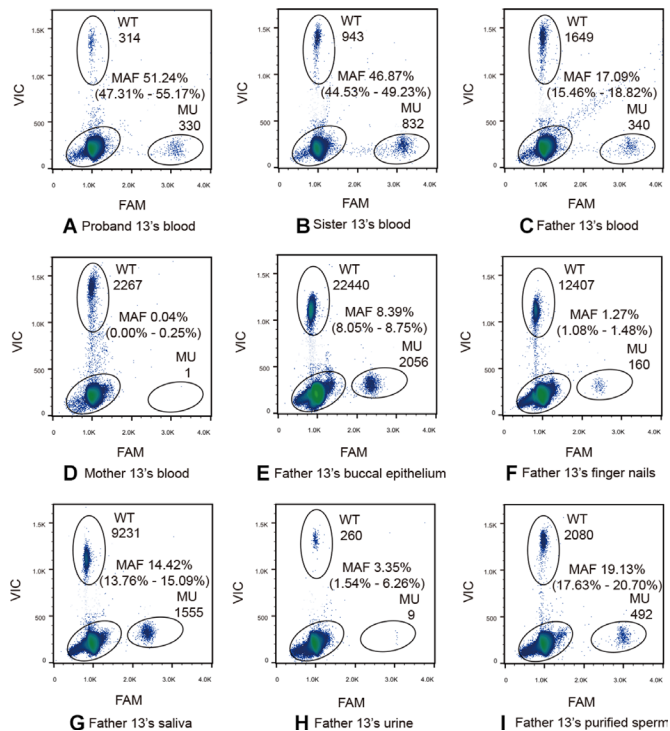
Figure 5 The mDDPCR results of Family 13. Detection of *PCDH19* c.370G>A mutation in Proband 13's blood, her sister's blood and their father's multiple tissues (blood, buccal epithelium, finger nails, saliva, urine and purified sperms). The wild-type and mutant population are circled in the top panels and bottom panels, respectively, with the % MUT indicated in the top right corner (MUT drops/total of WT+MUT droplets). MAF, mutant allelic fraction; mDDPCR, microdroplet digital PCR; WT, wild type.

this hypothesis, we quantified the mosaic ratio at the *PCDH19* locus using mDDPCR. The MAFs in the two mosaic male patients were 37.08%–98.46%. The mutant allele fractions in the two asymptomatic fathers were 4.16%–37.38% and 1.27%–19.13% in different tissues. In our cohort, the affected males have a higher quantity of mutations than the unaffected fathers, although the percentage of mutation varies from one tissue to the other. The blood MAFs in the two patients was closer to 50% compared with the two asymptomatic fathers. So we suspect that a male with a mutant percentage near to 50% in the brain seems more likely to be affected, while males with too high or too low percentage of mosaicism may not be affected. Although we assumed the clinical outcome of mosaic males was related to the mosaic MAFs, we could not identify a definite cut-off value. In Terracciano's two male patients,[7] the mosaic ratio was 10% and 90%, but in our cohort, the two asymptomatic fathers with 13% and 19% mosaicism in blood. However, the percentage of mosaicism in the brain cannot be extrapolated from the blood.[5]

Maternal mosaicism of *PCDH19* mutations has also been previously reported.[10] The mosaic mothers were affected or asymptomatic due to random X inactivation or the ratio of mutant versus wild-type protocadherin 19 protein. Thus, parental mosaicism should be considered when providing genetic counselling for couples who have one affected offspring. We successfully found two asymptomatic mosaic fathers by Sanger sequencing; therefore, the parents' Sanger sequencing results of other cohorts should be reanalysed to avoid negligence of mosaic situations.[19] However, Sanger sequencing could not detect mosaicism with a MAF <5% and could hardly distinguish heterozygous mutations from mosaic mutations in females. Mosaicism

is now more readily detectable and quantified by mDDPCR or other deep sequencing techniques in families with assumed de novo mutations by Sanger sequencing.[13] NGS is a powerful tool for mosaicism detection. Among the 14 males (10 males previously reported and 4 males reported in this study, online supplementary table S3) with somatic mosaic mutations in *PCDH19*, 9 (64%) were detected by targeted NGS. To quantify the mosaic ratio, we applied the more sensitive mDDPCR method which could detect MAF as low as 0.01% in our study.[13]

Somatic gene conversion from wild-type to mutant is known as 'forward mosaicism'.[35] On the other hand, spontaneous gene correction can happen in the opposite direction during mitosis, known as 'revertant mosaicism'.[35] Back mutation now becomes an important genetic mechanism to consider when explaining examples of a reversion of somatic cells to 'normal' in persons with a genetically determined abnormal phenotype.[36] In our study, male patient 25 had a mutant allele of more than 50%. As one male's *PCDH19* allele was inherited from his mother, the mutation was assumed to already be present in the oocyte prior to fertilisation. Back mutation was suspected to occur during the early development in this male patient, leading to postzygotic *PCDH19* mosaicisms. This suspected reversion mechanism may be a reason for male patients with mosiac *PCDH19* mutations. By contrast, spontaneous genetic reversion has been described as 'natural gene therapy' in some disease, such as Fanconi anaemia and dyskeratosis congenita.[37 38] In our study, the mother of patient 25 with *PCDH19* c.317T>A gonadal mosaicism had developed a teratoma. In a previous study, the *PCDH19* c.918C>T mutation found in a male mosaic patient had been previously reported in somatic tumour tissue.[7] Based on these findings, tissue limited mosaicism for *PCDH19* mutations may have a relationship with tumours and thus demand special attention.

Moreover, we identified one mosaic female proband in 11 families with apparent de novo *PCDH19* mutations using mDDPCR. Mosaicism can also be an important cause of phenotypic variation in some females.[11] We quantified three mildly affected or asymptomatic mothers in our cohort using mDDPCR. The mutant allele ratio was all nearly 50% and was thus ruled out for mosaicism. This finding suggests other genetic or non-genetic modifiers, rather than mosaicism, which may also be involved in the phenotype of females, such as X inactivation.[39]

Overall five mosaics, including two male patients and one female patient and two asymptomatic mosaic fathers were identified in our *PCDH19* gene study. This finding further demonstrated that *PCDH19* mutations could occur in males and females at any development stage. The observation of asymptomatic mosaic fathers suggested that the frequency of parental mosaicism is underestimated. Taken together, our data implied that parental mosaicism should be considered during genetic counselling. We hypothesised that the mosaic MAFs in males may be related to phenotype, although other genetic or non-genetic modifiers may also be involved.

Competing interests None declared.

Patient consent Parental/guardian consent obtained.

Ethics approval Institutional Review Board at Peking University (IRBPU), the Ethics Committee of Peking University First Hospital and the Ethics Committee of Wuhan Children's Hospital.

Provenance and peer review Not commissioned; externally peer reviewed.

Open access This is an open access article distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited, appropriate credit is given, any changes made indicated, and the use is non-commercial. See: http://creativecommons.org/licenses/by-nc/4.0/.

REFERENCES

1. Dibbens LM, Tarpey PS, Hynes K, Bayly MA, Scheffer IE, Smith R, Bomar J, Sutton E, Vandeleur L, Shoubridge C, Edkins S, Turner SJ, Stevens C, O'Meara S, Tofts C, Barthorpe S, Buck G, Cole J, Halliday K, Jones D, Lee R, Madison M, Mironenko T, Varian J, West S, Widaa S, Wray P, Teague J, Dicks E, Butler A, Menzies A, Jenkinson A, Shepherd R, Gusella JF, Afawi Z, Mazarib A, Neufeld MY, Kivity S, Lev D, Lerman-Sagie T, Korczyn AD, Derry CP, Sutherland GR, Friend K, Shaw M, Corbett M, Kim HG, Geschwind DH, Thomas P, Haan E, Ryan S, McKee S, Berkovic SF, Futreal PA, Stratton MR, Mulley JC, Gécz J. X-linked protocadherin 19 mutations cause female-limited epilepsy and cognitive impairment. *Nat Genet* 2008;40:776–81.
2. Depienne C, Trouillard O, Bouteiller D, Gourfinkel-An I, Poirier K, Rivier F, Berquin P, Nabbout R, Chaigne D, Steschenko D, Gautier A, Hoffman-Zacharska D, Lannuzel A, Lackmy-Port-Lis M, Maurey H, Dusser A, Bru M, Gilbert-Dussardier B, Roubertie A, Kaminska A, Whalen S, Mignot C, Baulac S, Lesca G, Arzimanoglou A, LeGuern E. Mutations and deletions in PCDH19 account for various familial or isolated epilepsies in females. *Hum Mutat* 2011;32:E1959–E1975.
3. van Harssel JJ, Weckhuysen S, van Kempen MJ, Hardies K, Verbeek NE, de Kovel CG, Gunning WB, van Daalen E, de Jonge MV, Jansen AC, Vermeulen RJ, Arts WF, Verhelst H, Fogarasi A, de Rijk-van Andel JF, Kelemen A, Lindhout D, De Jonghe P, Koeleman BP, Suls A, Brilstra EH. Clinical and genetic aspects of PCDH19-related epilepsy syndromes and the possible role of PCDH19 mutations in males with autism spectrum disorders. *Neurogenetics* 2013;14:23–34.
4. Depienne C, LeGuern E. PCDH19-related infantile epileptic encephalopathy: an unusual X-linked inheritance disorder. *Hum Mutat* 2012;33:627–34.
5. Depienne C, Bouteiller D, Keren B, Cheuret E, Poirier K, Trouillard O, Benyahia B, Quelin C, Carpentier W, Julia S, Afenjar A, Gautier A, Rivier F, Meyer S, Berquin P, Hélias M, Py I, Rivera S, Bahi-Buisson N, Gourfinkel-An I, Cazeneuve C, Ruberg M, Brice A, Nabbout R, Leguern E. Sporadic infantile epileptic encephalopathy caused by mutations in PCDH19 resembles Dravet syndrome but mainly affects females. *PLoS Genet* 2009;5:e1000381.
6. de Lange IM, Rump P, Neuteboom RF, Augustijn PB, Hodges K, Kistemaker AI, Brouwer OF, Mancini GMS, Newman HA, Vos YJ, Helbig KL, Peeters-Scholte C, Kriek M, Knoers NV, Lindhout D, Koeleman BPC, van Kempen MJA, Brilstra EH. Male patients affected by mosaic PCDH19 mutations: five new cases. *Neurogenetics* 2017;18:147–53.
7. Terracciano A, Trivisano M, Cusmai R, De Palma L, Fusco L, Compagnucci C, Bertini E, Vigevano F, Specchio N. PCDH19-related epilepsy in two mosaic male patients. *Epilepsia* 2016;57:e51–e55.
8. Thiffault I, Farrow E, Smith L, Lowry J, Zellmer L, Black B, Abdelmoity A, Miller N, Soden S, Saunders C. PCDH19-related epileptic encephalopathy in a male mosaic for a truncating variant. *Am J Med Genet A* 2016;170:1585–9.
9. Perez D, Hsieh DT, Rohena L. Somatic Mosaicism of PCDH19 in a male with early infantile epileptic encephalopathy and review of the literature. *Am J Med Genet A* 2017;173:1625–30.
10. Dibbens LM, Kneen R, Bayly MA, Heron SE, Arsov T, Damiano JA, Desai T, Gibbs J, McKenzie F, Mulley JC, Ronan A, Scheffer IE. Recurrence risk of epilepsy and mental retardation in females due to parental mosaicism of PCDH19 mutations. *Neurology* 2011;76:1514–9.
11. Terracciano A, Specchio N, Darra F, Sferra A, Bernardina BD, Vigevano F, Bertini E. Somatic mosaicism of PCDH19 mutation in a family with low-penetrance EFMR. *Neurogenetics* 2012;13:341–5.
12. Liu A, Xu X, Yang X, Jiang Y, Yang Z, Liu X, Wu Y, Wu X, Wei L, Zhang Y. The clinical spectrum of female epilepsy patients with PCDH19 mutations in a Chinese population. *Clin Genet* 2017;91:54–62.
13. Xu X, Yang X, Wu Q, Liu A, Yang X, Ye AY, Huang AY, Li J, Wang M, Yu Z, Wang S, Zhang Z, Wu X, Wei L, Zhang Y. Amplicon resequencing identified parental mosaicism for approximately 10% of "de novo" SCN1A Mutations in children with dravet syndrome. *Hum Mutat* 2015;36:861–72.
14. Duszyc K, Terczynska I, Hoffman-Zacharska D. Epilepsy and mental retardation restricted to females: X-linked epileptic infantile encephalopathy of unusual inheritance. *J Appl Genet* 2015;56:49–56.
15. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 1988;16:1215.
16. Saitsu H, Hoshino H, Kato M, Nishiyama K, Okada I, Yoneda Y, Tsurusaki Y, Doi H, Miyake N, Kubota M, Hayasaka K, Matsumoto N. Paternal mosaicism of an STXBP1 mutation in OS. *Clin Genet* 2011;80:484–8.
17. Xue J, Li H, Zhang Y, Yang Z. Clinical and genetic analysis of two chinese infants with mabry syndrome. *Brain Dev* 2016;38:807–18.
18. Wu J, Matthaei H, Maitra A, Dal Molin M, Wood LD, Eshleman JR, Goggins M, Canto MI, Schulick RD, Edil BH, Wolfgang CL, Klein AP, Diaz LA, Allen PJ, Schmidt CM, Kinzler KW, Papadopoulos N, Hruban RH, Vogelstein B. Recurrent GNAS mutations define an unexpected pathway for pancreatic cyst development. *Sci Transl Med* 2011;3:92ra66.
19. Djémié T, Weckhuysen S, von Spiczak S, Carvill GL, Jaehn J, Anttonen AK, Brilstra E, Caglayan HS, de Kovel CG, Depienne C, Gaily E, Gennaro E, Giraldez BG, Gormley P, Guerrero-López R, Guerrini R, Hämäläinen E, Hartmann C, Hernandez-Hernandez L, Hjalgrim H, Koeleman BP, Leguern E, Lehesjoki AE, Lemke JR, Leu C, Marini C, McMahon JM, Mei D, Møller RS, Muhle H, Myers CT, Nava C, Serratosa JM, Sisodiya SM, Stephani U, Striano P, van Kempen MJ, Verbeek NE, Usluer S, Zara F, Palotie A, Mefford HC, Scheffer IE, De Jonghe P, Helbig I, Suls A. EuroEPINOMICS-RES Dravet working group. Pitfalls in genetic testing: the story of missed SCN1A mutations. *Mol Genet Genomic Med* 2016;4:457–64.
20. Cohen AS, Wilson SL, Trinh J, Ye XC, Xc Y. Detecting somatic mosaicism: considerations and clinical implications. *Clin Genet* 2015;87:554–62.
21. Vadlamudi L, Dibbens LM, Lawrence KM, Iona X, McMahon JM, Murrell W, Mackay-Sim A, Scheffer IE, Berkovic SF. Timing of de novo mutagenesis–a twin study of sodium-channel mutations. *N Engl J Med* 2010;363:1335–40.
22. Krawczak M, Smith-Sorensen B, Schmidtke J, Kakkar VV, Cooper DN, Hovig E. Somatic spectrum of cancer-associated single basepair substitutions in the TP53 gene is determined mainly by endogenous mechanisms of mutation and by selection. *Hum Mutat* 1995;5:48–57.
23. Alvarado C, Beitel LK, Sircar K, Aprikian A, Trifiro M, Gottlieb B. Somatic mosaicism and cancer: a micro-genetic examination into the role of the androgen receptor gene in prostate cancer. *Cancer Res* 2005;65:8514–8.
24. Smith TA, Yau SC, Bobrow M, Abbs SJ. Identification and quantification of somatic mosaicism for a point mutation in a Duchenne muscular dystrophy family. *J Med Genet* 1999;36:313–5.
25. Ortega-Moreno L, Giráldez BG, Soto-Insuga V, Losada-Del Pozo R, Rodrigo-Moreno M, Alarcón-Morcillo C, Sánchez-Martín G, Díaz-Gómez E, Guerrero-López R, Serratosa JM. Grupo Español de Genética de las Epilepsias de la Infancia (GEGEI). Molecular diagnosis of patients with epilepsy and developmental delay using a customized panel of epilepsy genes. *PLoS One* 2017;12:e0188978.
26. Depienne C, Arzimanoglou A, Trouillard O, Fedirko E, Baulac S, Saint-Martin C, Ruberg M, Dravet C, Nabbout R, Baulac M, Gourfinkel-An I, LeGuern E. Parental mosaicism can cause recurrent transmission of SCN1A mutations associated with severe myoclonic epilepsy of infancy. *Hum Mutat* 2006;27:389.
27. Twigg SR, Matsumoto K, Kidd AM, Goriely A, Taylor IB, Fisher RB, Hoogeboom AJ, Mathijssen IM, Lourenco MT, Morton JE, Sweeney E, Wilson LC, Brunner HG, Mulliken JB, Wall SA, Wilkie AO. The origin of EFNB1 mutations in craniofrontonasal syndrome: frequent somatic mosaicism and explanation of the paucity of carrier males. *Am J Hum Genet* 2006;78:999–1010.
28. Peat RA, Baker NL, Jones KJ, North KN, Lamandé SR. Variable penetrance of COL6A1 null mutations: implications for prenatal diagnosis and genetic counselling in Ullrich congenital muscular dystrophy families. *Neuromuscul Disord* 2007;17:547–57.
29. Masliah-Plachon J, Auvin S, Nectoux J, Fichou Y, Chelly J, Bienvenu T. Somatic mosaicism for a CDKL5 mutation as an epileptic encephalopathy in males. *Am J Med Genet A* 2010;152A:2110–1.
30. Etheridge SP, Bowles NE, Arrington CB, Pilcher T, Rope A, Wilde AA, Alders M, Saarel EV, Tavernier R, Timothy KW, Tristani-Firouzi M. Somatic mosaicism contributes to phenotypic variation in Timothy syndrome. *Am J Med Genet A* 2011;155A:2578–83.
31. Spinner NB, Conlin LK. Mosaicism and clinical genetics. *Am J Med Genet C Semin Med Genet* 2014;166C:397–405.
32. Levenson D. Somatic mosaicism in parents may cause single-gene disorders in children: latest study calculates incidence of parental mosaicism higher than previous research suggests. *Am J Med Genet A* 2014;164A:viii–ix.
33. Steel D, Symonds JD, Zuberi SM, Brunklaus A. Dravet syndrome and its mimics: Beyond SCN1A. *Epilepsia* 2017;58:1807–16.
34. Polani PE. Somatic gene mosaicism and expressivity. *Trends Genet* 2001;17:253.
35. Fujita A, Waga C, Hachiya Y, Kurihara E, Kumada S, Takeshita E, Nakagawa E, Inoue K, Miyatake S, Tsurusaki Y, Nakashima M, Saitsu H, Goto YI, Miyake N, Matsumoto N. Different X-linked KDM5C mutations in affected male siblings: is maternal reversion error involved? *Clin Genet* 2016;90:276–81.
36. Ellis NA, Ciocci S, German J. Back mutation can produce phenotype reversion in Bloom syndrome somatic cells. *Hum Genet* 2001;108:167–73.
37. Kalb R, Neveling K, Nanda I, Schindler D, Hoehn H. Fanconi anemia: causes and consequences of genetic instability. *Genome Dyn* 2006;1:218–42.
38. Jongmans MC, Verwiel ET, Heijdra Y, Vulliamy T, Kamping EJ, Hehir-Kwa JY, Bongers EM, Pfundt R, van Emst L, van Leeuwen FN, van Gassen KL, Geurts van Kessel A, Dokal I, Hoogerbrugge N, Ligtenberg MJ, Kuiper RP. Revertant somatic mosaicism by mitotic recombination in dyskeratosis congenita. *Am J Hum Genet* 2012;90:426–33.
39. Dimova PS, Kirov A, Todorova A, Todorov T, Mitev V. A novel PCDH19 mutation inherited from an unaffected mother. *Pediatr Neurol* 2012;46:397–400.