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### Frequency of *CNKSR2* mutation in the X-linked epilepsy-aphasia spectrum

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## SUMMARY

Synaptic proteins are critical to neuronal function in the brain and their deficiency can lead to seizures and cognitive impairments. *CNKSR2* is a synaptic protein involved in Ras signaling-mediated neuronal proliferation, migration and differentiation. Mutations in the X-linked gene *CNKSR2* have been described in patients with seizures and neurodevelopmental deficits especially affecting language. In this study, we sequenced 112 patients with phenotypes within the epilepsy-aphasia spectrum (EAS) to determine the frequency of *CNKSR2* mutation within this complex set of disorders. We detected a novel nonsense mutation (c.2314 C>T; p.Arg712\*) in one Ashkenazi Jewish family, the male proband of which had a severe epileptic encephalopathy with continuous spike-waves in sleep (ECSWS). His affected brother also had ECSWS with better outcome, while their sister had childhood epilepsy with centro-temporal spikes. This mutation segregated in the three affected siblings in an X-linked manner, inherited from their mother who had febrile seizures. Although the frequency of point mutation is low, *CNKSR2* sequencing should be considered in families with suspected X-linked EAS because of the specific genetic counseling implications.

**KEY WORDS:** *CNKSR2*; epilepsy-aphasia spectrum; developmental delay; speech delay; Sanger sequencing

## INTRODUCTION

The Epilepsy-Aphasia-Spectrum (EAS) is a spectrum of disorders from the severe epileptic encephalopathy with continuous spike-waves in sleep (ECSWS) and Landau Kleffner syndrome (LKS) to the mild condition of childhood epilepsy with centro-temporal spikes. Some of the disorders show a male bias and there is evidence to suggest a genetic cause<sup>1</sup>. The NMDA subunit encoded by *GRIN2A* was the first gene associated with EAS<sup>2-5</sup>. Recently, the Ras-signaling protein encoded by *CNKSR2*<sup>6-10</sup>, was shown to cause phenotypes within the EAS spectrum; 4/5 unrelated probands had *CNKSR2* deletions discovered using chromosomal microarrays, suggesting loss of the protein at brain synapses to be pathogenic<sup>6; 7; 10</sup>. However, no study has systematically determined the contribution of *CNKSR2* point mutations to the epilepsy-aphasia spectrum.

## MATERIALS AND METHODS

### Subjects

We searched for DNA variation in *CNKS2* among our cohort of 112 EAS probands (62 male, 50 female) where there was no evidence of male-to-male transmission. There were 50 familial cases and 62 sporadics. The breakdown of phenotypes of this cohort is shown in Table 1. Genomic DNA was extracted from venous blood by standard methods<sup>11</sup>. A subset of the cohort has received testing for other genes associated with aphasia such as *GRIN2A*, for which they were negative. No individuals in the reported pedigree have been analysed on a gene panel or exome sequenced. The Human Research Ethics Committee (Project No. H2007/02961) of Austin Health, Melbourne, Australia, and Institutional Review Board of the Wolfson Medical Centre approved this study. Informed consent was obtained from all subjects or their parents or legal guardians.

### PCR and Sequencing

Coding exons and splice sites of the *CNKS2* gene were PCR amplified using specific primers designed to all isoforms using reference human gene transcripts (NCBI Gene; <http://www.ncbi.nlm.nih.gov/>). Primer sequences are available upon request. Amplification reactions were cycled using a standard protocol on a Veriti Thermal Cycler (Applied Biosystems, Carlsbad, CA). Bidirectional sequencing of all exons and flanking intronic regions including splice sites was completed with a BigDye<sup>TM</sup> v3.1 Terminator Cycle Sequencing Kit (Applied Biosystems), according to the manufacturer's instructions. Sequencing products were resolved using a 3730xl DNA Analyzer (Applied Biosystems). All sequencing chromatograms were compared to published cDNA sequence; nucleotide changes were detected using Codon Code Aligner (CodonCode Corporation, Dedham, MA).

## RESULTS

### Genetic Analysis

We detected a novel nonsense mutation (c.2314 C>T; p.Arg712\*) in the proband from one Ashkenazi Jewish family (Fig.1) that is not present in available public databases including the Exome Aggregation Consortium (ExAC), Exome Variant Server or 1000 Genomes. Although a variant

(p.Arg712Gln) is reported on ExAC to affect the same residue, this variant is not relevant because it is a missense change present in control individuals whereas we have found a novel nonsense mutation at the same residue. Segregation analysis revealed the mutation was present in all three affected siblings and was inherited in an X-linked manner from their mother who had febrile seizures. Samples from the mother's siblings, including the affected uncle, were unavailable for genetic analysis.

The p.Arg712\* mutation is located towards the C-terminal of the protein and is predicted to result in a truncated protein lacking the last 322 of 1034 residues. This portion of the protein includes a coiled-coil functional domain (residues 875 to 904; <http://www.uniprot.org/uniprot/Q8WXI2>). The p.Arg712 residue is also located only 6 residues away from a known short proline-rich motif (PPPP P2; residues 703-706) that has been shown to be essential for binding of *CNKS2* to WW domains of Vilse<sup>12</sup>, a protein previously reported to interact with the axon guidance receptor Robo1<sup>13</sup>. Disruption of this interaction may adversely affect synaptic activity. The other possibility is complete loss of the protein due to nonsense-mediated decay (NMD). We were unable to test for NMD because we could not obtain fresh blood samples from affected family members. All reported deletion or frameshift *CNKS2* mutations in EAS patients either include or are predicted to lead to loss of the C-terminal portion of the protein where our nonsense mutation is located<sup>6-10</sup>.

### Phenotypic Analysis

In the proband seizures began at 3.5 years with developmental and language delay occurring prior to seizure onset. Sleep-EEG demonstrated continuous bilateral centro-temporal or frontal spike and wave activity. Comorbidities included attention deficits and hyperactivity. At 4 years the proband's behaviour, cognition and language deteriorated and he became aphasic. At 12 years he was institutionalized with neuropsychiatric deterioration and remains institutionalized at age 18 years.

The proband's brother also had seizures onset at 3.5 years with developmental and language delay prior to seizure onset, continuous bilateral centro-temporal or frontal spike and wave activity on sleep-EEG (Fig.2, and attention deficits and hyperactivity. At age 12 years he was less severely affected than the proband, as indicated by useful language and attendance at regular school. However, he requires ongoing anti-epileptic medication.

Their sister had mild motor and language delay and seizure onset at 6 years; left centro-temporal spikes were recorded on EEG, consistent with a diagnosis of childhood epilepsy with centro-temporal spikes. At age 16 years, she was seizure free without medication and had mild learning difficulties.

The sibling's mother had febrile seizures without language or intellectual impairment. Their maternal uncle (DNA unavailable) had refractory seizures from 3.5 years of age, he had CSWS recorded on sleep-EEG and had a language and motor delay. He responded to the ketogenic diet and by five years old his EEG had normalised and the diet was discontinued. He had rare seizures over a few years, by 10 years he had ceased all medication. He has autism and mild-moderate intellectual disability.

## DISCUSSION

We confirm recent studies<sup>6-10</sup> reporting *CNKSR2* mutations in patients within the EAS and show the frequency is low (1/112). Although large deletions have been reported<sup>6; 7;11</sup>, they are unlikely to be present in our screening cohort since we amplified *CNKSR2* exonic DNA from all probands by PCR.

In our family, all affected males had onset of seizures at 3.5 years comparable to 2 or 2.5 years for four reported male cases but older than 8 day or neonatal onset in a further two male cases<sup>6; 10</sup>. Frequent or continuous spike and wave pattern on EEG has been documented in six reported cases, consistent with the three affected males from our family<sup>6; 9; 10</sup>. The vast majority of reported patients, including our family, have severe speech delay, often commencing prior to the time of seizure onset, which may continue and result in lifelong language loss<sup>10</sup>. Developmental and psychomotor delay are common features (again prior to seizures onset) as is intellectual disability<sup>6; 8</sup>. Finally, behavioral disturbances including severe attention deficit and hyperactivity have been frequently observed here and elsewhere<sup>10</sup>.

The previously reported cases of *CNKSR2* deficiency causing seizures have all been male with their genotype being inherited from unaffected carrier mothers. Here we report the first female with EAS and a *CNKSR2* mutation. The sister, in our family, has a less severe phenotype to her two brothers; this could be due to the fact the females are heterozygous and likely subject to X-inactivation, although the latter was not measured. Interestingly the mother, who also carries the point mutation, only had febrile seizures. Both FS and BECTS are common seizure disorders with the former affecting ~ 3% of children in the Western European population, while the latter accounts for ~ 10% of

focal epilepsies in children. CSWS is much rare although it has now been associated with *CNKSR2* mutation in several unrelated cases<sup>6-10</sup>. This is the first report of BECTS and FS in female patients carrying a *CNKSR2* mutation.

Our data suggest in cases with suspected X-linked EAS, specifically in males with CSWS, developmental delay, affected speech and early onset seizures *CNKSR2* gene sequencing should be performed in addition to standard clinical microarrays for detection of large deletions at the *CNKSR2* locus. Examinations of regulatory regions outside the coding and splice site regions interrogated here are an area for future research. Finding a pathogenic mutation in *CNKSR2* will help clinicians with diagnosis and genetic counselling, with implications for expressivity of both severe (e.g. CSWS), and milder (e.g. BECTS and FS) phenotypes, depending on whether the mutation is inherited by a male or female offspring.

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#### **DISCLOSURE OF CONFLICTS OF INTEREST**

Authors report grant funds that contributed to this project as outlined in the Acknowledgements section. S.F.B. discloses payments from UCB Pharma, Novartis Pharmaceuticals, Sanofi-Aventis, and Jansen Cilag for lectures and educational presentations, and a patent for *SCN1A* testing held by Bionomics Inc and licensed to various diagnostic companies. I.E.S. discloses payments from UCB Pharma, GlaxoSmithKline, Eisai, Athena Diagnostics and Transgenomics for lectures and educational presentations, and a patent for *SCN1A* testing held by Bionomics Inc and licensed to various diagnostic companies.

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## FIGURE LEGEND

**Figure 1: Ashkenazi Jewish family segregating the X-linked *CNKSR2* mutation.** A Two of the three affected siblings are males hemizygous for the mutation; the female sibling is heterozygous. The mutation was maternally transmitted; DNA from the affected uncle was not available. C, Wild-

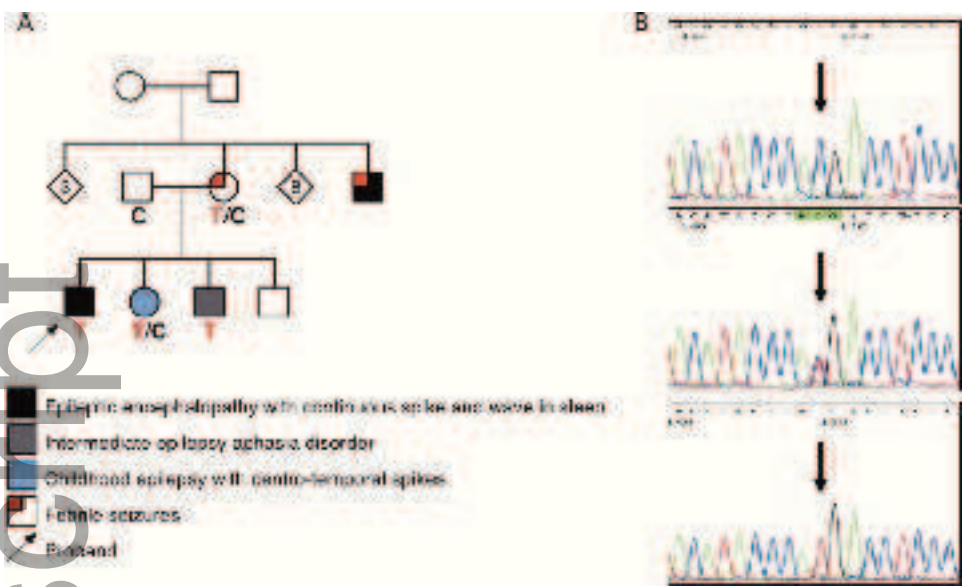
type allele; T, c.2314 C>T (p.Arg712\*) mutation. **B** Wild-type sequence in unaffected father (top). Heterozygous mutation in mother with febrile seizures (middle). Hemizygous mutation in male sibling with EAS (bottom).

**Figure 2: Centro-temporal or frontal spike and wave activity on sleep-EEG.** EEG from the proband's brother at 10.5 years showing continuous right sided focal epileptiform activity maximum in the temporal region with some involvement on the left.

#### TABLE

**Table 1 : Phenotypic breakdown of probands**

<b>Epilepsy-Aphasia-Spectrum diagnosis</b>	<b>Probands</b>
Childhood epilepsy with centro-temporal spikes	82
Intermediate Epilepsy Aphasia Disorder (IEAD)	12
Landau Kleffner Syndrome (LKS)	5
epileptic encephalopathy with continuous spike-waves in sleep (ECSWS)	13
<b>TOTAL</b>	<b>112</b>



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