# Revisiting the clinical impact of variants in EFHC1 in patients with different phenotypes of genetic generalized epilepsy

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**Abstract**

The most common form of genetic generalized epilepsy (GGE) is juvenile myoclonic epilepsy (JME), which accounts for 5 to 10% of all epilepsy cases. The gene EFHC1 is associated with JME. However, it remains debatable whether testing for EFHC1 mutations should be included in the diagnostic epilepsy gene panels. To investigate the clinical utility of EFHC1 testing, we studied 125 individuals: 100 with JME and 25 with other GGEs. We amplified and sequenced all EFHC1 coding exons. Then, we applied a revised version of the American College of Medical Genetics and Genomics (ACMG)/Association for Molecular Pathology(AMP) guidelines to predict the pathogenicity and benign impact of the variants. Mutation screening revealed 11 missense variants in 44 probands with JME (44%) and in 1 of the 7 individuals with generalized tonic clinic seizures on awakening (14%). Overall, only the variant c.685T>C was strictly classified as 'pathogenic' (1/11, 9%), five variants were classified as 'benign' (45%), and the remaining five (45%) were considered variants of uncertain significance (VUS). There is currently a limitation to test for genes that predispose an individual to complex, non-monogenic phenotypes. Thus, we consider EFHC1 to be a risk factor for JME but not currently useful for clinical purposes.

## INTRODUCTION

Identifying the genes that influence the risk for epilepsies is crucial to elucidate the mechanisms that underlie seizure susceptibility (Ottman et al., 2010). However, the complex relationship between genotype and phenotype poses considerable difficulties when evaluating the clinical utility of genetic testing (Ottman et al., 2010). Thus, classifying the pathogenicity of identified variants in complex disorders with any degree of certainty is often challenging (Cooper, Krawczak, Polychronakos, Tyler-Smith, & Kehrer-Sawatzki, 2013). New genetic technologies that involve massive parallel sequencing have influenced the diagnostic practices in patients with intractable epilepsy; there are several epilepsy gene panels that are currently commercially available. Still, choosing a specific panel can be problematic for clinicians (Chambers, Jansen, & Dhamija, 2016). Therefore, even using large-scale genomic tests, such as whole-exome sequencing and whole-genome sequencing, genetic diagnosis is restricted mainly to single-gene disorders (Boycott, Vastone, Bulma, & MacKenzie, 2013). Also, there has been limited success in identifying genes for complex epilepsies, such as the genetic generalized epilepsies (GGE; Greenberg & Stewart, 2014; Ottman et al., 2010).

The most common form of GGE is juvenile myoclonic epilepsy (JME), which accounts for 5 to 10% of all epilepsies (Camfield, Striano, & Camfield, 2013). The clinical presentation begins between the ages of 9 and 27 years; it is characterized by myoclonic seizures (Fisher et al., 2017), which may be followed by generalized tonic-clonic seizures (GTCS) and absence seizures (Leppik, 2003). One of the genes associated with JME is *EFHC1* , which encodes the EFHC1 protein, also known as myoclonin 1 (Medina et al., 2008). Although the function of the EFHC1 protein is still poorly understood, it is known to be associated with microtubules

and, consequently, involved in the regulation of cell division, as well as associated with the process of radial migration during the development of the central nervous system (CNS; Conte et al., 2009; de Nijs et al., 2009). It is believed that mutations in*EFHC1* significantly impair apoptotic activity, which could prevent the elimination of neurons with altered calcium homeostasis during the development of the CNS, leading to JME (de Nijs et al., 2009).

*EFHC1* is currently included in 53 tests for diagnostic purposes available in the Genetic Testing Registry (https://www.ncbi.nlm.nih.gov/gtr; accessed in January 2020). Recently, researchers have raised the possibility that some *EFHC1* variants might be pathogenic depending on specific genetic backgrounds in which they are introduced (Subaran, Conte, Stewart, & Greenberg, 2015). However, there is a lack of studies in patients with a more diverse ethnic background; most *EFHC1* variants have been found either in Hispanics, in patients from Central America, or in Japanese individuals.

Most importantly, there have been controversies as to whether genetic testing for *EFHC1* directly impacts therapeutic decision-making, treatment, outcome, or other aspects in the context of medical care for patients with GGEs. On the one hand, *EFHC1* has been implicated in JME (Bailey et al., 2017). On the other hand, *EFHC1* is not listed as an epilepsy-related genetic variant with implications for clinical management (Poduri, Sheidley, Shostak, & Ottman, 2014), and it has been advised that prediction of epilepsy susceptibility in individuals who harbor *EFHC1* variants must be handled with caution (Subaran et al., 2015). Therefore, it remains debatable whether *EFHC1* is clinically useful and should be included in the diagnostic gene panels for GGEs. Thus, our study aimed to contribute to this ongoing debate.

## METHODS

### Editorial policies and ethical considerations

The patients or their parents signed an informed consent form, which was approved by the research ethics committee on the clinical centers of the University of Campinas and the Federal University of Alagoas, Brazil.

### Patients

Our cohort comprised 125 individuals: 100 with JME, 10 with juvenile absence epilepsy (JAE), 7 with GTCS on awakening, 3 with childhood absence epilepsy (CAE), and 5 with unclassified GGE. These patients were regularly followed in the outpatient epilepsy clinic of the university hospitals at the University of Campinas and the Federal University of Alagoas, Brazil. They fulfilled the clinical criteria for GGEs, according to the International League Against Epilepsy (ILAE) guidelines (Fisher et al., 2017; Scheffer et al., 2017; Wilmshurst et al., 2015). Clinical and demographic features of these patients have been previously published (Betting et al., 2006; Gitai et al., 2012).

### Mutation screening

We obtained DNA samples for each patient from peripheral blood lymphocytes by standard procedures (Sambrook, Russell, & Fritsch, 2001). All 11 coding exons and intron-exon boundaries of *EFHC1*were amplified by polymerase chain reaction (PCR; primer sequences available upon request). Sanger sequencing was performed by capillary electrophoresis in an ABI 3500xL Genetic Analyzer using the BigDye® Terminator Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA, USA). Sequence variants were described according to the conventional nomenclature (den Dunnen & Antonarakis, 2001; den Dunnen, 2019) based on the full-length *EFHC1* isoform (NM_018100) and deposited in a public genomic database of GGEs (http://bipmed.iqm.unicamp.br/GGE).

### Pathogenic classification

We applied the American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP) guidelines (Richards et al., 2015) in our study. We assessed the predicted pathogenicity and benign impact of the variants found in our cohort. To do so, we revised the ACMG/AMP rules to determine which criteria apply to our framework for GGE-related *EFHC1* variants.

We performed a series of analyses for each identified *EFHC1* variant. To address whether the identified amino acid changes were already established as pathogenic or benign, we performed a literature search using the online search engine PubMed (https://www.ncbi.nlm.nih.gov/pubmed/) for the terms ´EFHC1 AND (mutation OR variants) AND epilepsy´ up to April 2019. We further assessed the frequency of the variants found in *EFHC1* in databases of individuals from different populations: BIPMed (http://bipmed.org/; Secolin et al., 2019), ABraOM (http://abraom.ib.usp.br/; Naslavsky et al., 2017), NHLBI Exome Sequencing Project (ESP) (http://evs.gs.washington.edu/EVS/), gnomAD (http://gnomad.broadinstitute.org/), and 1000 Genomes (http://www.internationalgenome.org/). We also investigated the*EFHC1* variants found in our cohort in 100 unrelated Brazilian individuals without a family history of epilepsy. To verify whether the prevalence of these variants in affected individuals was statistically increased over controls, we performed the unconditional exact homogeneity/independence test (Z-pooled method, one-tailed). If the odds ratio (OR) calculated is 1.00, then there is no association between the variant and the risk for the disease. On the other hand, values greater than 1.00 indicate that the variant increased the odds of having the disease (Bailey et al., 2017). To avoid the possibility of population stratification, we performed separate tests for each of the control groups that contained admixed Brazilian individuals (our 100 individuals control group, as well as the BIPMed and AbraOM databases).

In order to predict the deleterious effect of *EFHC1* missense variants in protein function, we used 13 of the 16 computer algorithms recommended by the ACMG/AMP guidelines: FATHMM (http://fathmm.biocompute.org.uk; Shihab et al., 2015), Condel (http://bg.upf.edu/condel; González-Pérez & Lopez-Bigas, 2011), MutationTaster (http://www.mutationtaster.org; Schwarz, Rodelsperger, Schuelke, & Seelow, 2010), PANTHER (http://www.pantherdb.org/tools; Mi, Muruganuian, & Thomas, 2013), SNPs&GO (http://snps.biofold.org/snps-and-go/snps-and-go.html; Calabrese Capriotti, Fariselli, Martelli, & Casadio, 2009), MutPred2 (http://mutpred.mutdb.org; Pejaver et al., 2017), PROVEAN (http://provean.jcvi.org; Choi, Sims, Murphy, Miller, & Chan, 2012), CADD (http://cadd.gs.washington.edu; Rentzsch, Witten, Cooper, Shendure, & Kircher, 2019), PolyPhen2 (http://genetics.bwh.harvard.edu/pph2; Adzhubei, Jordan, & Suyaev, 2013), MutationAssessor (http://mutationassessor.org/r3; Reva, Antipin, & Sander, 2011), SIFT (http://siftdna.org; Sim et al., 2012), Align GVGD (http://agvgd.hci.utah.edu; Tavtigian et al., 2006), and PhD-SNP (http://snps.biofold.org/phd-snp/phd-snp.html; Capriotti, Calabrese, & Cadadio, 2006).

## RESULTS

Mutation screening in *EFHC1* in our cohort of GGE patients revealed 11 heterozygous missense variants. They were found in 44 of the 100 probands with JME (44%), and one of the variants was also identified in 1 of the 7 individuals with GTCS on awakening (14%). Table 1 shows all 11 variants, along with their predicted protein repercussions and the clinical features of the carriers. All these variants have been deposited and are publicly available at http://bipmed.iqm.unicamp.br/GGE.

We revised and adapted the ACMG/AMP guidelines to our framework. For the pathogenic classification, we eliminated the following four criteria:**PM1** (variant in mutational hot spot or well-studied functional domain without benign variation) because no mutational hot spots have been reported for *EFCH1* , and the variants found in patients with GGEs are scattered throughout the gene; **PM3** (variant detected in *trans* with a pathogenic variant), which is exclusive for recessive disorders; **PP2** (missense variant in gene with low rate of benign missense variants and pathogenic missenses common) because *EFHC1* has a low Z score for missense variants (0.14 according to gnomAD), indicating that the deviation of observed counts is not far from the expected number, and thus the gene is tolerant to missense variants; and **PP4** (patient's phenotype or family history highly specific for the gene) because GGEs are genetically heterogeneous (Zifkin, Andermann, & Andermann, 2005). We then organized the remaining 12 suitable criteria for a comprehensive classification scheme (Figure 1). We used the corresponding evidence of pathogenicity to meet the ACMG/AMP rules for combining criteria to classify variants (Table 2).

For the benign classification, we considered two ACMG/AMP criteria not applicable to our framework: **BS2** (variant observed in a healthy adult with full penetrance expected at an early age) because GGE-

related *EFHC1* variants do not have full penetrance (Suzuki et al., 2004); and **BP1** (missense variant in a gene for which primarily truncating variants are known to cause disease) because truncating variants in *EFHC1* is rarely associated with JME (Bailey et al., 2017). We used the remaining 10 criteria in another classification scheme (Figure 2), an action that fulfills the ACMG/AMP rules for combining criteria to classify genetic variants (Table 2).

We assessed the different criteria for each of the 11 *EFHC1* variants found in our cohort. From the literature search, we observed that two variants, namely c.1765G>A and c.1820A>G, had not been reported. Next, we assessed the allele frequency of the variants in databases of individuals from different populations and computed the values (Table S1). All 11 variants were identified in at least one database. Nine of the 11 variants (82%) presented allele frequencies higher than 1% in at least one subpopulation, and six of them (6/11, 54%) had allele frequencies higher than 5%. Furthermore, we calculated the OR and statistical significance (*P* values) of the association between the *EFHC1* variants found in patients with GGE in our cohort versus in a group of 100 control subjects of Brazilian origin and in two population databases of Brazilian individuals (BIPMed: Secolin et al., 2019; and ABraOM: Naslavsky et al., 2017) using an unconditional exact test (Z-pooled, one-tailed). The allele frequencies and test results are shown in Table 3. Moreover, we used 13 computer algorithms to estimate the deleterious effects of the identified variants (Table S2).

Finally, we classified the 11 variants identified in our cohort according to the ACMG/AMP guidelines (Table S3). The variants c.662G>A and c.685T>C were the only ones classified as 'pathogenic.' However, c.662G>A also meets the criteria for 'benign.' Six of the 11 variants were classified as 'benign,' and the remaining variants are considered variants of uncertain significance (VUS).

## DISCUSSION

Variant interpretation is currently one of the most significant challenges in genomic medicine (Wright et al., 2019). Frequently, the causal relationship between the genotype, the identified variant, and the disease phenotype is not evident. This phenomenon results in ambiguous, erroneous, or incomplete interpretation of the genetic tests. An uncertain test result will not provide useful information to clarify the diagnosis, assist in treatment or management, or help in disease prevention (Richards et al., 2015). With this premise in mind, we revisited the criteria for interpretation of the clinical impact of *EFHC1* variants in a cohort of patients with GGEs. This information is likely to provide new information in the assessment of the clinical utility of *EFHC1* testing.

Mutation screening in our cohort revealed that *EFHC1* variants were almost exclusively in JME patients (10/11); only one of them was also identified in an individual with GTCS on awakening. However, we acknowledge the limited number of patients with other GGE phenotypes included in the present study. Few studies have investigated *EFHC1* variants in GGEs other than JME (Stogmann et al., 2006; Subaran et al., 2015). These studies also included a limited cohort of non-JME phenotypes (37 and 23 individuals, respectively), with variants considered potentially pathogenic reported in only two patients with JAE and one with unclassified GGE (Stogmann et al., 2006). Thus, the relatively low frequency of potentially pathogenic variants in *EFHC1* in subjects with common GGEs suggests that it might not be the leading cause of these epilepsies.

All variants identified in our study are missense; they were found in patients with a family history of epilepsy (n = 20) as well as sporadic patients (n=25), a phenomenon that has also been observed in previous studies (Annesi et al., 2007; Bai et al., 2009; Jara-Prado et al., 2012; Ma et al., 2006; Medina et al., 2008; Raju et al., 2017; Subaran et al., 2015; Stogmann et al., 2006; Suzuki et al., 2004; Thounaojam et al., 2017; von Podewils et al., 2015). Furthermore, we found that overall, patients carrying variants in *EFHC1* did not present distinct clinical characteristics compared to those without it. Previous studies also did not identify an apparent correlation between molecular findings and clinical features (Annesi et al., 2007; Thounaojam et al., 2017), although von Podewils et al. (2015) reported an association of the variants c.545G>A, c.685T>C and c.881G>A with early-onset GTCSs, a higher risk of status epilepticus, and a decreased risk of bilateral myoclonic seizures in series. Hence, we cannot exclude the possibility that additional studies in larger cohorts

might highlight subtle phenotypic differences in JME patients with and without specific genetic variants.

We found that the amino acid residue changes that result from the missense variants are distributed throughout the EFHC1/myoclonin protein (Figure 3): in the three DM10 domains, in the region between the first two domains, in the EF-hand motif, and in the C-terminal region. Therefore, as described previously, we found no preferred region for the occurrence of *EFHC1* variants in patients with JME and other GGEs (Bailey et al., 2017). Notably, our study is one of the few to report variants in the EF region: p.E589K and p.N607S, both of which are novel.

To further advance the classification of GGE-related *EFHC1* variants, we reviewed the ACMG/AMP guidelines, structured them into a comprehensive classification scheme, and applied them to our framework. We performed a literature review of variants that had already been established as either pathogenic or benign. Nine of the 11 variants found in the present study were previously reported in the literature. The variants c.662G>A and c.685T>C were reported as 'pathogenic' and found in patients with JME (Annesi et al., 2007; Bai et al., 2009; Bailey et al., 2017; Ma et al., 2006; Suzuki et al., 2004; Raju et al., 2017; von Podewils et al., 2015), although c.685T>C was not considered pathogenic in another report (Stogmann et al., 2006). Functional assays demonstrated that both variants reduce the effects of cell death, prevent the apoptosis of neurons with precarious calcium homeostasis during SNC development (Suzuki et al., 2004), induce defects in the mitotic spindle, and affect the radial morphology of glial and migratory neurons (de Nijs et al., 2009). In addition, both variants were found to co-segregate with affected individuals in families with JME (Suzuki et al., 2004).

The variants c.887G>A and c.896A>G were previously reported by Bailey et al. (2017) in patients from Brazil and classified as VUS, but no further information was provided. The variants c.475C>T, c.475C>G, c.545G>A, c.1343T>C, c.1855A>C are widely recognized as polymorphisms, and thus they are not considered pathogenic (Annesi et al., 2007; Ma et al., 2006; Stogmann et al., 2006; Subaran et al., 2015; Suzuki et al., 2004; Thounaojam et al., 2017). Functional studies showed that they do not affect cell death (de Nijs et al., 2009; Suzuki et al., 2004).

Moreover, we obtained the allele frequency of the *EFHC1* variants in different population databases. We observed that the variation in the allelic frequency is dependent on the ethnic composition of the investigated population. Only the variants c.896A>G and c.1765G>A do not have allele frequencies higher than 1% in any of the investigated subpopulations (Table S1), and these two variants were absent from our group of 100 controls as well both databases of Brazilian individuals (BIPMed and AbraOM; Table 3). The allele frequencies of c.685T>C, c.887G>A, and c.1820A>G are only over 1% in specific subpopulations (c.685T>C: South Asian; c.887G>A and c.1820A>G: African; Table S1). The remaining six variants (c.475C>T, c.475C>G, c.545G>A, c.662G>A, c.1343T>C, and c.1855A>C) present allele frequencies higher than 1% in different populations.

To verify whether the frequency of *EFHC1* variants in affected individuals is increased over controls, we screened 100 Brazilian individuals without a family history of epilepsy and performed an unconditional exact test. In addition, we performed the same test using two independent databases with genomic information on Brazilian individuals (BIPMed e ABraOM) to overcome the possibly insufficient sample size of our control group. Bailey et al. (2017) employed this approach with race-matched population groups from the ExAC database, but, unfortunately, the populations in the ExAC database do not match the Brazilian population.

We found a statistically significant association with JME for the variant c.685T>C when comparing the allele frequencies of our cohort to those in the ABraOM database (OR: 6.17; 95% confidence interval: 1.24–30.77; $P$ value: 0.0245). Three variants (c.896A>G, c.1765G>A, and c.1820A>G) were not found in our control group or the Brazilian population databases; thus, the OR value was infinite ([?]). For the remaining variants (c.475C>T, c.475C>G, c.545G>A, c.662G>A, c.887G>A, c.1343T>C, and c.1855A>C), we found no association between the variant and the risk for GGE.

To estimate the effect of potentially deleterious changes on protein function, we used the *in silico* prediction algorithms recommended by the ACMG/AMP (Richards et al., 2015). There was no consensus in these

analyses—we did not observe concordance among the results of the 13 utilized algorithms for the investigated variants. Therefore, we were unable to use this evidence; the ACMG/AMP guidelines state that all of the *in silico* programs must agree on the prediction. This finding is in sharp contrast with what we have recently observed in the analysis of another epilepsy-related gene, *SCN1A,* in the context of Dravet syndrome (Gonsales et al., 2019). Indeed, as opposed to *SCN1A* ,*EFHC1* is a gene that is tolerant to variation. This potential can be inferred by its probability of being loss-of-function intolerant (pLI), calculated as 0.000, and its Z scores (deviation of observed counts from the expected number) of 0.46 and 0.14 for synonymous and missense variants, respectively (http://gnomad.broadinstitute.org). For*SCN1A* , a gene considered to be intolerant to variation, these values are much higher: pLI = 1.000, synonymous Z score = 0.88, and missense Z score = 5.22 (http://gnomad.broadinstitute.org).

The lack of consensus in the prediction analysis can be attributed to differences in the parameters used by the different algorithms (Sun & Yu, 2019; Walters-Sen et al., 2015). Also, we hypothesize that because the phenotype in most GGE patients is not as severe as in Dravet syndrome, it is possible that the putative genetic changes do not cause drastic disruptions in protein function. This factor would lead to inaccurate predictions. A recent study that evaluated the limitations in computational methods revealed that prediction models are usually excessively dependent on the conservation feature of the variants, a factor that results in predictive errors (Sun & Yu, 2019). The overlooked disease susceptibility of genes might also explain the failures of the computational tools. Interestingly, the limitations in the predictive power of the currently available *in silico*algorithms to analyze *EFHC1* variants were seen even for amino acid substitutions that were previously shown to cause functional abnormalities in biological assays (Suzuki et al., 2004). Indeed, as mentioned above, the variants c.662G>A and c.685T>C, which was found in 1 and 3 of our patients with JME, respectively, were previously studied by Suzuki et al. (2004) and found to affect the apoptotic activity of neurons with precarious calcium homeostasis.

Applying the proposed modified classification guidelines to the*EFHC1* variants from our cohort, only the variants c.662G>A and c.685T>C were classified as 'pathogenic.' However, c.662G>A also met the criteria to be classified as 'benign.' In cases when the criteria for benign and pathogenic variants are contradictory, the ACMG/AMP rules for combining criteria states that the variant should be classified as VUS (Richards et al., 2015). Thus, only variant c.685T>C can strictly be classified as 'pathogenic' (1/11, 9%).

It is noteworthy that the variant c.662G>A only met one benign criterion—the stand-alone BA1—due to an allele frequency of 5.3% in one African subpopulation. Indeed, all six variants classified as 'benign' (6/11, 55%) met the stand-alone criteria BA1 because they present an allele frequency greater than 5% in at least one database subpopulation. Therefore, the evidence-based population frequency promoted a substantial increase in the classification scores, which in some cases could have been overly weighted, especially when analyzing genes of minor effect. This phenomenon would induce an increased susceptibility rather than a major effect.

Ethnicity might have an important influence in defining which genetic factors are implicated in diseases with complex inheritance, including GGEs (Subaran et al., 2015). Thus, different genetic backgrounds would present distinct epilepsy susceptibility genes. In this scenario, one recently reported possibility is that *EFHC1* variants might be pathogenic when they are found in specific genetic backgrounds (Subaran et al., 2015). Interestingly, we found variants in our patients originating from the Northeast part of Brazil that are common only in specific populations: c.662G>A, c.887G>A, and c.1820A>G, each with a higher allele frequency in populations of African ancestry. A study that investigated the global ancestry of Brazilians showed that the genetic composition of the populations from the specific region where the probands harbor these variants are originated (the Northeast region) is closer to the Europeans (Saloum de Neves Manta et al., 2013). It is important to highlight that the underrepresentation of non-European ancestry groups in population databases poses an additional challenge to the interpretation of genetic variants (Petrovski & Goldstein, 2016). A better population match would improve the application of population frequency criteria for underrepresented ethnicities.

In contrast to Mendelian disorders, common or complex inheritance diseases might not have one single major

causative gene. Nevertheless, multiple genetic variants contribute to a small effect on disease risk. Hence, reduced penetrance and small effect size are possible explanations for why healthy individuals might harbor pathogenic variants (Cooper et al., 2013; Wright et al., 2019). Penetrance refers to the proportion of individuals in a population with a disease-related genotype that manifests the disease phenotype, while a small effect size implies that the variant has a low impact on the multifactorial etiology of the disease (Cooper et al., 2013). Therefore, some variants are insufficient to cause disease on their own and require interaction with other genetic and/or environmental factors to surpass an estimated threshold into a pathogenic phenotype (Cooper et al., 2013). In this context, *EFHC1* might be considered a partially penetrant gene. Even among disorders for which this concept has been well-established, such as cancer syndromes, the implications of low penetrance or small effect size when considering a gene suitable for testing in the clinical setting has been discussed (Ellsworth, Turner, & Ellsworth, 2019; Wendt & Margolin, 2019). For instance, variants found in the highly penetrant susceptibility genes *BRCA1* and *BRCA2* for breast cancer are clinically actionable (Ellsworth et al., 2019). However, definitive clinical recommendations cannot be drawn for lower risk genes (Wendt & Margolin, 2019), and effective management and therapeutics strategies are still required for patients who harbor variants in other genes (Ellsworth et al., 2019). Although it is known that multiple gene variants are necessary to produce the GGE phenotype, there are still no accepted models on a presumed polygenic inheritance regarding the type of variants and the involved genes (Mullen, Berkovic, & Commission, 2018). Moreover, the clinical management of the GGEs is not altered by the result of the genetic test. This fact suggests that it should be considered only in the research context (Mullen et al., 2018).

Another crucial debate regarding genetic testing is the challenge that clinicians face when reporting the results to the patients or the parents, especially when a VUS is found. These variants have undefined clinical significance, and it is a consensus that they should not be used in clinical decision-making (Richards et al., 2015). Patients report anxiety symptoms, worries, and uncertainty in response to a VUS result (Makhnoon, Shirts, & Bowen, 2019). Moreover, in the case of genetic testing performed in children, there is the possibility that the parents will misinterpret the test results, a phenomenon that would lead to unnecessary anxiety due to excessive medical attention (Wynn et al., 2018). Thus, given that almost half of the variants found in our cohort are VUS (5/11, 45%), this potential represents a significant concern when considering *EFHC1* for clinical genetic testing.

## CONCLUSIONS

There is still a significant limitation in the medical interpretation of genetic testing for complex, non-monogenic phenotypes. Our study shows that almost half of the *EFHC1* variants found in patients with GGEs remained as VUS after applying the modified classification proposed in this study using the ACMG/AMP guidelines. In addition, the only variant that could be classified as 'pathogenic' (c.685T>C) was found in only 3% of the JME patients in our current study and in 1–5% in the reported literature (Annesi et al., 2007; Podewils, 2015; Raju et al., 2017; Stogmann et al., 2006). Our results, together with previous evidence, indicate that *EFHC1* variants are currently best classified as a risk factor—not a causal, major gene—for JME and other GGEs. Given that the interpretation of genetic testing results for *EFHC1* variants is complex and offers little information for clinical decision-making, we suggest that the inclusion of *EFHC1* in gene panels for genetic testing should be limited to research purposes.

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### Conflict of Interest Statement

All authors declare no conflicts of interest related to the work reported in this manuscript.

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## TABLES

**Table 1.** Eleven *EFHC1* variants found in our cohort of 125 individuals with genetic generalized epilepsies (GGEs).

| DNA change | Amino acid change | Patient ID | Phenotype | Gender | Seizure onset (years |
|---|---|---|---|---|---|

| DNA change | Amino acid change | Patient ID | Phenotype | Gender | Seizure onset (years |
|---|---|---|---|---|---|
| NM_018100.3:c.475C>T | NP_060570.2:p.(Arg159Trp) | 2 | JME | M | 17 |
| | | 13 | JME | F | 12 |
| | | 18 | JME | M | 16 |
| | | 22 | JME | F | 14 |
| | | 24 | JME | M | 18 |
| | | 26 | JME | M | NI |
| | | 29 | JME | F | 14 |
| | | 39 | JME | F | NI |
| | | 41 | JME | M | NI |
| | | 42 | JME | F | 5 |
| | | 47 | JME | F | 19 |
| | | 52 | JME | F | 15 |
| | | 60 | JME | F | 9 |
| | | 78 | JME | M | 15 |
| | | 82 | JME | F | 14 |
| | | 93 | JME | F | 12 |
| | | 97 | JME | F | 20 |
| NM_018100.3:c.475C>G | NP_060570.2:p.(Arg159Gly) | 71 | JME | M | 12 |
| NM_018100.3:c.545G>A | NP_060570.2:p.(Arg182His) | 9 | JME | F | NI |
| | | 21 | JME | F | 17 |
| | | 28 | JME | F | 13 |
| | | 43 | JME | F | 11 |
| | | 46 | JME | F | 19 |
| NM_018100.3:c.662G>A | NP_060570.2:p.(Arg221His) | 65 | JME | M | 9 |
| NM_018100.3:c.685T>C | NP_060570.2:p.(Phe229Leu) | 4 | JME | M | 7 |
| | | 16 | JME | F | NI |
| | | 23 | JME | M | NI |
| NM_018100.3:c.887G>A | NP_060570.2:p.(Arg296His) | 56 | JME | M | 14 |
| | | 58 | JME | M | 14 |
| NM_018100.3:c.896A>G | NP_060570.2:p.(Lys299Arg) | 28 | JME | F | 13 |
| NM_018100.3:c.1343T>C | NP_060570.2:p.(Met448Thr) | 4 | JME | M | 7 |
| | | 21 | JME | F | 17 |
| | | 44 | JME | F | NI |
| | | 62 | JME | M | 14 |
| | | 66 | JME | F | 7 |
| | | 69 | JME | M | 21 |
| | | 76 | JME | M | 4 |
| | | 99 | JME | F | 16 |
| NM_018100.3:c.1765G>A* | NP_060570.2:p.(Glu589Lys) | 87 | JME | M | 11 |
| NM_018100.3:c.1820A>G* | NP_060570.2:p.(Asn607Ser) | 60 | JME | F | 9 |
| NM_018100.3:c.1855A>C | NP_060570.2:p.(Ile619Leu) | 2 | JME | M | 17 |
| | | 19 | JME | F | 21 |
| | | 32 | JME | F | 14 |
| | | 34 | JME | F | 12 |
| | | 38 | JME | F | NI |
| | | 43 | JME | F | 11 |
| | | 48 | JME | M | 14 |

| DNA change | Amino acid change | Patient ID | Phenotype | Gender | Seizure onset (years |
|---|---|---|---|---|---|
| | | 56 | JME | M | 14 |
| | | 58 | JME | M | 14 |
| | | 68 | JME | M | 13 |
| | | 70 | JME | M | 3 |
| | | 89 | JME | M | NI |
| | | 104 | GTCSa | F | 11 |

ID: identification; JME: juvenile myoclonic epilepsy; GTCSa: generalized tonic-clonic seizures on awakening; M: male; F: female; My: myoclonic seizures; GTC: generalized tonic-clonic; A: absence; NI: not informed. *novel variants.

**Table 2.** Guidelines for combining different criteria to classify *EFHC1* variants into 'pathogenic,' 'likely pathogenic,' benign,' 'likely benign,' or variants of uncertain significance (VUS).

| Classification | Evidence of pathogenicity or benign impact |
|---|---|
| Pathogenic | 1 Very Strong + 1 to 4 Strong |
| | 1 Very Strong + 2 to 4 Moderate |
| | 1 Very Strong + 1 Moderate + 1 Supporting |
| | 1 Very Strong + 2 or 3 Supporting |
| | 2 to 4 Strong |
| | 1 Strong + 3 or 4 Moderate |
| | 1 Strong + 2 Moderate + 2 or 3 Supporting |
| Likely pathogenic | 1 Very Strong + 1 Moderate |
| | 1 Strong + 1 or 2 Moderate |
| | 1 Strong + 2 or 3 Supporting |
| | 3 or 4 Moderate |
| | 2 Moderate + 2 or 3 Supporting |
| Benign | 1 Stand-alone |
| | 2 or 3 Strong |
| Likely benign | 1 Strong + 1 Supporting |
| | 3 to 5 Supporting |
| VUS | Other criteria shown above are not met |
| | Contradictory criteria for pathogenic and benign |

**Table 3.** Allele frequencies of *EFHC1* variants found in our cohort of genetic generalized epilepsy (GGE) patients in 100 control subjects and population databases of Brazilian individuals.

| Nucleotide change | AF in relation to phenotype | Control subjects (n = 100) | Control subjects (n = 100) | Control subjects (n = 100) | BIPMed (n = 258) | BIPMed (n = 258) | BIPMed (n = 258) | AbraOM (n = 609) | AbraOM (n = 609) |
|---|---|---|---|---|---|---|---|---|---|
| | | AF | OR | *P* value | AF | OR | *P*-value | AF | OR |
| NM_018100.3:c.47[5C>T] | 8.5% (JME) | 7.5% | 1.15 | 0.3966 | 11.4% | 0.72 | 0.1450 | 14.5% | 0.55 |
| NM_018100.3:c.47[5C>G] | 0.5% (JME) | 0.5% | 1.00 | 0 | 0.6% | 0.86 | 0.5243 | 1.2% | 0.41 |

| Nucleotide change | AF in relation to phenotype | Control subjects (n = 100) | Control subjects (n = 100) | Control subjects (n = 100) | BIPMed (n = 258) | BIPMed (n = 258) | BIPMed (n = 258) | AbraOM (n = 609) | AbraOM (n = 609) |
|---|---|---|---|---|---|---|---|---|---|
| NM_018100.3:c.54[illegible] | 2.5% (JME) | 4.0% | 0.62 | 0.2645 | 4.3% | 0.58 | 0.1452 | 4.2% | 0.59 |
| NM_018100.3:c.66[illegible] | 0.5% (JME) | 0.5% | 1.00 | 0 | 0.6% | 0.86 | 0.5243 | 0.2% | 2.04 |
| NM_018100.3:c.68[illegible] | 1.5% (JME) | 0.5% | 3.03 | 0.1883 | 0.4% | 3.91 | 0.1219 | 0.2% | 6.17 |
| NM_018100.3:c.88[illegible] | 1.0% (JME) | 0.0% | [?] | 0.1058 | 0.2% | 5.19 | 0.1422 | 0.4% | 2.45 |
| NM_018100.3:c.89[illegible] | 0.5% (JME) | 0.0% | [?] | 0.2645 | 0.0% | [?] | 0.1205 | 0.0% | [?] |
| NM_018100.3:c.13[illegible] | 4.0% (JME) | 4.0% | 1.00 | 0 | 5.8% | 0.68 | 0.1976 | 7.8% | 0.49 |
| NM_018100.3:c.17[illegible] | 0.5% (JME) | 0.0% | [?] | 0.2645 | 0.0% | [?] | 0.1205 | 0.0% | [?] |
| NM_018100.3:c.18[illegible] | 0.5% (JME) | 0.0% | [?] | 0.2645 | 0.0% | [?] | 0.1205 | 0.0% | [?] |
| NM_018100.3:c.18[illegible] | 6.0% (JME) 7.0% (GTCSa) | 4.5% | 1.35 3.50 | 0.2657 0.1888 | 5.4% | 1.11 2.90 | 0.4557 0.2935 | 4.1% | 1.49 3.89 |

AF: allele frequency; OR: odds ratio; JME: juvenile myoclonic epilepsy; GTCSa: generalized tonic-clonic seizures on awakening. *P*- values were calculated using an unconditional exact test (Z-pooled, one-tailed). Population databases: of Brazilian individuals (http://bipmed.org/), AbraOM (http://abraom.ib.usp.br/). $^{*}P < 0.05$.

## FIGURE LEGENDS

**Figure 1.** Pathogenic classification scheme applied to the genetic generalized epilepsy (GGE)-related *EFHC1* variants.

**Figure 2.** Benign classification scheme applied to the genetic generalized epilepsy (GGE)-related *EFHC1* variants.

**Figure 3.** Predicted location of variants found in the EFHC1/myoclonin protein in patients with juvenile myoclonic epilepsy (JME) and other genetic generalized epilepsies (GGEs). DM: Domain; EFH: EF-Hand.

## SUPPLEMENTARY MATERIAL

**Table S1.** Population allele frequency of the genetic generalized epilepsy (GGE)-related missense *EFHC1* variants found in our cohort.

**Table S2.** Results of the 13 computer algorithms used to predict the effects of the *EFHC1* missense variants found in our cohort of genetic generalized epilepsy (GGE) patients.

**Table S3.** Classification of the genetic generalized epilepsy (GGE)-related missense *EFHC1* variants found in our cohort.

| | |
|---|---|
| **1. Type of variant:** | |
| Null variant (nonsense, frameshift, canonical ±1 or 2 splice sites, initiation codon, single or multiexon deletion) **(PVS1)**. | **Very strong evidence** |
| Variant that changes protein length (in-frame deletions/insertions and stop losses) **(PM4)**. | **Moderate evidence** |
| Missense variants (see further criteria). | |
| Synonymous/silent variant (see benign classification). | |
| **2. Revision of literature information about the variant:** | |
| Amino-acid change described as pathogenic according to the guidelines, regardless of the nucleotide change **(PS1)**. | **Strong evidence** |
| Well-established functional studies showing a deleterious effect **(PS3)**. | **Strong evidence** |
| Same amino-acid residue previously described as pathogenic according to the guidelines but caused by different missense changes **(PM5)**. | **Moderate evidence** |
| Reputable source reports variant as pathogenic, but evidence not available to perform independent evaluation **(PP5)**. | **Supporting evidence** |
| **3. Inheritance and family studies:** | |
| *De novo* variant (not present in parents) with paternity and maternity confirmed **(PS2)**. | **Strong evidence** |
| *De novo* variant without paternity and maternity confirmed **(PM6)**. | **Moderate evidence** |
| Variant co-segregates with the disease in multiple affected family members **(PP1)**. | **Supporting evidence** (Stronger evidence with increasing segregation data) |
| **4. Frequency of the variant in controls/population:** | |
| Prevalence in affected statistically increased over controls **(PS4)**. | **Strong evidence** |
| Variant absent or with low allele frequency below in population databases **(PM2)**. | **Moderate evidence** |
| **5. Prediction of the deleterious effects using computer algorithms:** | |
| Multiple lines of computational evidence supporting a deleterious effect on the gene product **(PP3)**. | **Supporting evidence** |

| | |
|---|---|
| **1. Type of variant:** | |
| Synonymous (silent) variant with non-predicted splice impact **(BP7)**. | **Supporting evidence** |
| Variant that changes protein length (in-frame deletions/insertions) in a repetitive region without a known function **(BP3)**. | **Supporting evidence** |
| Other non-synonymous variants (see pathogenic classification). | |
| **2. Revision of literature information about the variant:** | |
| Well-established functional studies show no deleterious effect **(BS3)**. | **Strong evidence** |
| Reputable source reports variant as benign, but evidence not available to perform independent evaluation **(BP6)**. | **Supporting evidence** |
| **3. Inheritance and family studies:** | |
| Lack of segregation with the disease in affected members of a family | **Strong evidence** |
| **4. Frequency of the variant in controls/population:** | |
| Allele frequency is >5% in population databases **(BA1)**. | **Stand-alone evidence** |
| Allele frequency is greater than expected for the disorder **(BS1)**. | **Strong evidence** |
| **5. Prediction of the deleterious effects using computer algorithms:** | |
| Multiple lines of computational evidence suggesting no impact on the gene product **(BP4)**. | **Supporting evidence** |
| **6. Other observations:** | |
| Observed in *trans* with a pathogenic dominant variant or in *cis* with a pathogenic variant in any inheritance pattern **(BP2)**. | **Supporting evidence** |
| Found in a case with an alternate molecular basis for disease **(BP5)**. | **Supporting evidence** |