Deletions or duplications in KCNQ2 can cause benign familial neonatal seizures

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LETTER TO JMG

Deletions or duplications in *KCNQ2* can cause benign familial neonatal seizures

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Background: Benign familial neonatal seizures are most often caused by mutations in the voltage-gated potassium channel subunit gene *KCNQ2*. More than 60 mutations have been described in BFNS families, approximately half of which lead to protein truncation. The hypothesis of this study was that deletion or duplication of ⩾1 exons of *KCNQ2* could cause BFNS in cases without coding or splicing mutations.

Methods: Multiplex ligation-dependent probe amplification (MLPA) was used to test a group of 21 unrelated patients with clinical features consistent with either BFNS, benign familial neonatal–infantile seizures or sporadic neonatal seizures, for exonic deletions and duplications.

Results: Three deletions and one duplication mutation were identified in four familial cases and cascade testing of their available family members showed that the mutations segregated with the phenotype in each family. The junction fragment for one of the deletions was amplified by PCR and sequenced to characterise the breakpoint and verify that a deletion had occurred.

Conclusions: Submicroscopic deletions or duplications of *KCNQ2* are seen in a significant proportion of BFNS families: four of nine (44%) cases previously testing negative for coding or splice site mutation by sequencing *KCNQ2* and *KCNQ3*. MLPA is an efficient second-tier testing strategy for *KCNQ2* to identify pathogenic intragenic mutations not detectable by conventional DNA sequencing methods.

Benign familial neonatal seizures (BFNS; OMIM 121200) and benign familial neonatal–infantile seizures (BFNIS; OMIM 607745) are autosomal dominant seizure disorders of early infancy. In BFNS, affected neonates have onset of seizures in the first few days of life and offset usually occurs by a few months of age.[1] BFNS is caused in most families by mutations in the voltage-gated potassium channel subunit genes *KCNQ2* (OMIM 602235) and *KCNQ3* (OMIM 602232),[2–4] and to date, >60 mutations in these genes have been reported in BFNS families. Most of these mutations are in *KCNQ2*.[5 6] In contrast, the age of seizure onset in BFNIS varies from the neonatal period to 13 months of age.[7] BFNIS, which is caused by mutations of the voltage-gated sodium channel subunit gene *SCN2A* (OMIM 182390), is rarer than BFNS, with 11 families described to date.[7–10] Although there is phenotypic overlap between these two disorders, they can be diagnosed on clinical grounds but with the probability of correct diagnosis increasing with the number of affected family members for whom the age of seizure onset is accurately known.

Approximately half of the *KCNQ2* mutations described in BFNS are truncations, splice site defects, or deletions or insertions of a small number of bases causing frameshifts. All these are predicted to lead to haploinsufficiency, as no functional protein can be produced from the mutated allele. Larger deletions and duplications affecting ⩾1 exons of *KCNQ2* would also be predicted to cause BFNS. The chance finding of a submicroscopic deletion that removed an allele of a microsatellite marker closely linked to *KCNQ2*, causing apparent aberrant segregation, was originally exploited to identify *KCNQ2* as a BFNS gene.[4] This deletion was later found to remove the last nine exons of the *KCNQ2* gene and 22 kb of the 3′ genomic sequence.[11] However, no other mutations altering the copy number of ⩾1 contiguous exons have subsequently been described because this type of mutation is not detectable using standard, systematic PCR-based mutation screening and sequencing methods.

The development of multiplex ligation-dependent probe amplification (MLPA) allows the relatively rapid and inexpensive screening of patients for deletions, duplications and other copy number variants affecting any number of contiguous exons in a selected gene by testing for changes in the copy number of each exon.[12] We used this technology to screen for intragenic copy number variations in *KCNQ2* in a group of unrelated patients in whom *KCNQ2* and *KCNQ3* mutations had been previously excluded by direct sequencing.

METHODS

Patients and DNA samples

In total, 21 probands were tested, of whom 18 had neonatal seizures. Of these 18 probands, 8 had classic BFNS, 7 had sporadic neonatal seizures and 3 had familial neonatal seizures with atypical features such as refractory ongoing epilepsy and developmental delay. The patients with neonatal seizures had previously been tested by direct sequencing for mutations in all coding exons of *KCNQ2* and exons 5 and 6 of *KCNQ3*. Three probands were from families diagnosed with BFNIS and these patients had also been sequenced for all coding exons of *SCN2A*. The initial clinical diagnosis of BFNS versus BFNIS in families with both neonatal and early infantile onset (less than 6 months) was determined as follows. Where the majority of cases had a neonatal onset and a single case or small number of cases had early infantile onset, BFNS was diagnosed. Where early infantile and neonatal onset were in similar proportions, or early infantile predominated, a clinical diagnosis of BFNIS was made. DNA was extracted from peripheral blood samples using standard methods. The study was approved by the ethics committee of Austin Health.

Multiplex ligation-dependent probe amplification

Samples were prepared for MLPA by repurifying 1 μg of DNA using the Qiaquick PCR purification kit (Qiagen, Valencia, California, USA). MLPA was carried out using the SALSA

Abbreviations: BFNIS, benign familial neonatal-infantile seizures; BFNS, benign familial neonatal seizures; MLPA, multiplex ligation-dependent probe amplification; OMIM, Online Mendelian Inheritance in Man

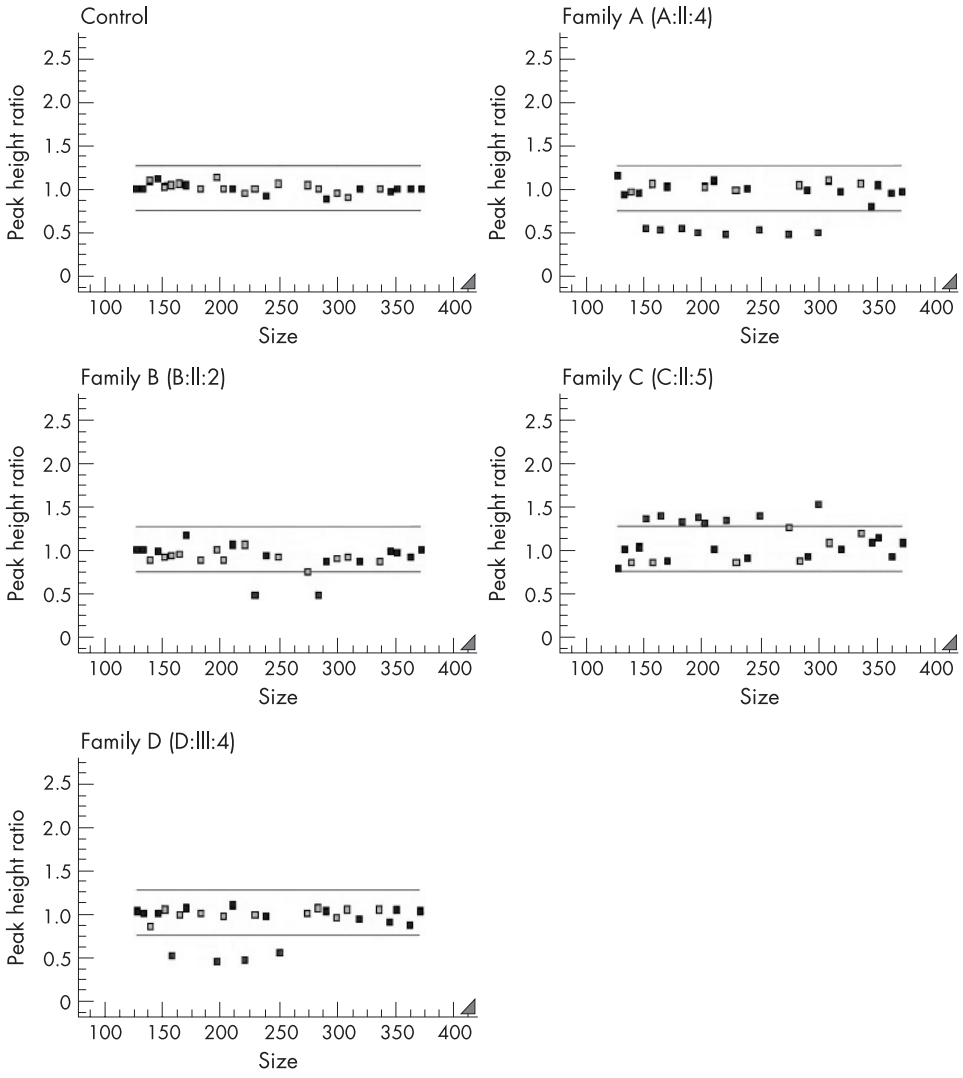

Figure 1 Scatter plots generated from the analysis of MLPA data using GeneMarker software. The five panels show the results for a control sample and a member of each of the four families (respectively: A:II:4; B:II:2; C:II:5; D:III:4) with deletions or duplications involving *KCNQ2*. The points represent the comparative peak height ratios for each probe in the MLPA kit compared with normal samples, as calculated by GeneMarker software. A ratio <0.75 indicates a deletion and >1.25 indicates a duplication of the exon; these thresholds are indicated by the parallel solid lines on each plot. The light grey points within the normal range represent results for *KCNQ2* probes and the dark grey points within the normal range are results for control probes specific to other regions of the human genome.

MLPA kit P166 *KCNQ2* (MRC Holland, Amsterdam, The Netherlands) according to the manufacturer's instructions. This kit contains probes specific for 14 of 17 exons and the promoter of *KCNQ2* (probes for exons 2, 8 and 14 are not included) and 13 control probes specific for other regions of the human genome. Automated analysis, using GeneMarker V.1.5 software (SoftGenetics, State College, Pennsylvania, USA) comparing peak heights was carried out on files directly imported from an ABI 3100 Avant DNA analyser (Applied Biosystems, Foster City, California, USA).

Long-range PCR and sequencing of products

Long-range PCR to search for amplifiable junction fragments was carried out using Platinum Taq High Fidelity DNA Polymerase (InvitrogenCarlsbad, California, USA). PCRs were set up and run according to the manufacturer's instructions. Reactions contained between 40 ng and 100 ng of template DNA. The reactions were cycled for 35 cycles with an extension time of 4 minutes and a 5-minute final extension following cycling. Products were separated by electrophoresis on 0.8% agarose gels and visualised by ethidium bromide staining.

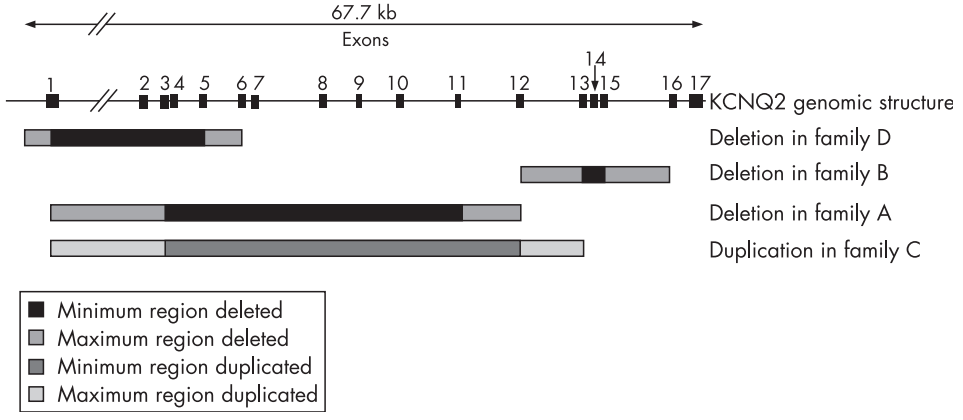

Figure 2 Genomic structure of *KCNQ2* and the regions affected by the deletion or duplication in each family.

Products for sequencing were purified using the Qiaquick PCR purification kit (Qiagen). Sequencing reactions were carried out using BigDye V. 3.1 (Applied Biosystems) and analysed on an ABI 3730 capillary sequencer (Applied Biosystems).

RESULTS

Three deletions and a duplication were identified in 4 of the 21 patients tested (fig 1). Of the 14 unsolved familial cases, deletions or duplication mutations involving multiple exons occurred in 3 classic BFNS families and 1 initially suspected on clinical grounds of being BFNIS. No mutations were present in the remaining seven sporadic cases of neonatal seizures.

The three deletions detected all removed multiple contiguous exons of *KCNQ2*: in family A, exons 3–11 were deleted; in family B, exons 13–15; and in family D exons 1–5. The duplication in family C involved an extra copy of exons 3–12, inserted in unknown orientation (fig 2). All available relatives of these patients were tested by MLPA. The respective deletions were detected in nine affected members in family A and all four affected members and one obligate carrier (D-III-2) in family D. The deletion in family B was detected in both affected members but was not seen in either of the unaffected members of the first generation of this family and therefore represents a de novo mutation. Segregation of 12 highly polymorphic microsatellite markers was consistent with the pedigree as given. Four affected members of family C were tested, and the duplication was present in all of them. The pedigrees of the four families are shown in figure 3.

Of the four families with mutations, two (B and C) were typical BFNS families on clinical grounds. Family D was initially ascertained with one confirmed neonatal onset and one definite infantile onset case and was tentatively diagnosed as BFNIS; subsequently, two more relatives with neonatal onset were ascertained. This, and the molecular result showing the

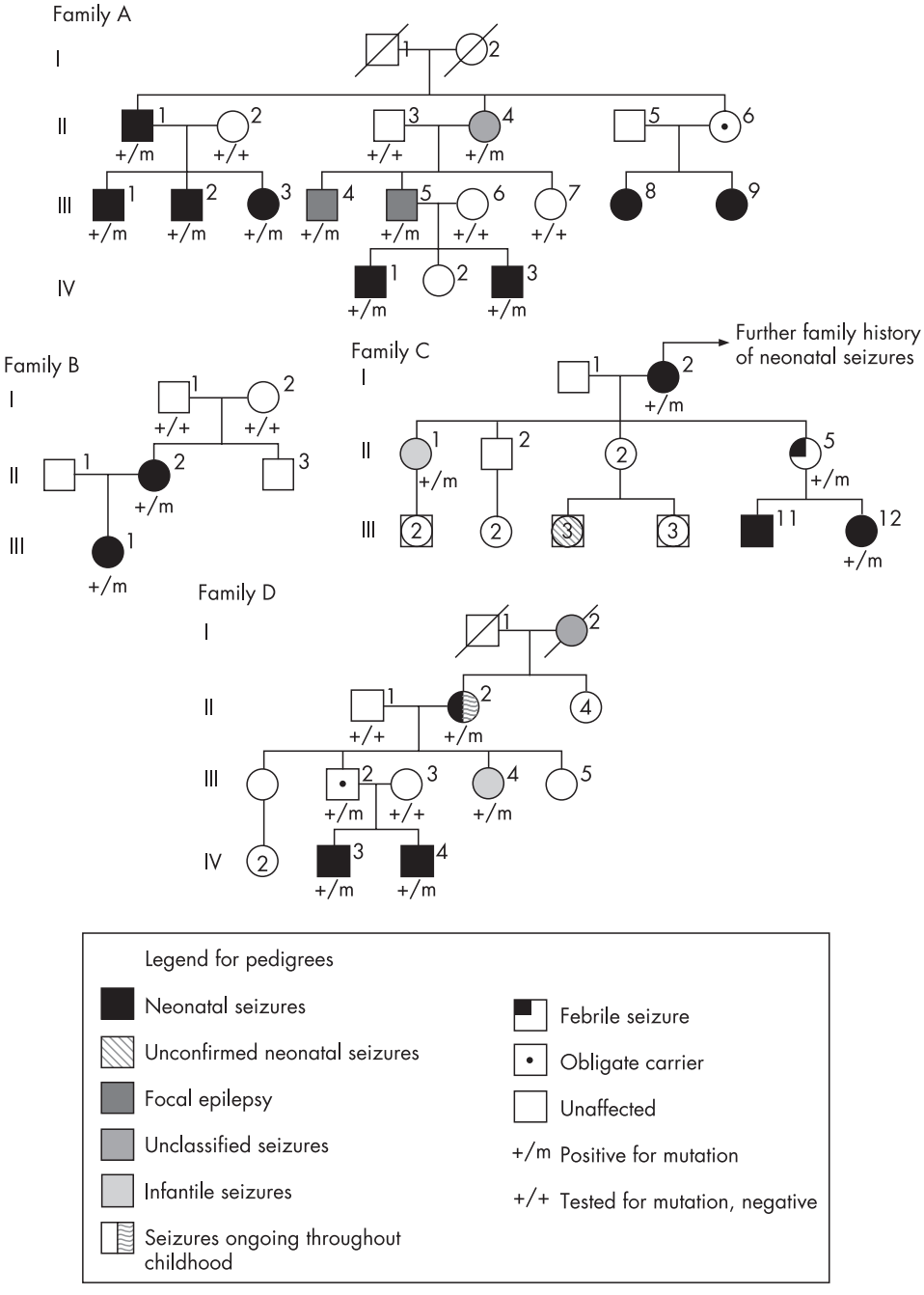

Figure 3 Pedigrees of the four families with *KCNQ2* MLPA detected microchromosomal abnormalities.

Table 1 Clinical details of affected family members

Patient ID	Sex	Age	Onset	Offset	Clinical features
A:II:1	M	61 years	Neonate	12 months	
A:II:4	F	58 years	37 years	50 years	Neonatal history not known. Five nocturnal seizures
A:III:1	M	34 years	7 days	10 months	Four seizures
A:III:2	M	31 years	<1 month	<12 months	Ten seizures
A:III:3	F	29 years	3 days	6 months	
A:III:4	M	37 years	26 years	30 years	No neonatal seizures. Six PSSG in 6 weeks
A:III:5	M	36 years	20 years	Not known	No neonatal seizures. Six PSSG
A:IV:1	M	Not known	9 days	9 days	
A:IV:3	M	7 months	4 days	5 days	Single cluster of 8 seizures
B:II:2	F	32 years	2 days	2 days	Two seizures
B:III:1	F	7 months	2 days	9 days	Nine seizures occurring in three clusters
C:I:2	F	57 years	Neonatal	<4 months	
C:II:1	F	40 years	4 months	Ongoing	Cluster of seizures occurring every 1–2 hours over 2–3 days. PSSG in adolescence and adulthood
C:II:5	F	37 years	10 months	10 months	Single seizure during febrile illness
C:III:11	M	15 years	7 days	7 days	Single cluster of seizures
C:III:12	F	3 years	5 days	3 years	Five clusters over 4 months. Further seizures when unwell
D:II:2	F	55 years	5 days	12 years	Tonic–clonic seizures
D:III:4	F	27 years	4 months	4 months	Single cluster of 4 seizures
D:IV:3	M	1.5 years	5 days	4 months	Nine seizures occurring in four clusters
D:IV:4	M	2 months	5 days	5 days	Single cluster of 5 seizures

PSSG, partial seizure secondarily generalised.

deletion of *KCNQ2* exons 1–5, led to the definitive diagnosis as BFNS. Family A has eight members who had neonatal seizures with onset of seizures in the first month of life. Seizures were focal clonic or generalised tonic–clonic with apnoea and all ceased by 12 months of age. An additional three family members (A:II:4, A:III:4 and A:III:5) had seizures commencing in adulthood. Two members had focal seizures that secondarily generalised, while the third had several nocturnal tonic–clonic seizures that could not be classified. None of these three members experienced seizures in the neonatal period (although the mother of A:II:4 was not available for questioning). The clinical details of the affected members of the four families are summarised in table 1.

As there were no prior samples with *KCNQ2* deletions available to validate the MLPA procedure, we chose one case to independently confirm the result. LR-PCR was carried out to characterise the breakpoints of the smallest deletion, which was in family B. The primers 5′-GCTTCCTCTCTGAAGTGT GTCAG-3′ and 5′-TACTGCAGGAGAGACTCTCAACAG-3′ were used to amplify a product spanning both deletion breakpoints for the deletion that excised exons 13–15. The expected normal PCR product from these primers is 3849 bp in length. This product was not successfully amplified; however, a smaller product of approximately 800 bp in length was amplified from the homologue affected by the deletion from two affected members of family B (fig 4A). This confirmed the presence of the deletion detected by MLPA and indicated that the size of the deletion in family B was approximately 3 kb. The product amplified from individual B:II:2 was sequenced to determine the exact excision points for the deletion, the exact size of the deletion and if additional nucleotides were incorporated during the DNA repair that rejoined the sequence (fig 4B). The deletion in family B was IVS12-768 to IVS15+572 del3018bp, effectively validating the MLPA result. No additional nucleotides were added to the sequence during DNA repair. The exact size of the PCR product was 831 bp.

DISCUSSION

The occurrence of submicroscopic deletions and duplications at the intragenic level of resolution was predicted for *KCNQ2*, based on the high proportion of known truncating mutations in the gene. MLPA was used to screen a group of families

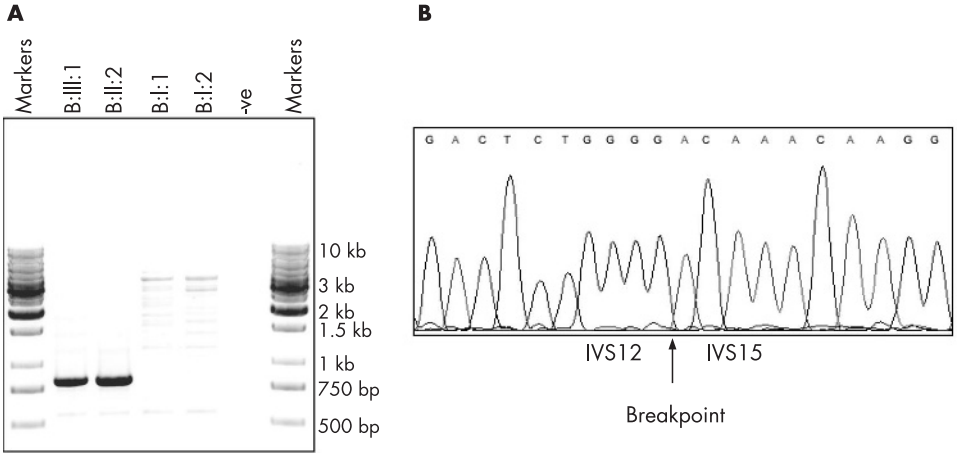

Figure 4 Amplification and characterisation of the breakpoint in family B. (A) 0.8% agarose gel of LR-PCR products from four members of family B and a negative (water) control. The markers used on this gel were the GeneRuler 1 kb DNA ladder (Fermentas). (B) Part of the sequencing trace from the product amplified from patient B:II:2, showing the deletion breakpoint, indicated by the arrow.

tentatively diagnosed on clinical grounds as BFNS or BFNIS, and a group of patients with sporadic neonatal seizures, in whom *KCNQ2* mutations had not previously been identified by PCR-based techniques. Submicroscopic deletions and a duplication mutation were detected in three of eight likely BFNS families and one of three possible BFNIS families, definitively diagnosing the latter as BFNS based on the molecular result and bringing the total number of BFNS families in the study to nine. The same mutations were detected in all affected relatives of each proband. No mutations were detected in the sporadic cases with neonatal seizures. The detection rate for deletions and duplications in patients with BFNS who had previously tested negative by sequencing for *KCNQ2* and *KCNQ3* mutations was 44%.

For one mutation, the deletion breakpoints were characterised. The DNA repair in this case was not accompanied by nucleotide insertions. A 4 bp nucleotide insertion was previously characterised as part of the DNA repair process associated with an *SCN1A* deletion detected by MLPA.[13] Few such breakpoints have been characterised.

All four mutations detected are very likely to be pathogenic, causing the loss of one functional copy of the *KCNQ2* gene, and therefore haploinsufficiency. This mechanism has been suggested for BFNS[2] and is the likely result of the numerous truncating mutations of *KCNQ2* identified in BFNS families. The KCNQ2 protein forms part of the inhibitory M-channel and therefore reduction in its activity, resulting from either missense mutations or haploinsufficiency, is expected to increase neuronal excitability and therefore seizure susceptibility.

Two families (C and D) each contained a single person who had onset of seizures at 4 months of age. BFNS families with *KCNQ2* mutations have been reported in which occasional members had seizure onset between 1 and 8 months of age.[11] Family C has an extensive family history of seizures, all commencing in the neonatal period, therefore the single individual with later seizure onset did not modify our diagnosis of BFNS (see Methods), but was considered to fall within this previously observed variation. However, family D is much smaller, initially with only two confirmed affected members. One had neonatal onset and one had infantile onset, leading to the initial diagnosis of BFNIS. This illustrates that the clinical distinction between BFNS and BFNIS is difficult in small families with both neonatal and infantile onsets. A family with a *KCNQ2* mutation has previously been reported in which the onset of seizures occurred between 2 and 4 months of age, consistent with a familial diagnosis of BFNIS.[14] Together with our data, this further highlights the phenotypic overlap between families with *KCNQ2* and *SCN2A* mutations and the need to incorporate genetic testing as part of the patient investigation if a definitive clinicomolecular syndromal diagnosis is required. Detection of the *KCNQ2* defect in family D and the ascertainment of two more affected members with neonatal seizure onset suggested that the diagnosis of BFNS for the family is more likely.

Family A has two members who did not have observed neonatal seizures, confirmed by a reliable maternal history, but did have focal seizures commencing in adulthood. Their mother also had seizures in adult life, but her neonatal history was unknown. These three people (A:II:4, A:III:4 and A:III:5) all have the BFNS-related *KCNQ2* intragenic deletion. The mutation may be responsible, at least in part, for the partial epilepsy in these members as an alternative phenotype.

Other than the families included in this study, we have examined 15 BFNS families in whom missense or truncating mutations were previously detected by sequencing (Biervert *et al*[2], Richards *et al* [15] and unpublished data). Submicroscopic deletions or duplications were detected in four of the nine BFNS families that were studied here by MLPA, bringing the total number of BFNS families for whom we have identified mutations to 19 out of a total of 24 studied. These numbers suggest that submicroscopic deletions and duplications in *KCNQ2* account for a significant proportion of unsolved familial cases in which mutations are not detected by direct sequencing: over one-fifth of all *KCNQ2* mutations and one-sixth of BFNS families.

A small proportion of probable BFNS families remains unsolved. These could be caused by pathogenic mutations in the promoter or introns of *KCNQ2*, or additional genetic heterogeneity. The promoter and most of the intronic sequences are not usually covered by PCR-based mutation screens, but mutations in these regions could lead to downregulation of transcription or incorrect splicing of the *KCNQ2* transcript. There may also be deletions or promoter mutations in *KCNQ3*, although mutations leading to haploinsufficiency are less likely in *KCNQ3* given that all the mutations described to date in this gene are missense mutations.[5] Another possibility is the presence of at least one other BFNS gene, the existence of which is likely to be confirmed only if a family is identified that is large enough for exclusion of linkage to both *KCNQ2* and *KCNQ3* or by demonstration of linkage to another region of the genome. A final possibility is that a proportion of cases could be polygenic, which might include large copy number variations affecting a number of susceptibility genes related to channel function or expression, including *KCNQ2* and *KCNQ3*.

These results suggest that MLPA is an important second-tier testing strategy for suspected cases of BFNS or BFNIS without previously identified *KCNQ2, KCNQ3* or *SCN2A* coding or splice-site mutations. Submicroscopic deletions and duplications of *KCNQ2* may account for a significant proportion of these cases.

Key points

- Multiplex ligase-dependent probe amplification was used to detect submicroscopic deletions and duplications in *KCNQ2* in a group of patients with benign familial neonatal seizures, benign familial neonatal-infantile seizures or other neonatal seizures.
- Mutations were identified in four of nine benign familial neonatal seizures patients screened.
- Submicroscopic deletions and duplications in *KCNQ2* are seen in approximately one-sixth of families with benign familial neonatal seizures.

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Authors' affiliations

S E Heron, K Cox, J C Mulley, Department of Genetic Medicine, Women's and Children's Hospital, North Adelaide, South Australia, Australia
Sarah E Heron, John C Mulley, School of Paediatrics and Reproductive Health, University of Adelaide, Adelaide, South Australia, Australia
B E Grinton, S F Berkovic, I E Scheffer, Department of Medicine (Neurology), University of Melbourne and Austin Health, Heidelberg, Victoria, Australia
S M Zuberi, Fraser of Allander Neurosciences Unit, Royal Hospital for Sick Children, Yorkhill, Glasgow, UK

S Kivity, R Straussberg, Schneider Children's Medical Center, Petaq Tikva, Israel
Z Afawi, Department of Neurology, Tel Aviv Sourasky Medical Center, Tel Aviv, Israel
I E Scheffer, Department of Paediatrics, University of Melbourne, Royal Children's Hospital, Melbourne, Victoria, Australia
J C Mulley, School of Molecular and Biomedical Science, University of Adelaide, Adelaide, South Australia, Australia

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Correspondence to: Ms S Heron, Department of Genetic Medicine, Women's and Children's Hospital, 72 King William Road, North Adelaide SA 5006, Australia; sarah.heron@cywhs.sa.gov.au

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