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Large scale calcium channel gene rearrangements in episodic ataxia and hemiplegic migraine: implications for diagnostic testing

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ABSTRACT

Background: Episodic ataxia type 2 (EA2) and familial hemiplegic migraine type 1 (FHM1) are autosomal dominant disorders characterised by paroxysmal ataxia and migraine, respectively. Point mutations in *CACNA1A*, which encodes the neuronal P/Q-type calcium channel, have been detected in many cases of EA2 and FHM1. The genetic basis of typical cases without *CACNA1A* point mutations is not fully known. Standard DNA sequencing methods may miss large scale genetic rearrangements such as deletions and duplications. The authors investigated whether large scale genetic rearrangements in *CACNA1A* can cause EA2 and FHM1.

Methods: The authors used multiplex ligation dependent probe amplification (MLPA) to screen for intragenic *CACNA1A* rearrangements.

Results: The authors identified five previously unreported large scale deletions in *CACNA1A* in seven families with episodic ataxia and in one case with hemiplegic migraine. One of the deletions (exon 6 of *CACNA1A*) segregated with episodic ataxia in a four generation family with eight affected individuals previously mapped to 19p13. In addition, the authors identified the first pathogenic duplication in *CACNA1A* in an index case with isolated episodic diplopia without ataxia and in a first degree relative with episodic ataxia.

Conclusions: Large scale deletions and duplications can cause *CACNA1A* associated channelopathies. Direct DNA sequencing alone is not sufficient as a diagnostic screening test.

Episodic ataxia type 2 (EA2) and familial hemiplegic migraine type 1 (FHM1) are dominant disorders caused by mutations in *CACNA1A* on chromosome 19p1.3.[1,2] This gene encodes the α subunit of the P/Q-type calcium channel,[3] the principal channel involved in neurotransmission at central nerve terminals.[4] Episodic ataxia type 2 (EA2) is characterised by paroxysmal attacks of cerebellar dysfunction resulting in incoordination, slurred speech and ataxic gait.[5–7] Epilepsy has also been reported as part of the phenotype in some cases.[8,9] Familial hemiplegic migraine type 1 (FHM1) is a variant of migraine with aura resulting in attacks of unilateral hemiplegia and/or hemisensory loss.[10] Nonsense and missense mutations, which result in a premature stop codon or aberrant splicing, account for many cases of EA2[11–13] while missense mutations altering conserved amino acid residues tend to associate with FHM1[14] but also cause EA2.[15]

A significant proportion of EA patients with clinical features typical of EA2 do not have point mutations in *CACNA1A*. Mutations in two additional genes (*CACNB4* and *SLC1A3*) have been reported in a small number of patients with EA2-type features. A mutation in *CACNB4*, encoding the β subunit of the P/Q-type calcium channel, has been described in one family with an EA2 type phenotype (termed EA5) and also in another family with juvenile myoclonic epilepsy.[16] *SLC1A3*, encoding the excitatory amino acid transporter EAAT1, has been associated with EA2-like features in three families (termed EA6).[17,18] Two additional genes have been associated with FHM: *ATP1A2*[19–21] and *SCN1A*[22] cause FHM types 2 and 3, respectively. However, many patients with typical clinical features of EA2 and FHM1 do not have point mutations in these genes. These observations suggest that either other genes are responsible or that they are due to unidentified genetic defects in *CACNA1A*. Defects such as those in introns, alternatively spliced exons and regulatory regions may not be detected by standard mutation screening strategies.

In the present study, we used multiplex ligation dependent probe amplification (MLPA) to screen for large scale genetic rearrangements in the *CACNA1A* gene in a series of patients who had clinical EA or hemiplegic migraine. Our aim was to investigate if large deletions and duplications in the gene can cause these disorders.

METHODS

Patients

Ethical approval was obtained from the UCLH ethics committee. A total of 53 cases, including the cohort described in Graves and Hanna,[24] were included in the current study. Samples from patients with a clinical diagnosis of EA2 or FHM were referred from England, Republic of Ireland, and Denmark. These patients had been previously sequenced for mutations in the coding regions of *CACNA1A* and no disease-causing mutations were identified. Critical evaluation of patient data was carried out in accordance with published criteria.[5,24]

MLPA analysis

The MLPA *CACNA1A* test kit (SALSA P279_A1) is manufactured by MRC-Holland, Amsterdam. The kit contains probes for exons 1, 4, 6–8, 10, 11, 13, 16, 18, 20, 24, 25, 27, 29, 31, 33, 35, 38, 39, 40, 43, 46 and

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47 of *CACNA1A*. MLPA was performed according to manufacturers' instructions and the amplified fragments were analysed using an ABI 3730xl capillary sequencer (Applied Biosystems, Foster City, California, USA) and the GeneMarker v1.70 software (SoftGenetics, State College, Pennsylvania, USA). Individual peaks corresponding to each exon (*CACNA1A* as well as control genes) were identified based on the difference in migration relative to the size standards (LIZ 500, Applied Biosystems). The GeneMarker analysis software uses the MLPA ratio method to determine the deviation of each allele peak, relative to the average deviation of all peaks. This method standardises the data so that the median point within the dataset is considered to be 1 and calculates the deviation of each peak from this median as a ratio. A peak with a ratio <0.75 indicates a deletion while a peak with a ratio >1.25 indicates a duplication.

DNA sequencing

Automated direct DNA sequencing was performed on PCR products using primers that amplified each exon of *CACNA1A* which were found to be deleted or duplicated using MLPA analysis. Samples were sequenced using the ABI Big Dye Terminator Sequencing Kit version 1.1 on an ABI 3730xl capillary sequencer.

RESULTS

Patients from eight unrelated families were found to have rearrangements in *CACNA1A*. Six had a clinical diagnosis of attacks with features typical of EA2, one had sporadic hemiplegic migraine, and one patient had episodic diplopia. In three patients the data indicated that at least a single exon deletion was present (table 1, figs 1 and 2). In all cases the presence of single nucleotide polymorphisms (SNPs) under the probe binding site was excluded by direct DNA sequencing. None of the deletions or the single duplication were detected in a panel of 180 normal control chromosomes. The frequency of rearrangement mutations in our patient cohort is significantly different when compared to a control population ($p<0.001$; Fisher's exact test), supporting the view that our results are not false positives occurring by chance only in the patient disease group and not in the control group. The mutations have not been reported before in the literature.

Family 1

Episodes of ataxia began in the proband at the age of 39 years with stereotyped attacks of unsteadiness and dysarthria lasting a few hours. There were no precipitants for attacks but sleep helped their resolution. The attacks were completely abolished by acetazolamide. Interictal examination revealed square wave jerks, gaze evoked nystagmus and jerky pursuits, but no limb ataxia. Family history indicated dominant inheritance with the proband's daughter and father also affected (fig 3A). The daughter reported stress induced attacks of unsteadiness, dysarthria and nausea which lasted several hours. She responded well to acetazolamide. Examination revealed gaze evoked nystagmus. The proband's father is deceased but had a history consistent with EA2.

MLPA identified a heterozygous deletion of exon 4 of *CACNA1A* in the proband and in his daughter (fig 1B). Probes for exons 1 and 6 gave normal results, indicating that the maximum length of the deletion is 146131 base pairs (fig 2).

Family 2

The proband was from a family with eight affected individuals, spanning four generations exhibiting a classical phenotype of EA2 (fig 3B). She developed attacks from the age of four years. These consisted of diplopia, slurred speech, unsteadiness and headaches. Excitement, stress and tiredness triggered episodes. Examination at the age of 59 years revealed cerebellar signs including gaze evoked nystagmus, cerebellar dysarthria, finger nose ataxia and a marked broad based gait. Since the age of 30 there has been persistent gait ataxia and dysarthria.

Table 1 Summary of clinical and genetic findings in families with *CACNA1A* rearrangements detected by multiplex ligation dependent probe amplification

Family index case	Dx	Age onset (years)	Episodic features	Episode duration	Interictal examination	FH	Additional features	MRI brain	Response to ACZ	Exon del/dup	Maximum length of del/dup (bp)
1	EA2	39	Ataxia, dysarthria	Few hours	SWJ, GEN JP	Daughter/father affected	Headaches	–	Good	del: exon 4	146131
2	EA2	4	Diplopia, dysarthria ataxia, headache	24 h	GEN, dysarthria, ataxic gait	8 affected	Persistent cerebellar syndrome	–	Good	del: exon 6	35778
3	ED	Lifelong	Diplopia, oscillopsia	Few hours	GEN	Father had EA	–	Normal	None	dup: exon 6	35778
4	EA2	5	Vertigo, dysarthria ataxia	Hours to days	SWJ, GEN JP	No	Epileptiform EEG	–	None	del: exon 27	7474
5	EA2	1.5	Ataxia, vomiting	Few hours	GEN, JP Ataxic gait	No	–	–	Good	del: exons 20–38	86155
6	EA2	10	Ataxia, vertigo, vomiting	Several hours	GEN, DDK	No	Absence seizures	Cerebellar atrophy	Initial response	del: exons 39–47	Unknown; predicted to be18225
7	SHM	17	LOV in right hemifield, right sided hemiplegia	30 min	Normal	No	–	Normal	ACZ declined	del: exons 39–47	18225
8	EA2	2.5	Ataxia, vertigo, nausea	Few hours	DDK	No	–	Normal	ACZ declined	del: Exons 39–47	18225

ACZ, acetazolamide; bp, base pairs; DDK, dysdiadochokinesis; del, deletion; dup, duplication; Dx, diagnosis; EA, episodic ataxia; ED, episodic diplopia; FH, family history; GEN, gaze evoked nystagmus; JP, jerky pursuit; LOV, loss of vision; SHM, sporadic hemiplegic migraine; SWJ, square wave jerks.

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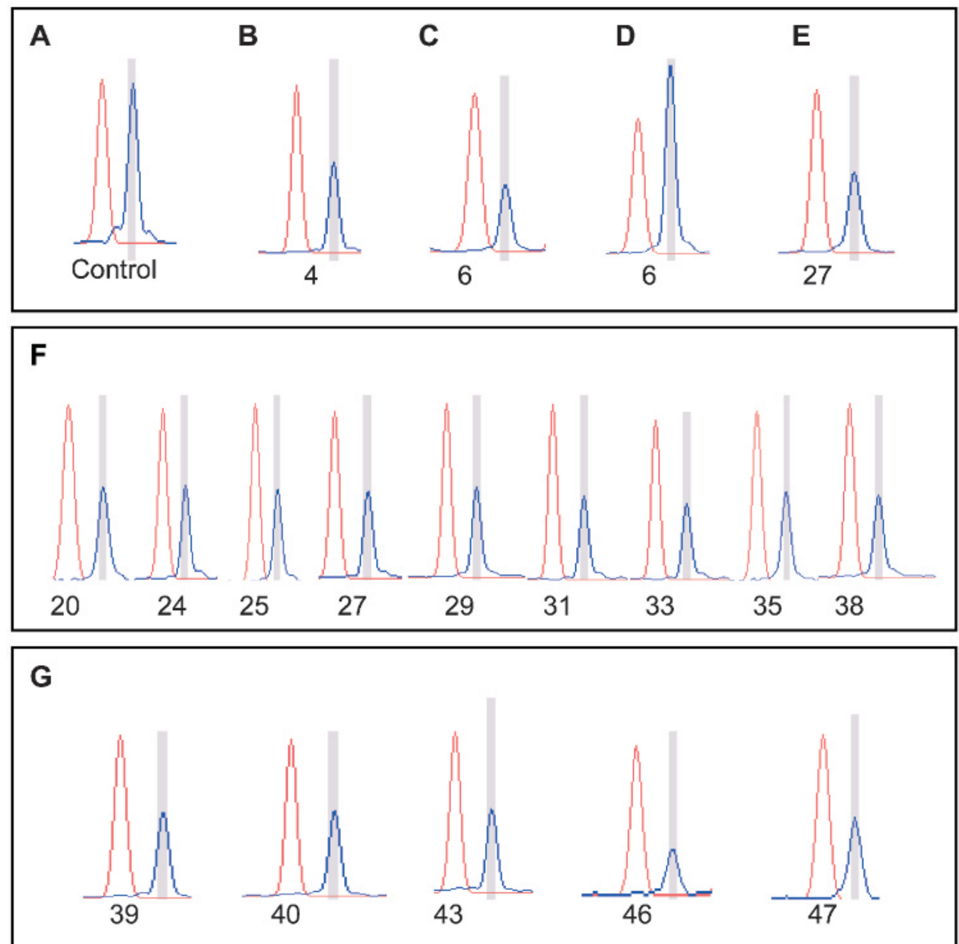

Figure 1 Multiplex ligation dependent probe amplification traces showing deletions and duplications of *CACNA1A* exons. The red peak is a control peak of known copy number to which the patient DNA (blue peak) is compared and a ratio calculated. A peak with a ratio <0.75 indicates a deletion while a peak with a ratio >1.25 indicates a duplication. Numbers correspond to exons in *CACNA1A*. A: Normal control; B: deletion of exon 4 (family 1; ratio: 0.43); C: deletion of exon 6 (family 2; ratio: 0.55); D: duplication of exon 6 (family 3; ratio: 1.39); E: deletion of exon 27 (family 4; ratio: 0.550); F: deletions of exons 20 (0.54), 24 (0.55), 25 (0.53), 27 (0.53), 29 (0.52), 31 0.48), 33 (0.53), 35 (0.48) and 38 (0.52) seen in family 5; G: deletions of exons 39 (0.52), 40 (0.54), 43 (0.52), 46 (0.31) and 47 (0.50) seen in family 6 (a similar trace was detected in families 7 and 8).

Although the age of onset and disease severity varied among the other affected family members, all experienced similar symptoms to that of the proband. All affected members showed a good response to acetazolamide.

Linkage analysis identified a common haplotype at 19p13 that segregated with disease status in the family (supplemental fig 1). However, DNA sequencing of the entire coding and flanking regions of *CACNA1A* in the proband did not identify a point mutation. In contrast, MLPA identified a heterozygous deletion of exon 6 of *CACNA1A* in the proband (fig 1C). There was no probe for exon 5, but exons 4 and 7 were present. Based on these data, the maximum size of the deletion is predicted to be 35778 base pairs (fig 2). The deletion was confirmed to segregate with the EA2 phenotype and was found in all affected family members and was absent in one unaffected family member.

Family 3

The index case described lifelong "spasms" of his eyes by which he meant attacks of double vision and oscillopsia, which occurred up to twice a day and lasted 1–2 h. These attacks were often triggered by sudden head movements. He denied other motor or sensory symptoms. There was no history of ataxia but he did report dizziness which occasionally accompanied his attacks of double vision. On examination he had gaze evoked nystagmus. There were no cerebellar signs in his limbs and no gait ataxia. Magnetic resonance imaging (MRI) of his brain was normal and he did not respond to acetazolamide. His father had excitement induced attacks of ataxia and slurred speech which would last 1 h, during which he reportedly had oscillopsia and nystagmus.

MLPA identified a heterozygous duplication of exon 6 of the *CACNA1A* gene in the proband (fig 1D). The maximum length of the duplication is predicted to be 35778 base pairs (fig 2).

Family 4

The index case experienced stereotyped attacks of EA2 from the age of 5 years. Episodes consisted of vertigo, slurred speech and incoordination in her arms and legs. A throbbing headache and vomiting accompanied half her attacks which lasted from a few hours to 3 days. Sleep often either aborted or lessened the severity. Interictally, she described occasional oscillopsia. There was no family history of episodic ataxia or migraine. When examined at 18 years, she had square wave jerks in the primary position with gaze evoked nystagmus and jerky pursuit movements. There was mild gait ataxia. An electroencephalogram (EEG) demonstrated a mild excess of irregular slow wave bursts with spike wave transients suggestive of epileptiform paroxysms. However, an EEG taken at the time of an attack remained in its normal wake state, although the bursts were a little more frequent. There was no history to suggest seizures. Unusually, there was no beneficial response to acetazolamide.

A heterozygous deletion of exon 27 of *CACNA1A* was detected by MLPA (fig 1E). Probes for exons 25 and 29 gave normal results, indicating that the maximum length of the deletion is 7474 base pairs (fig 2).

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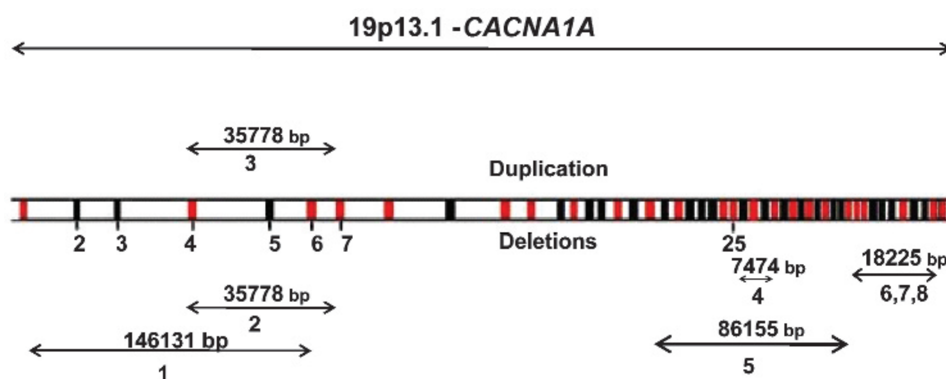

Figure 2 Maximum extent of the deletions and duplication detected using multiplex ligation dependent probe amplification (MLPA). Red squares indicate exons for which a probe is included in the MLPA analysis. Letters represent index cases of families as they appear in the main text.

Family 5

From the age of 18 months, the index case experienced attacks of unsteadiness and vomiting which lasted up to 6 h and occurred twice a month. There was no relevant family history. Interictal examination revealed jerky pursuit movements, gaze evoked nystagmus and a mild broad based gait. There was a good response to acetazolamide.

MLPA identified heterozygous deletions of exons 20 to 38 of *CACNA1A* (fig 1F). As exons 18 and 39 were present in the proband, the maximum length of the deletion is predicted to be 86155 base pairs. This large deletion is predicted to result in either a truncated protein product or degradation of the mRNA transcript.

Family 6

Episodes of "clumsiness" began at the age of 10 years in the index case. There was sudden onset of dizziness and the inability to sit or stand and associated nausea. Examination showed reduced balance on tandem walking and mild dysdiadochokinesis. At the age of 11, she experienced typical absence seizures with ictal EEG recordings consistently showing 3 Hz generalised spike and wave discharges. A few months later, she experienced attacks of vertigo, nausea and vomiting. Initially they occurred weekly and would last up to 4 h. Sleep would help attack resolution. Examination during one such episode revealed ataxia, problems with sitting balance and gaze evoked nystagmus. Initial treatment with acetazolamide resulted in a marked improvement, although attacks were not completely abolished. Over the years, the attack frequency varied from twice monthly to periods of 6 months when she was attack-free. The duration of the attacks diminished with age. An MRI of the brain at 12 years was normal, although subsequent scans at the age of 16 and 18 years demonstrated slight atrophy of the cerebellum, and in particular the vermis. There was no family history of EA2.

Family 7

When assessed at age 37 years the index case gave a 20 year history of frequent episodic neurological symptoms affecting her right side. Attacks comprised loss of vision in her right hemifield, weakness and numbness of the right side of her face, right arm and leg. These attacks generally lasted between 15–30 min. The episodes varied in frequency from twice monthly to three or four times per year. They were usually not accompanied by headaches, nausea, vomiting or photophobia. She also described as having typical migraine with nausea and vomiting which occurred separately from the hemiplegic attacks, but these were infrequent. Neurological examination was entirely normal as was an MRI of the brain. The attacks were considered to be consistent with hemiplegic migraine. Her mother suffered from migraine, but did not have attacks of weakness or abnormal sensation. Her paternal grandfather was described to have had several strokes in his 40s but further details were not available.

Family 8

In the index case, attacks of unsteadiness, dizziness and nausea occurred from the age of 2.5 years. These were precipitated by exertion and relieved by sleep, and would occur on average once a week. She has recently begun to complain of headaches. There was no family history of episodic ataxia but her mother and sister complained of migraine without aura or weakness. Interictal examination at the age of 8 years revealed mild dysdiadochokinesis with poor balance but no other cerebellar signs. EEG and brain MRI were both normal. Acetazolamide has not been prescribed to date.

In the index cases of families 6, 7 and 8, MLPA probe analysis was consistent with a heterozygous deletion of exons 39–47 of *CACNA1A* (fig 1G). Exon 38 was present in all three cases and

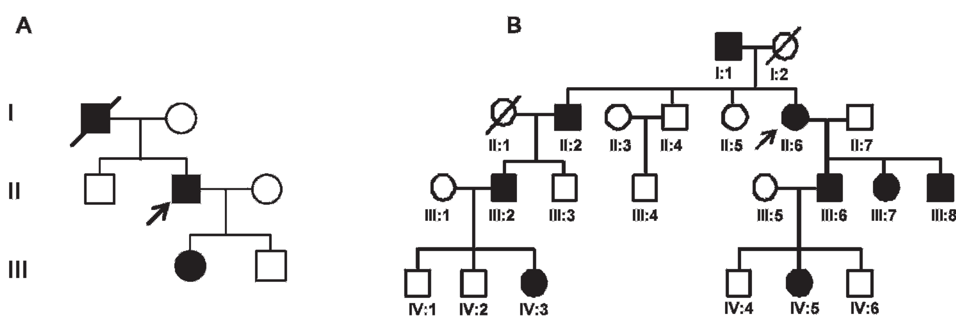

Figure 3 Families 1 (A) and 2 (B). Filled symbols represent affected individuals. Arrows represent the proband.

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although the 3' limit of the deletion is yet to be characterised, two of the patients (7 and 8) were heterozygous for a CAG repeat present at the end of exon 47, downstream of the location of the MLPA probe (data not shown). Therefore the maximum length of the deletion in these patients is predicted to be 18225 base pairs (fig 2). We suspect the breakpoint of the deletions will be the same in all three patients and therefore assume the size of the deletion to be the same in the third patient, who was homozygous for the CAG repeat, but until the breakpoints of the deletion are mapped, this cannot be confirmed. However, in all three cases, the absence of multiple exons is likely to result in degradation of the mRNA transcript.

DISCUSSION

An increasing number of point mutations in *CACNA1A* have been reported to cause EA2 and FHM1.[1 6 10 11 14] However, a significant number of patients with typical phenotypes do not harbour point mutations in *CACNA1A*. Sequencing of additional candidate genes, *SCL1A3* and *CACNB4*, failed to identify mutations in part of our cohort.[23] A deletion mutation mechanism has been described in other ion channel genes such as in *SCN1A*, which causes severe myoclonic epilepsy of infancy (SMEI).[25] Recently, a large deletion in *CACNA1A* was reported in the two affected members of a family with EA2.[26]

In the present study we identified *CACNA1A* rearrangements in eight families. These rearrangements are likely to be pathogenic. Their location within the channel suggests they would remove or duplicate highly conserved regions of the channel protein. It is possible that the deletions in the index case of family 5 which spans at least exons 20 to 38 and in families 6, 7 and 8, which removes all exons downstream of exon 38, would result in premature truncation of the channel protein. Alternatively these multi-exon deletions may result in mRNA transcripts that undergo nonsense mediated decay, but this needs further evaluation. In the index cases of families 1, 2, 3 and 4, in whom rearrangements of at least one exon have been identified, the fate of the mRNA transcript will depend on whether the mutations affect the reading frame. If in-frame, such large rearrangements are likely to interfere with correct protein assembly, folding and trafficking. In family 1, the deletion spanned exon 4 which encodes the S4 voltage sensor segment of domain I, removal of which is likely to have deleterious effects on kinetic parameters such as the voltage dependence of activation. The segregation of the deletion in family 2 in eight affected individuals and its absence in an unaffected member also supports pathogenicity. Segregation was also seen in family 1. The rearrangements were not identified in a group of 180 normal control chromosomes. We estimated that it is very unlikely our observations are false positives as we did not see any rearrangements in the control group ($p<0.001$, Fisher exact test).

The phenotypic spectrum observed in this study included EA2, hemiplegic migraine and episodic diplopia. The finding of a large scale deletion in the index case of family 7 who presented with hemiplegic migraine is of interest as to date only small deletions have been described in this disorder.[27] Although FHM is generally considered to be caused by gain of function mutations, some groups have argued that FHM may arise from loss of function mutations.[28 29] Our findings suggest that a deletion mutation mechanism, which is more likely to result in a loss of function, may underlie the phenotype in family 7. Parental samples from the index case of family 7 were not available for testing. Our findings, together with our recent identification of a second hemiplegic migraine patient with the same deletion (see Addendum), suggest that patients with hemiplegic migraine should be screened for deletions.

In family 3 we identified the first reported exonic duplication in *CACNA1A*. The index case presented with an episodic eye movement disorder without ataxia or dysarthria. Eye movement abnormalities including diplopia and oscillopsia are documented in EA2 patients[30] but are not commonly reported in isolation. The index case's father was reported to have typical EA2, suggesting phenotypic variability among members of the same kindred.

Three patients were found to have deletions of exons 39–47. Their diverse ethnic backgrounds (Irish, Indian, and Danish) would argue against a founder effect. This recurrent large deletion may reflect regional intragenic propensity to recombination. The only previously described large deletion was considered to have arisen through homologous recombination of *Alu* sequences.[26]

Patients 3 and 4 did not respond to acetazolamide. In our experience nearly all patients with clinical episodic ataxia appear to respond very well to acetazolamide, but there is subset of patients in whom it has very little effect. This might be a result of the specific mutations, but the reason remains unclear.

The identification of a deletion in *CACNA1A* in the large family with EA2 (family 2) highlights the importance of screening for deletions in *CACNA1A* as part of the molecular characterisation of EA2. Our linkage analysis had previously identified a common haplotype at 19p13 that segregated with disease status but sequencing had failed to identify any mutations in *CACNA1A*. Segregation of the deletion with the disease phenotype supports the view that this deletion is disease-causing.

Since MLPA probes for all exons are not yet available, a negative MLPA result in this study does not exclude deletions or duplications of the remaining exons. However, the positive finding of large scale rearrangements in *CACNA1A* in eight unrelated families provides new evidence that such mutations can be associated with episodic ataxia, episodic diplopia, and with hemiplegic migraine. In our cohort of EA2 families, in whom we have now identified pathogenic mutations in *CACNA1A*, 12% have a large scale rearrangement.

This study has expanded the mutation spectrum in *CACNA1A* to include large scale rearrangements. Our observations have implications for DNA diagnosis. To date most groups have used direct DNA sequencing which may miss these

Key points

- The aim of this study was to screen a series of patients with a clinical phenotype of episodic ataxia or hemiplegic migraine for large scale rearrangements in the *CACNA1A* gene.
- Using MLPA we initially identified four single exonic deletions and four multi-exonic deletions in eight families.
- Three families had multiple deletions of exons 39–47 of *CACNA1A*. In addition, we have since identified two unrelated patients who also harbour deletions of exons 39–47 (see addendum) suggesting that this region of the gene may be a hot spot for recombination.
- Our findings suggest that screening for rearrangements in *CACNA1A* should complement direct sequencing of the gene in *CACNA1A* associated channelopathies.

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rearrangements. We suggest that screening for large scale rearrangements by rapid techniques such as MLPA should be considered as a first line approach for genetic diagnostic testing of *CACNA1A* associated channelopathies. Our findings indicate that large scale gene rearrangements are an important cause of *CACNA1A* associated neurological disease.

Addendum

Since the original submission of this manuscript we have identified a further two unrelated patients with deletions of exons 39 to 47. One patient has sporadic hemiplegic migraine and experiences hemisensory and hemimotor disturbance, while the second patient has episodic ataxia and epilepsy. This brings the total number of unrelated individuals harbouring rearrangements to 10 identified in this study, eight of which have multiple consecutively deleted exons.

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