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Using experimental models to provide insights into mechanism of genetic generalised epilepsy

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Abstract

EFHC1 gene mutations have been described in patients with juvenile myoclonic epilepsy (JME) and other types of idiopathic generalized epilepsy. We generated *Efhc1*-deficient mouse and found that the mouse showed spontaneous myoclonus and increased susceptibility to a convulsant, pentylenetetrazol, further supported and confirmed that *EFHC1* is the gene for JME. Myoclonin1 protein encoded by EFHC1 is well expressed in prenatal choroid plexus and postnatal ependymal cell cilia. In consistent with this, the *Efhc1*-deficient mouse showed slowed beating frequency of ependymal cilia and enlarged ventricles. Recent report also described that myoclonin1 was expressed in neurons and their mitotic spindles and midbody, but our re-investigation suggested that those signals in neurons were non-specific.

IDENTIFICATION OF EFHC1 GENE MUTATIONS IN PATIENTS WITH JUVENILE MYOCLONIC EPILEPSY

Juvenile myoclonic epilepsy (JME) is characterized by adolescent onset myoclonic jerks on awakening, grand mal clonic tonic-clonic and tonic clonic seizures, and less frequent absence seizures. JME is one of the most common epilepsies that is responsible for 3% to 12% of all known epilepsies.[1] Electroencephalography reveals 15-30Hz multispikes during myoclonic and tonic-clonic convulsions. By using genetic linkage analyses on Mexican JME families, we previously mapped and narrowed one of the chromosomal loci harboring genes responsible for JME down to chromosome 6p12.[2] After extensive gene searches[3-5], we finally identified the gene for JME on 6p12, named *EFHC1* (EF-hand domain containing 1).[6] The human *EFHC1* encodes a 640 amino acid non-ion channel protein "myoclonin1" that harbors three tandemly repeated DM10 domains, a motif of unknown function, and one EF-hand calcium-binding motif at the carboxyl terminus. *EFHC1* mRNA was observed in multiple tissues including the brain in Northern blot analyses.[6] Ikeda *et al*. reported that mouse myoclonin1 is expressed at tracheal cilia, and sperm flagella.[11] We also reported that mouse myoclonin1 protein was dominantly expressed in prenatal choroid plexus, and in the cilia of ependymal cells lining the wall of ventricles at postnatal stages.[12]

Successive mutation studies by other groups reported *EFHC1* heterozygous missense mutations in a JME Caucasian family[7] and Italian JME families.[8] In addition to the mutations in JME, Stogmann *et al*. described *EFHC1* mutations in other types of idiopathic epilepsies; juvenile absence epilepsy, cryptogenic temporal lobe epilepsy, and an unclassified idiopathic epilepsy.[9] Furthermore, we recently reported additional *EFHC1* missense mutations in the full-length as well as truncation mutations in a short isoform of *EFHC1* in Mexican and Japanese patients.[10] In addition to the original full-length myoclonin 1, the *EFHC1* gene also encodes a short isoform of myoclonin 1 (278 amino acids) that harbors only one DM10 domain without an EF-hand motif, and a unique carboxyl-terminal end.[4] We identified heterozygous frameshift and nonsense mutations in the part of EFHC1 transcript encoding the unique carboxyl-terminal end of the myoclonin1 short isoform in 3 JME families (2 families from Honduras and one from Mexico).[10]

SPONTANEOUS MYOCLONIC EPILEPSY AND INCREASED SEIZURE SUSCEPTIBILITIES IN MOUSE WITH EFHC1-DEFICIENCY

To further address the putative relevance of *EFHC1* in epilepsies, we generated and characterized *Efhc1*-deficient mice.[13] Most of the mice were normal in outward appearance and both sexes were found to be fertile. However, the ventricles

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of the brains were significantly enlarged in the null mutants but not in the heterozygotes. Although the ciliary structure was normal, the ciliary beating frequency was significantly reduced in null mutants. In adult stages, both the heterozygous and null mutants developed frequent spontaneous myoclonus. Furthermore, the threshold of seizures induced by pentylenetetrazol was significantly reduced in both heterozygous and null mutants.[13] All the above mentioned results support our contention that *EFHC1* is a gene responsible for epilepsies.

EFHC1/MYOCLONIN1 SIGNALS AT MITOTIC SPINDEL AND MID BODY IN NEURONS ARE NON-SPECIFIC?

Recently, de Nijs *et al*. reported that myoclonin1 interacts with microtubules, and regulates cell division and cortical development.[14] In their study, the suppression of *EFHC1* via *ex vivo* electroporation of shRNA in rat brain induced abnormal (suppressed) radial migration of neurons, cell division, and cell cycle exit. However, these features are too drastic when compared to that of our *Efhc1*-deficient mouse[13], and their results may have to be confirmed by additional experiments. We carefully re-investigated their results by using the same polyclonal antibody mRib72-pAb they used[11,14] together with the *EFHC1* homozygous null mutant mouse, *Efhc1* (-/-), that we generated[13] and the anti-myoclonin1 monoclonal antibody (6A3-mAb).[12] In western blot analyses of mouse brain and lung tissue lysates, the 6A3-mAb successfully detected a 75 kDa band of myoclonin1 in wild-type mouse (WT) and this band well disappeared in *Efhc1* (-/-). The mRib72-pAb also detected the 75 kDa band with faint intensity in lung of WT (hardly detected in brain), and the band disappeared in *Efhc1* (-/-). However, mRib72-pAb detected additional bands with much higher densities, and these bands remained in *Efhc1* (-/-). In western blots of cultured neurosphere cell (neural stem cells) lysates from WT and *Efhc1* (-/-), 6A3-mAb again successfully detected the 75 kDa band of myoclonin1 in WT and the band well disappeared in *Efhc1* (-/-). The mRib72-pAb detected multiple sized bands in WT those remained in *Efhc1* (-/-), and hardly detected the 75 kDa myoclonin1 band in any of the lysates. These results suggest that mRib72-pAb detects not only 75 kDa myoclonin1 but also other proteins with high affinities.

Immunocytochemistry on cultured neurosphere cells from WT mouse showed that the mRib72-pAb surely developed signals at cytoplasm and at mitotic spindles during cellular mitosis as reported in their study. However, these mRib72-pAb signals also remained in *Efhc1* (-/-). In dividing HEK cells, mRib72-pAb signals were observed at the intercellular bridge, midbody, but the signals remained in RNAi treated HEK cells. 6A3-mAb did not show any signals at the intercellular bridge, mitotic spindles and centriole in HEK cells. Immunohistochemistry on mouse brain sections revealed that mRib72-pAb showed signals in cerebral cortical cells from WT as described in de Nijs's study, but those signals again remained in *Efhc1* (-/-). 6A3-mAb did not show any signals in cerebral cortex. Meanwhile, both mRib72-pAb and 6A3-mAb showed intense signals at the cilia of ependymal cells lining the ventricles in WT and these signals well disappeared in *Efhc1* (-/-). These results indicate that mRib72-pAb well detects myoclonin1 at ependymal cell cilia, but also suggest that mRib72-pAb signals at mitotic spindle, midbody, and cells at cerebral cortex reported previously were nonspecific and not 75 kDa EFHC1/myoclonin1.

We also investigated whether *Efhc1* (-/-) mouse has any abnormalities in cerebro-cortical progenitors, locomotion of postmitotic neurons, or radial migration by using antibodies for SOX2 (marker for progenitor cells), phospho-Histone H3 (PH3; marker for mitotic cells), and brain lipid-binding protein (BLBP; marker for radial glia) those were used in their study. We did not observe any marked differences in the number of SOX2, PH3, and BLBP-positive cells between WT and Efhc1 (-/-). We also performed TUNEL assay on brain sections of *Efhc1* (-/-), however it revealed no differences between WT and *Efhc1* (-/-). These results suggest that the elimination of myoclonin1 may not affect mitotic spindle structure, M-phase progression and cell cycle exit of cerebral cortical progenitors, radial glia scafold organization and radial migration of postmitotic neurons, and may not increase apoptosis.

These our results suggest that previously-reported mRib72-pAb signals at mitotic spindles and midbody were nonspecific and the elimination of myoclonin1, at least the 75 kDa full-length, may not critically affect cell division and neuronal migration during cortical development in mouse. Further investigations are required to clarify the pathological cascade between deficiency of myoclonin1 and the seizure phenotypes.

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