Next Generation Sequencing Identifies Novel $\textit{CACNA1A}$

Gene Mutations in Episodic Ataxia Type 2

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Episodic Ataxia type 2 (EA2) is a rare autosomal dominantly inherited neurological disorder characterized by recurrent disabling imbalance, vertigo and episodes of ataxia lasting minutes to hours. EA2 is caused most often by loss of function mutations of the calcium channel gene \textit{CACNA1A}. In addition to EA2, mutations in \textit{CACNA1A} are responsible for two other allelic disorders: familial hemiplegic migraine type 1 (FHM1) and spinocerebellar ataxia type 6 (SCA6). Herein, we have utilised Next Generation Sequencing (NGS) to screen the coding sequence, exon-intron boundaries and UTRs of five genes where mutation is known to produce symptoms related to EA2, including \textit{CACNA1A}. We performed this screening in a group of 31 unrelated patients with EA2 symptoms. Both novel and known mutations were detected through NGS technology, and confirmed through Sanger sequencing. Genetic testing showed in total 15 mutation bearing patients (48%), of which 9 were novel mutations (6 missense and 3 small frameshift deletion mutations) and six known mutations (4 missense and 2 nonsense). These results demonstrate the efficiency of our NGS-panel for detecting known and novel mutations for EA2 in the \textit{CACNA1A} gene, also identifying a novel missense mutation in \textit{ATP1A2} which is not a normal target for EA2 screening.

\textbf{KEY WORDS}: AmpliSeq Custom Panel; \textit{CACNA1A}; Episodic Ataxia type 2; Next Generation Sequencing.
Introduction

Episodic Ataxia type 2 (EA2) is an autosomal dominantly inherited paroxysmal cerebral disorder that demonstrates variable expressivity and starts in childhood or early adolescence (age range 2-32 years). EA2 is characterized by episodes of ataxia, vertigo and nausea lasting minutes to hours. The frequency of attacks ranges from once a year to four times a week. Attacks can be triggered by stress, exertion, caffeine, alcohol, fever, and heat. Acetazolamide (Griggs et al., 1978) and 4-aminopyridine (Strupp et al., 2004) have been shown to be effective in treating EA2, particularly in reducing attack frequency and severity.

The \textit{CACNA1A} gene (MIM: 601011) which covers 300 Kb with 47 exons, is located at chromosome 19p13 (Kramer et al., 1995, Teh et al., 1995) and is the only gene in which mutations are known to cause EA2 (Ophoff et al., 1996). The gene codes for the $\alpha_{1A}$ pore-forming subunit of Ca2+ voltage-gated Cav2.1 channels and is widely expressed throughout the central nervous system (CNS) (Mori et al., 1991, Westenbroek et al., 1995). It is involved in a variety of Ca2+ dependent processes, including mediating the entry of Ca2+ ions into excitable cells, muscle contraction, hormone or neurotransmitter release, and gene expression (Tsien et al., 1991).

Mutations in the \textit{CACNA1A} gene have been found to be responsible for three disorders with autosomal dominant inheritance: episodic ataxia type 2 (EA2; MIM: 108500), familial hemiplegic migraine type 1(FHM1; MIM: 141500) and spinocerebellar ataxia type 6 (SCA6; MIM: 183086). Clinical overlap between the three disorders in terms of symptoms has been previously reported (Jodice et al., 1997, Mantuano et al., 2003, Romaniello et al.,
Nonsense mutations (Ophoff et al., 1996), deletions (Labrum et al., 2009, Riant et al., 2008) and missense mutations in CACNA1A gene have all been found to lead to loss-of-function of recombinant human CaV2.1 channels in heterologous expression systems found to cause EA2.

Since the 1970s, most DNA diagnosis is undertaken by ‘gold standard’ traditional DNA Sanger sequencing. This is an accurate but slow and expensive means of diagnosis. Additionally, in order to avoid extensive costs to patients or health systems, the practice typically involves screening only regions where mutations are known, or more likely, to occur, limiting the ability of testing to identify unusual mutations causing disease. Those who fail initial screenings must thus go through additional rounds of testing. Therefore, testing for EA2 using Sanger sequencing is difficult, time consuming and expensive due to the number and size of sequences investigated. In contrast, Next Generation Sequencing (NGS) approaches have opened the door for massive parallel sequencing of targeted genes, as well as whole exome and whole genome sequencing. The AmpliSeq custom panel technique used here is an appropriate method to screen mutations in genes which cause clinically overlapping disorders.

We have developed an AmpliSeq custom panel comprising the genes most related to episodic ataxia type2 and FHM in terms of causation of symptoms (CACNA1A, ATP1A2, SCN1A, NOTCH3 and KCNK18) in order to improve mutation detection. We screened a cohort of 31 unrelated patients with clinical diagnosis of EA2 for whom primary Sanger sequencing had failed to find a causative mutation using the Ion Torrent NGS platform.
MATERIALS AND METHODS

Patients

The Genomics Research Centre (GRC) diagnostics clinic began diagnostic testing in 1999 and to date has tested approximately 730 patients for Episodic Ataxia type 2 (EA2), Familial Hemiplegic Migraine (FHM) and cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL). In the early stages of the clinic’s operation, causative mutations were identified using the traditional Sanger sequencing approach. During this period, DNA testing was undertaken in 167 EA2 patients according to the request of certified clinical neurologists around Australia and New Zealand. Of the 167 patients that had been screened previously for mutations in selected exons in CACNA1A gene, few were found to have genetic aberrations. This prompted the development of the NGS test. From our 167 patients, 31 index cases underwent advanced testing, due to the presence of clinical EA symptoms without detection of genetic mutations by primary Sanger sequencing.

Migraine Panel Characteristics and Ion Torrent Sequencing

In the development of the panel, we chose 5 genes [Error! Reference source not found.] that were known to be involved in monogenic neurological disorders, including EA2. The ampliseq.com web designer was utilized to design primer pools to our specified genomic regions, according a reference human genome (hg 19 for our study). The primers for this panel of genes covers exonic regions, exon-intron boundaries as well as 3’ and 5’ Untranslated Regions (UTRs). In total, this amounts to 34.9 kb of sequencing region, with the
primers allowing for sequencing of 91.97% of our original target regions, using 274 primer
pairs in two highly multiplexed reactions requiring only 10ng/reaction of input DNA per
sample.

In brief, sequencing of the amplified regions for the 31 genomic DNA samples were
then conducted using the Ion AmpliSeq library kit (Life Technologies, Cat. no. 4480441 Rev
A.0), followed by template preparation using the Ion OneTouch instrument (Life
Technologies, Catalog Number 4480974 Rev 5.0), and finally were sequenced on the Ion
Torrent Personal Genome Machine (PGM) using the sequencing 200 v2 kit protocols (Life
Technologies, Catalog Number 4482006 Rev 3.0), all according to manufacturer instructions.
One of 16 unique barcodes (Ion Xpress) was used to provide identification for each sample
on a particular sequencing reaction. Library quality and quantity was assessed using the
Agilent DNA High Sensitivity Bioanalyzer Kit. Two Ion 316 v2 Chips were used for
sequencing allowing multiplexing of the 16 barcoded samples. The number of samples used
per chip provided scope for around 100x read depth of coverage at each bases, allowing
accurate detection of mutations.

NGS Sequencing Analysis

Data from the PGM runs were processed initially using the Ion Torrent platform-
specific pipeline software (Torrent Suite v4.0.2) to generate sequence reads, trim adapter
sequences, filter, and remove lower-quality reads according to the BED file provided by
AmpliSeq designer. Generated sequence files were aligned to the human complete genome
(hg19). DNA and protein sequences from NGS and later Sanger sequencing were compared
with the NCBI reference sequences (Pruitt et al., 2012), and the UCSC genome browser
All rs ID numbers, locations, allele frequencies and genotypes for known variants were determined based on SNPs reported in dbSNP database (Sherry et al., 2001) and further verified in the 1000 Genomes data sets. All variants detected were visually confirmed using the Integrative Genome Viewer (IGV2.3) software (Thorvaldsdottir et al., 2013) and Ion Reporter Software (Life Technologies) were used to annotate the variants.

All potentially causative mutations were further investigated by conventional Sanger Sequencing using standard protocols (Roy et al., 2012). Forward and reverse primer sequences for the validations are listed in [Error! Reference source not found.]. PCR products were used as templates for sequencing with BigDye Terminator reagents on a 3500 DNA sequencing Analyzer (Life Technologies). Sanger sequence traces were aligned to the gene-specific reference sequence (NCBI build) using BLAST and CLUSTAL W, and visualised for manual verification of mutations and variants with Chromas 2.33 software.

To predict the damaging effect of non-synonymous single nucleotide substitutions on protein structure, function, or phenotype, we used the available online tools, such as SIFT (Ng and Henikoff, 2001) (http://sift.jcvi.org/www/SIFT), Polyphen2 (Adzhubei et al., 2010) (http://genetics.bwh.harvard.edu/pph2/) and finally Mutation Taster (Schwarz et al., 2010) (http://www.mutationtaster.org/). Each software package, was used to estimate the pathogenicity effect of every suspected variant, such as exonic nonsynonymous/synonymous, intronic and variants in the 5’ and 3’ untranslated regions, though SIFT and Polyphen2 only estimate pathogenicity based on coding sequence changes.
Where patients were also family members seeking confirmation of mutation status, segregation analysis for the available family members was conducted to further determine the impact of novel mutations/variants with the phenotype.

**Results**

**Sequencing output analysis**

Genomic DNA from 31 patients with episodic ataxia type 2 phenotypes were sequenced using a NGS approach. Resultant Ion Torrent PGM sequence data was analysed using the Ion Torrent platform- specific software Torrent Suite v4.0.2 as detailed above. Sequencing via PGM generated an average sequencing of 3,136,750 total reads per Ion 316 Chip, 455 Mb total bases detected, and 450 Mb of final readable data with 99% of total bases aligned to the human complete genome (hg19) and an average amplicon size of 147bp.

The complete summary of data for each detected functional variant, mutation and amino acid neutral novel variant can be found in Error! Reference source not found..

**Variants analysis**

Our panel sequences five genes associated with different neurological disorders including EA2, which all have overlapping phenotypes as part of our comprehensive diagnostics tool.

After variant filtering, annotation, and interpretation, 127 different variants were identified among the 31 patients in the five sequenced genes with an average of 40
variants/patient. The bulk of these are unremarkable common SNPs and are not for clinical interest. In the following subsections, we will discuss the more unusual variants detected in these cases.

Identification of known and novel mutations

Nucleotide changes resulting in changes in highly conserved amino acid residues and predicted to be pathogenic with at least two of the prediction tools used, were considered to be mutations.

In total, 15 of 31 (48.4%) of our patients carried probable disease-causing non-synonymous, nonsense and small frameshift deletion mutations were identified in CACANA1A and ATP1A2 genes [Error! Reference source not found.]. As expected on the basis of the requested EA2 test, 14 of the 15 identified mutations were detected in the CACNAIA gene and only one was unexpectedly detected in ATP1A2 gene.

Among the 14 detected mutations in the CACNAIA gene [Error! Reference source not found., Error! Reference source not found.&Error! Reference source not found.], five were previously described disease-causing mutations: two missense mutations in exon 12 p.Glu533Lys (Scoggan et al., 2006), p.Gly540Arg (Rajakulendran et al., 2010); two missense mutations in exon 6 and 13 (Cases13, 30), p.Asp302Asn (Burk et al., 2014) p.Arg583Gln (rs121908217) (Battistini et al., 1999, Cleves et al., 2010) and; the two nonsense mutations found in exon 27 and 37, p.Trp1448Ter (Jen et al., 2004) and p.Tyr1853Ter (Giffin et al., 2002).
The remaining 8 mutations in CACNA1A were previously undescribed and estimated by the *in silico* tools as pathogenic. In the patients tested, there were three novel frameshift deletions: Case 17, c.928_931delACTG p.Thr310fs*5; Case 3, c.1672-1_1675deletionGGTTA Val558Serfs*13; and case 8, c.1799_1800delTC p.Leu600fs*41 detected in exon 6, 13 and 14 respectively. Other than the frameshifts, there were: Five novel non-synonymous mutations scattered throughout five different exons: in exon3 (Case 28) p.Gly162Val; in exon6 (Case 29) p.Arg279Cys; in exon8 (Case 24) p.Arg387Gly; in exon9 (Case 27) p.Gly411Trp; and in exon34 (Case 9) p.Leu1749Pro.

Interestingly, one novel mutation was identified in one of the 31 EA2 patients screened (Case 18) in exon13 of the ATP1A2 gene and (c.1709C>T, p.Thr570Met) and not the CACNA1A gene. Like the mutations detected in CACNA1A this ATP1A2 mutation was predicted to be deleterious by the three prediction tools used here.

**Identification of novel variants**

In addition, to the novel and known mutations identified in these cases, there were a number of novel non amino-acid changing variants identified. First, a synonymous novel variant in exon7 c.984C>T found in the same patient (Case 13) who was carrying a known mutation (p.Asp302Asn) and both the synonymous and non-synonymous variants were confirmed with Sanger in the index case and the available DNA of the only one family member also sent for testing (a brother). Similarly Case 29, was found to have a novel missense mutation (p.Arg279Cys) in CACNA1A gene in exon 6 along with novel potentially damaging 3’UTR variant [Error! Reference source not found.].
Moreover, there were three unique variants identified in three different patients, the first (Case 19) was a predicted damaging synonymous variant detected in the \textit{NOTCH3} gene which shares the same chromosome as the \textit{CACNA1A} gene, while an previously unknown, but predicted to be tolerated intronic variant was detected in \textit{CACNA1A} in one patient (Case 6) and finally, a new 3’UTR variant was detected (Case 10) in the \textit{ATP1A2} gene, also predicted to be tolerated by Mutation Taster software.

\textbf{Identification of rare SNPs}

In 10 of 16 cases for whom our panel couldn’t detect known or unknown mutations/damaging variants, several rare SNPs were detected, which are listed in [Error! Reference source not found.]. All have variable pathogenic effect when analysed by Mutation Taster software, most of them were unique to patients without any known or novel pathogenic genetic aberration identified in the five screened genes.

\textbf{Mutation validation and segregation analysis}

All known and novel mutations were further validated and confirmed with conventional Sanger sequencing [Error! Reference source not found.]. In four of the 15 cases (Cases: 8, 13, 29, and 30), DNA samples from one of the patient’s parents or siblings was also requested for genetic testing in addition to the index case. Upon detection of the mutation in the index cases, these family members were also tested to help confirm mutation pathogenicity. Segregation of the individual mutations with similar phenotype as the index
case as noted in the testing request was confirmed within all four families (see schematic pedigree for novel mutations in Error! Reference source not found.).

In summary, the mutational spectrum for EA2 patients comprised 6 known and 9 unknown mutations. Among them are three new small deletions (CACNA1A p.Thr310fs [4bp]; Val558Serfs*13 [5bp]; and p.Leu600fs [2bp]) and five new missense mutations all predicted to cause EA2.

Clinical Context

The clinical features varied among the 31 tested cases (18 males and 13 females). Frequency of attacks ranged from weekly to yearly attacks, and duration of attacks ranged from 30 minutes to hours, 12/31 had family history of a similar phenotype to the index case. Some cases had more extensive clinical symptoms than others [see Error! Reference source not found.] with examples of some of these outlined below:

For case 6 (a 6 year old male); he presented with a clinical picture suggesting EA2. He had an episode of ‘encephalitis’ at 13 months of age associated with marked ataxia, which resolved over 3 months. Since then, he has had 4-5 episodes of ataxia associated with fever which resolved over several days. In the last described episode, he had severe ataxia with fever, marked truncal and gait ataxia and past pointing. He had a normal MRI result, but no response to Acetazolamide. A novel single base-pair exchange was identified in the CACNA1A gene (located in intron 44) in this patient, which is predicted to affect the CACNA1A protein through changes to mRNA splicing sites.
In case 8 (an 11 year old female), there were episodes of ataxia since the age of 10 months, dizziness; visual symptoms; unsteadiness (inability to walk); nausea; vomiting and occasional headaches reported during the attacks, with complete resolution of symptoms typically taking 1-2 hours. Her mother (44 years of age) had similar attacks starting at an age of 5 years, improving as she got older, though pregnancies exacerbated her symptoms. During recent episodes of ataxia, she was unable to walk and suffered severe dysarthria. Both daughter and mother’s symptoms were relieved by Acetazolamide. A small frameshift deletion \( p.\text{Leu600fs*41} \) [2bp] was identified using the NGS panel in the index case (daughter) and confirmed by Sanger sequencing in both the index case and her mother.

In case 10 (a 37 year old female), there was at least a 3 year history of episodic acute onset ataxia associated with nausea and vomiting. She also had a history of migraine and recent daily attacks were reported. A new single nucleotide variant (SNV) in the 3’UTR in \( \text{ATP1A2} \) gene was detected in this patient.

In case 13, familial periodic ataxia (paralysis) and migraine were described in both index case (a 28 year old male), his brother (35 years of age) as well as their mother and all were known to respond to Acetazolamide. The brother was also diagnosed as having Charcot-Marie-Tooth (CMT) neuropathy; pes cavus and with inability to walk on his heels but no muscle wasting. The amino acid changing \( p.\text{Asp302Asn} \) mutation and the new synonymous single nucleotide variant (Asn328Asn) in \( \text{CACNA1A} \) gene were both confirmed in both brothers.

In case 19 (a 38 year old female), typical vertigo and abnormal nerve excitability were reported suggesting a clinical diagnosis of episodic ataxia or migraine vertigo. Although no
mutation was identified in this case, a novel non amino acid changing variant was detected in the *NOTCH3* gene at the nucleotide position c.5640C>T (Val1880Val), which was computationally indicated to be potentially damaging.

**Discussion**

Autosomal dominant episodic ataxia type 2 (EA2) results from mutations of the *CACNA1A* gene, covering 300 kb with 47 exons. EA2 is caused by a wide range of mutations in *CACNA1A*, localized on chromosome 19p, which encodes the pore-forming $\alpha_{1A}$ subunit of the Cav2.1 $\text{Ca}^{2+}$ channel (Ophoff et al., 1996). This subunit comprises four repeated domains (I-IV), and each domain contains six transmembrane regions (S1-S6) comprising a pore loop between S5 and S6. In our tested patients, 5 of 15 mutations detected were involved in the pore loop regions of Domains I, III, and IV of the protein. Of these, three mutations were missense mutations in Cases 9, 13, and 29, one was a truncation mutation (Case 17) and one was a previously known nonsense mutation (p.Trp1448Ter) (Case 5) (Jen et al., 2004). It is worth noting that these mutations were spread throughout the gene. This reinforces the difficulty in screening a few exons which harbor “hot spots” for mutation in the *CACNA1A* gene, an element compounded by symptomatic overlap between EA2 and other diseases.

EA2 is mainly characterized by episodes of ataxia, vertigo and nausea lasting for minutes to hours, but a variety of overlapping clinical features with other dominant disorders like FHM1 and SCA6 have been previously described (Jodice et al., 1997, Mantuano et al., 2003, Romaniello et al., 2010), such as dysarthria, diplopia, hemiplegia and headache (Jen et
Al., 2004). Attacks are triggered by emotional stress, exertion, caffeine or alcohol in similar ways to these disorders and even common migraine. The issue with overlapping symptoms potentially confusing decisions relating to the clinical diagnosis of EA2 and treatment are exemplified in Case 18, in whom we identified a new missense mutation in the ATP1A2 gene (p.Thr570Met), instead of CACNA1A where one was expected to be found. This patient suffers from highly similar symptoms to EA2 (hence this being the requested test), and all episodes of their illness were resolved by using Acetazolamide treatment. This case’s result opens the door to potential clinical confusion in the precise differential diagnosis of EA2 from FHM type 2, with response to acetazolamide not being a reliable indicator of having EA2. This also leads to potential to sequence incorrect genes to identify mutations, and while this may not always lead to delays in treatment, it does have the potential to limit family planning options for patients and the ability to diagnose other family members.

Moreover, two of the known mutations presented here in two clinically diagnosed EA2 patients p.R583Q and p.Y1853* were previously described to be associated with both Familial Hemiplegic Migraine (FHM) and Episodic Ataxia2 (EA2). In 1999, Battistini et al (Battistini et al., 1999) reported two sisters with p.Arg583Gln mutation who have typical hemiplegic migraine attacks associated with confusion and fever, accompanied with progressive cerebellar ataxia. In 2010, the same mutation was reported by Cleves et al (Cleves et al., 2010) in two sisters experiencing episodic ataxia without hemiplegia and confusion. In 2002, Giffin et al described a 3 year old male with FHM and ataxia symptoms linked to a nonsense mutation (p.Tyr1853Ter) in exon 37 in CACNA1A gene, the attacks being unresponsive to treatment with acetazolamide (Giffin et al., 2002). In a similar overlapping case, the mutation p.Asp302Asn (reported here in Case 13) was reported in 2014 by Jaffer et al in a patient with episodic ataxia (www.scribd.com/doc/111991160/Untitled).
while the same mutation was described by Bürk et al (2014) in a German patient with dominant cerebellar ataxia and absence of recurrent ataxic episodes (Burk et al., 2014).

For the 16 patients for whom our panel failed to detect known or unknown mutation, we detected three previously unknown variants: in Case 6, an intronic single nucleotide variant (SNV) in CACNA1A gene; in Case 19, a synonymous variant in the NOTCH3 gene; and in Case 10, a single nucleotide variant found in the 3’untranslated region of ATP1A2. These variants have unknown pathogenicity, and all but Case 19’s variant were predicted to be tolerated by the \textit{in silico} tools.

In addition, rare SNPs (<1% minor allele frequency) were also detected in 10/16 cases, some of which might be involved with a patient’s individual phenotype. For instance, in Case 31, the unique amino acid changing p.Arg1917Cys at c.5749C>T (rs121917956) in the SCN1A gene is predicted to have a damaging effect when using PolyPhen-2 and Mutation Taster programs but is categorized by NCBI as a variant with an allele of uncertain significance. Therefore, further assessment for deleterious or disease causing effects using \textit{in silico} methods of all novel variants might help to confirm the phenotype-genotype correlation. In addition, family segregation analyses may be needed to confirm the contribution of these variants to the patient’s phenotype. This may be especially important as such rare polymorphisms may effectively represent low penetrance mutations whose presence in disease carriers has gone unnoticed due to variable phenotype expression.

Alternatively, it is possible that these rare polymorphisms do not contribute to disease phenotype and there are mutations in other genes responsible for their illnesses. Given our discovery of a gene causing a disease with EA2 symptoms in a patient bearing an ATP1A2
mutation, this seems likely, and perhaps the use of Whole Exome or Genome Sequencing approaches will enhance the probability of identifying new genes and/or mutations responsible for EA2 to allow future improvement of EA2 diagnostics.

In terms of a diagnostics approach, the imprecise definitions of symptoms for the clinical diagnosis of EA2 and the large number of exons in the CACNA1A gene, make obtaining a clear clinical diagnosis using direct genetic testing using conventional Sanger sequencing for molecular diagnosis extremely difficult. Indeed, our detection of mutations in ATP1A2 potentially causing EA2 like symptoms strongly indicates that multiple gene screening could be a clinically valuable approach. Indeed, one might reasonably screen the other familial hemiplegic migraine genes FHM2 (ATP1A2), FHM3 (SCN1A) along with FHM1 (CACNA1A) in case these are responsible for EA2-like symptoms in a patient.

With the number of the mutations identified in 15 of 31 (48.4%) of the EA2 patients tested our newly developed custom panel provides an improved diagnostics tool compared to traditional exon-by-exon sequencing widely used in laboratories to identify the genetic aberrations of patients sharing overlapping symptoms with EA2, such as FHM and SCA6. However, these results also indicated that there may be other EA2 genes yet to be identified and to be included on future diagnostic arrays.

In comparison to panels with a very large number of genes or to Whole Exome Sequencing, the small number of genes in a disease-specific gene panel (such as our NGS multi-gene panel) allows a significant increase of coverage on target sequences and high read depth of all bases. This is a great help to reliably detect disease-causing mutations, as well as to limiting the detection and validation of large numbers of unclassified variants in different
genes which might not relate to the patient’s phenotypes. Moreover, high read depth for all
the 15 mutations identified (>200x coverage depth) [Table3] and 100% concordance of the
mutations detected by NGS panel with Sanger sequencing provides high confidence in
mutation detection. This may enable elimination of Sanger sequencing to confirm the
presence of the mutations if the coverage read depth is ≥100x, with Sanger confirmation
perhaps reserved for less reliably detected mutations. Therefore, massively parallel
sequencing of small panel genes should be considered as a screening tool to detect clear
monogenic mutations of neurological disorders in which there is phenotypic and genotypic
heterogeneity. Indeed, in an environment where whole exome and potentially whole genome
sequencing is becoming more affordable, multi-gene panels may become the first stage in a
genes-to-exome-to-genome approach to replace the older exon-by-exon sequencing using
Sanger methods as the clinical standard method for genetic disease diagnosis.

Conclusions

We have developed a custom panel comprising 5 genes for daily routine genetic
testing of Episodic Ataxia type 2 (EA2). The mutational spectrum identified in this study
included fifteen different mutations (6 known and 9 unknown). Among them are three small
deletions (CACNA1A p.Thr310fs*5 [4bp]; Val558Serfs*13 [5bp]; and p.Leu600fs*41 [2bp]
which disrupt the reading frame and result in a premature stop of the CACNA1A protein at
amino acid positions 314, 570, and 640 respectively, these were identified in exons 6, 13,
and 14). The remaining CACNA1A mutations were simple amino-acid substitutions.
Additionally, a previously unknown mutation was identified in the ATP1A2 gene
p.Thr570Met in association with EA2 symptoms, confirming the difficulty to differentiate between the clinical features of EA2 and FHM for a precise clinical diagnosis.

Based on this report, we felt that transitioning to an NGS platform that performs parallel sequencing enables much more cost-effective diagnosis and a more comprehensive diagnostics test, involving an interrogation of all implicated and related genes simultaneously. This provides the opportunity to identify novel and unexpected mutations increasing diagnostic capability. This approach reduces the difficulty for clinicians in choosing the genes to investigate due to the symptom overlap in relation to severe migraine related disorders, with the added consequence of keeping patient costs down.

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**Figure Legends**

**Figure 1:** Sequences of the eight novel genetic variants in CACNA1A gene identified by next generation sequencing. Five heterozygous exonic missense point mutations (A) in exon 3; (B) in exon 6; (D) in exon 8; (E) in exon 9; and (H) in exon 34. Three small frameshift deletion mutations (C) in exon 6; (F) in exon 13; and (G) in exon 14.

**Figure 2:** Mutations in the α 1 A subunit of the voltage-gated Cav2.1 Ca 2+ channel encoded by EA2 CACNA1A gene. The protein is located in the plasma membrane and contains four repeated domains (I-IV), each encompassing six transmembrane segments (S1-6). ○ = Known mutations, ● = Novel mutations. Numbers in the symbol correspond to the mutation listed in order.