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BRIEF COMMUNICATION

A novel homozygous truncating variant of *NECAP1* in early infantile epileptic encephalopathy: the second case report of EIEE21

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Abstract

We report the second case of early infantile epileptic encephalopathy (EIEE) arising from a homozygous truncating variant of *NECAP1*. The boy developed infantile-onset tonic-clonic and tonic seizures, then spasms in clusters. His electroencephalogram (EEG) showed a burst suppression pattern, leading to the diagnosis of Ohtahara syndrome. Whole-exome sequencing revealed the canonical splice-site variant (c.301 + 1 G > A) in *NECAP1*. In rodents, Necap1 protein is enriched in neuronal clathrin-coated vesicles and modulates synaptic vesicle recycling. cDNA analysis confirmed abnormal splicing that produced early truncating mRNA. There has been only one previous report of a mutation in *NECAP1* in a family with EIEE; this was a nonsense mutation (p.R48*) that was cited as EIEE21. Decreased mRNA levels and the loss of the WXXF motif in both the families suggests that loss of NECAP1 function is a common pathomechanism for EIEE21. This study provided additional support that synaptic vesicle recycling plays a key role in epileptogenesis.

II-3, a 16-month-old boy, is the third child of a healthy first-cousin Eurasian parents in Malaysia (Fig. 1a). He was delivered by cesarean section following premature labor at 32-weeks-gestational age. Development was profoundly delayed with no progress in milestones. At the corrected gestational age of 3 months, he developed generalized tonic-clonic and clonic seizures, a blank stare, and recurrent episodes of apnea. His EEG showed a burst suppression pattern both in awake and sleep states (Supplemental figure S1). Furthermore, the spasms occurred in clusters 7–8 times per day. The seizures were difficult to control and resistant to phenytoin, phenobarbitone, pyridoxine, and valproaic acid, but he partially responded to anti-epileptic drugs, such as topiramate and vigabatrin, which decreased the seizure frequency to 2–3 times per day. He mainly had focal to bilateral tonic-clonic seizures at the last follow-up review. His clinical manifestations are summarized in Table 1.

The genetic causes of EIEE are highly heterogeneous, with pathogenic variants in 69 genes currently documented in OMIM as EIEE entries (www.omim.org). The proportion of EIEE attributed to each gene also varies. Several genes, such as *STXBP1*, *SCN1A*, *CDKL5*, and *ARX* are prevalent in EIEE reports, whereas other genes are rarely associated with EIEE.

To date, only one consanguineous Saudi Arabian family has been reported with a *NECAP1* pathogenic variant. This family presented with a homozygous *NECAP1* (adaptiN Ear-binding Coat-Associated Protein 1) nonsense variant (p.R48*) linked to an EIEE phenotype [1]. Since then, no confirmatory studies have been reported.

In this study, we found a novel *NECAP1* variant at the canonical + 1 splice site. Whole-exome sequencing was initially performed for the affected boy (II-3) and variants were screened based on an autosomal recessive model [2],

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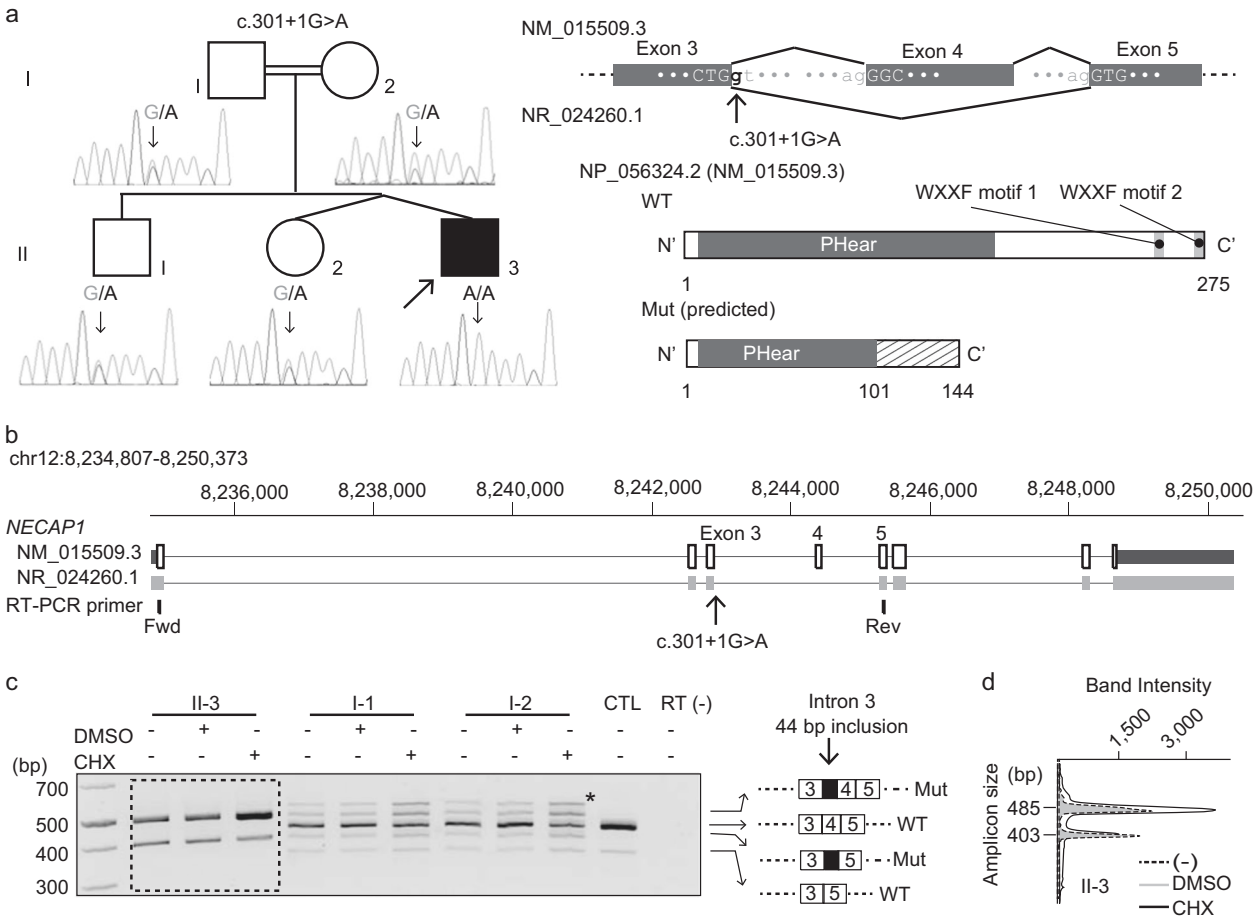

Fig. 1 Canonical splice site variant of *NECAP1* in a patient with Ohtahara syndrome. **a** Familial pedigree of the *NECAP1* variant (left). The c.301 + 1 G > A substitution was confirmed by Sanger sequencing. Schematic of the alternative use of exon 4 in two different *NECAP1* transcripts (right), indicating the protein-coding (NM_015509.3) and the non-coding (NR_024260.1) transcripts. The domain structure of the NECAP1 protein is depicted as documented in UniProtKB (https://www.uniprot.org/uniprot/). WXXF motif 1 (252–255) and WXXF motif 2 (272–275) are shown in gray. c.301 + 1 G > A causes abnormal splicing, which in turn leads to the frameshift of NM_015509.3. This alters glycine 101 to aspartic acid, which creates a new reading frame with a premature termination codon (p.Gly101Aspfs*45). **b** The gene structure of *NECAP1* as documented in the NCBI RefSeq genes track (GRCh37/hg19). Arrow indicates the splicing variant, c.301 + 1 G > A. The genomic positions of the forward (Fwd) and reverse (Rev) primers are shown as black boxes. **c** *NECAP1* transcript analysis. RT-PCR was performed using the total RNA of II-3, I-1, I-2, and an unrelated control individual (CTL). RNA was extracted from the patient-derived lymphoblast cells treated with dimethyl sulfoxide or cycloheximide (DMSO and CHX, respectively). RT (−), no reverse transcription. *, wild-type/mutant heteroduplex molecules. The c.301 + 1 G > A substitution resulted in a 44-bp intron 3 inclusion in both NM_015509.3 and NR_024260.1. **d** Intensities of the 485-bp and 403-bp bands from the RT-PCR of NM_015509.3 and NR_024260.1 from II-3, respectively, as quantified by Image Lab (Bio-Rad, Hercules, CA, USA). Three lanes in **c** (dotted box) were used for this quantification. The level of the abnormal transcript variant of NM_015509.3 (485 bp) increased two-fold when treated with CHX, indicating that nonsense-mediated mRNA decay was involved. No difference was observed for the CHX-treated noncoding NR_024260.1 transcript (403 bp)

as described in Supplemental methods. *NECAP1* variant (c.301 + 1 G > A) was the top candidate. This variant was not present in the Genome Aggregation Database (gnomAD, http://gnomad.broadinstitute.org/.) or in our in-house Japanese exome controls ($n = 575$). We confirmed a homozygous *NECAP1* variant in the affected boy (II-3) by Sanger sequencing. His parents, unaffected brother, and sister (I-1, I-2, II-1, and II-2) were all heterozygous for c.301 + 1 G > A (Fig. 1a). This variant resides on the runs of homozygosity region of chromosome 12 (Supplemental figure S2). II-3 had similar clinical features to that of the EIEE21 cases reported by Alazami, et al. [1]. Patients in both the families are characterized by EIEE, profound global developmental delay, and muscular hypotonia, whereas dysmorphic features, visual impairment, hearing impairment, and abnormal metabolite profiles were absent (Table 1). However, the two families did show some differences in their epileptic phenotype (in terms of seizure types and EEG patterns) and brain MRI results. II-3 developed generalized tonic-clonic seizures as well as tonic

Table 1 Clinical features of individuals with *NECAP1* variants

	II-3	Alazami et al.
Variant (NM_015509.3)	c.301 + 1G > A	c.142C > T
Sex	Male	Two males and two females
Consanguinity	+ (First cousins)	+ (First cousins)
Clinical diagnosis	Ohtahara syndrome	Infantile epileptic encephalopathy
Seizure		
Age of onset	3 m (corrected)	Early infancy
Seizure types at onset	Recurrent apnea episodes, tonic-clonic seizures, tonic seizures	Fragmented multifocal clonic and tonic seizures
EEG findings	Burst suppression pattern at 5 m	Generalized epileptiform discharges
Course of seizure	Asymmetric tonic posturing, spasms in clusters	Generalized tonic seizure
Response to treatment	Intractable	Intractable
Development	Profound global delay	Profound global delay
Neurological signs		
Axial hypotonia	+	+
Appendicular hypertonia	−	+
Brain MRI	Normal at 5 m	Non-specific generalized brain atrophy ($n = 2$)
Dysmorphic features	−	−
Metabolic workup	Normal	Normal
Visual impairment	−	−
Hearing impairment	−	−

cDNA and protein changes are based on the reference cDNA sequence, NM_015509.3

+ present, − absent, *m* months, *N.D.* not described

seizures with a burst suppression pattern on his EEG, whereas the Saudi Arabian family showed multifocal clonic and tonic seizures with generalized epileptiform discharges on their EEG. Brain MRI was unremarkable in II-3 at the time of initial onset of the seizures but non-specific generalized brain atrophy was reported in at least two of the four affected children of the Saudi Arabian family [1]. Information regarding age at the time of analysis was not fully provided for the Saudi Arabian cases, while MRI was performed at the corrected age of 3 months in our case. These phenotypic differences may reflect transitioning of the epileptic phenotype during similar courses of the same pathological conditions in the two families. II-3 may yet develop brain atrophy at a later stage.

The c.301 + 1 G > A variant likely affects splicing in both the *NECAP1* isoforms, the NM_015509.3 and the NR_024260.1 (Fig. 1a, b). This prediction was tested by reverse transcription PCR (RT-PCR) using total RNA extracted from the patient-derived lymphoblastoid cells. In an unrelated control individual, two amplicons attributable to NM_015509.3 and NR_024260.1 were observed (Fig. 1c). In contrast, abnormal transcripts were generated in II-3 as indicated by two distinct bands that were 44 bp larger than normal (Fig. 1c). Paternal and maternal samples both showed a heterozygous pattern (two normal transcripts, two aberrant transcripts, and one heteroduplex molecule). The formation of artificial heteroduplex DNA molecules, consisting of the wild-type and mutant DNA, was confirmed using the T7 endonuclease I cleavage reaction (Supplemental figure S3). Aberrant transcripts were further characterized by TA cloning followed by Sanger sequencing. This revealed that c.301 + 1 G > A resulted in a 44-bp intron 3 inclusion and activation of a cryptic 5′ splice site, which cause premature termination of NM_015509.3 (Supplemental figure S4). In general, such transcripts are preferentially degraded by nonsense-mediated mRNA decay. In fact, the mutated transcripts are significantly elevated after cycloheximide (CHX) treatment, suggesting that such decay is indeed involved (Fig. 1c, d). Residual transcripts may be translated but are functionally incompetent because the WXXF motif is deleted, and this motif is critical for binding the core clathrin-coated vesicle (CCV)-organizing protein, AP-2 (Fig. 1a) [3, 4]. Assembly of NECAP1/AP-2 complexes facilitate recruitment of other CCV accessory proteins for synaptic vesicle recycling [4]. After nascent CCV formation, the vesicle is pinched off from the presynaptic membrane by dynamin 1 (encoded by *DNM1*) [5]. Hence, NECAP1 and DNM1 work along the same molecular pathway at different stages of vesicle recycling, possibly exerting similar defects on neuronal synapses by their mutations. In fact, *DNM1* is mutated in EIEE31 [6–8]. More recently, heterozygous

variants in *CLTC*, which encodes the clathrin heavy chain 1 protein, were found to cause global developmental delay, intellectual disability (ID), and hypotonia with or without epilepsy [9]. The phenotype is likely less severe and heterogeneous in cases of *CLTC* abnormalities. Synaptic vesicle recycling may be more uniformly and severely impaired by homozygous truncating variants in *NECAP1* or dominant-negative *DNM1* variants in patients with severe epilepsy [10].

In conclusion, we confirmed a rare genetic cause of the autosomal recessive form of Ohtahara syndrome. This evidence underscores the importance of synaptic vesicle recycling in addition to vesicle fusion in neurodevelopment.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This study was approved by the Institutional Review Boards of Yokohama City University School of Medicine.

Informed consent Written informed consent was obtained from all participants.

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