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Highlights

- *EHFC1* mutations are observed in 33.3% of JME patients of Turkish origin.
- The most common mutation in JME patients was R182H, which was associated with higher risk of disease.
- The 3'UTR mutation was found to have lower risk against JME.

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EFHC1 gene mutation profile of Turkish JME patients and its association with disease risk

Kezban Aslan-Kara[1*], Ebru Dündar- Yenilmez[2], Elçin Ateş[1], Mustafa Muhlis Alparslan[2], Taylan Peköz[1], Hacer Bozdemir[1], Abdullah Tuli[2]

[1] Çukurova University Faculty of Medicine, Department of Neurology
[2] Çukurova University Faculty of Medicine, Department of Medical Biochemistry

***Kezban Aslan –Kara (Professor of Neurology, MD)**
Mail address: kezbanaslan@hotmail.com
ORCID number: **0000-0002-3868-9814**

***Ebru Dündar-Yenilmez (Associate Professor of Medical Biochemistry, MD)**
Mail address: edundaryenilmez@gmail.com
ORCID number:0000-0002-5519-3773

Elçin Ateş (Resident of Neurology, MD)
Mail address: elcinbas@gmail.com
ORCID number:0000-0003-3125-0507

Mustafa Muhlis Alparslan (Resident of Medical Biochemistry, MD)
Mail address: Mustafa.m.alparslan@gmail.com

Taylan Peköz (Assistant Professor of Neurology, MD)
Mail address: taylanpekoz@gmail.com
ORCID number:0000-0001-9126-9192

Hacer Bozdemir (Professor of Neurology, MD)
Mail address: hbozdemir@mail.cu.edu.tr
ORCID number:0000-0001-8402-4277

Abdullah Tuli (Professor of Medical Biochemistry, MD)
Mail address: tuliabd@gmail.com
ORCID number: **0000-0002-6475-1087**

*Equal Contributor

***Corresponding author:** Kezban Aslan –Kara
Address: Çukurova University, School of Medicine, Department of Neurology
Adana, Sarıçam-Adana
01330, Turkey
Telephone: 0-322-3386060/3206 **E-mail:** kezbanaslan@hotmail.com

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ABSTRACT

Objectives: Juvenile myoclonic epilepsy (JME) is a common form of generalized epilepsy with an important genetic component. This cohort study aimed to examine the frequency of *EFHC1*

gene variants in Turkish JME patients and a healthy control group and evaluate the association between these mutations and disease risk.

Methods: We screened 72 JME patients with a mean age of 31.8 ± 9.9 (20-65) years and 35 controls with a mean age of 29.1 ± 7.6 (17-50) years from southern Turkey using direct sequencing analyses.

Results: *EFCH1* single nucleotide variants were detected in 24 of 72 JME patients and 3 of 35 controls. The most common mutations were R182H in JME patients (p=0.010) and 3′UTR in the control group (p<0.001). The R182H mutation is a common variant in JME (95% CI: 1.232-76.580, p=0.031) and the 3′UTR mutation may be associated with lower risk of JME in the Turkish population (95% CI: 13.89-166.67, p<0.001).

Significance: Our results indicate that *EFHC1* gene variants carry a risk for JME and the 3′UTR variant may have a protective role against JME in the Turkish population. Screening for other genes is needed to further clarify the genetic inheritance of JME in Turkish patients.

Key words: Juvenil Myoclonic Epilepsy, EFHC1, genetic mutation

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Introduction

Juvenile myoclonic epilepsy (JME) is one of the most common epilepsy syndromes among the genetic generalized epilepsies (GGEs). Although it has been described using different names for many years, it was most recently defined in 1989 by the International League Against Epilepsy (ILAE) as an epilepsy syndrome usually seen in adolescents that is characterized by seizures with bilateral, single or repetitive, arrhythmic, irregular myoclonic jerks (predominantly in the upper limbs) without loss of consciousness during jerks. Symptoms are described as myoclonic seizures (MY) with sudden loss of tonus and falls, which are later accompanied by generalized tonic-clonic seizures (GTC), and less commonly absence seizures (AS). Seizures typically occur immediately upon waking in the morning and are aggravated by sleep deprivation. Typical electroencephalographic (EEG) findings are generalized irregular spike-wave (GSW) and polyspike-wave (GPSW) discharges that are often photosensitive [1, 2].

The prevalence of JME is 5-10% of all epilepsy cases and 15-20% of idiopathic generalized epilepsy (IGE); its estimated incidence is 1 in 100,000 [3, 4]. Numerous twin studies starting with those of William G. Lennox in the 1940s demonstrated over 90% concordance of IGEs in identical twins, and genetic penetrance was predicted in IGEs [5].

Electroclinical syndromes include childhood absence epilepsy (CAE), juvenile absence epilepsy (JAE), and JME. Epidemiological studies have shown that genetic factors are more likely to be present in the siblings of patients with these syndromes than in other epilepsies [4, 6, 7]. Genetic studies of GGEs have supported evidence implicating channelopathy disorders, ion channel disorders, gene mutations indicating recurrent microdeletions, and most recently, genes identified by next generation sequencing [5, 8, 9]. The JME gene was initially localized to the 6p12–p11 chromosomal region [10]. Suzuki et al. first examined the *EJM1* gene in JME patients, but later detected five disease-associated missense mutations of the EF hand-containing calcium-binding (*EFHC1*) gene at the same location in 6 Mexican JME families that were not present in 382 control individuals, and proposed *EFHC1* as the gene responsible for JME [11]. *EFHC1* gene mutations were later identified in different ethnic groups, including Austrian, Italian, Mexican, Indian, and Dutch populations [12-16]. To date, only one JME case linked to the *EFHC1* gene location has been reported in the Turkish population [17].

There are no previous genetic studies conducted in JME patients in Turkey. This study aimed to determine the frequency of *EFHC1* gene variants and the risk of disease development associated with these gene variants in Turkish JME patients.

Patients and Methods

This prospective cross-sectional study was conducted in the neurology department of Çukurova University Faculty of Medicine. Patients who were followed up in the epilepsy outpatient clinic and met the study criteria were included. Informed consent was obtained from each participant and the study protocol was approved by the Çukurova University Faculty of Medicine Ethics Committee (decision: April 1, 2016/52).

The study population consisted of 72 patients with JME and a control group of 35 individuals who presented to the Department of Neurology of Çukurova University Faculty of Medicine Hospital between January 2017 and January 2019. The control subjects were recruited from hospital staff of similar age and gender who had no personal or family history of epilepsy. A detailed history of the type and frequency of seizures was obtained from patients and parents at the time of examination and by reviewing the patients' medical records.

Sample Selection Criteria

Inclusion criteria were as follows:

1. Having a diagnosis of JME based on the criteria of the Commission on Classification and Terminology of the International League Against Epilepsy [2]
2. Being literate and able to sign the informed consent form
3. Having no lesional abnormality that may cause of seizure on cerebral imaging (non-specific findings or variations did not excluded)
4. Having no symptoms of systemic, metabolic, or degenerative disease.

DNA Preparation and EFHC1 Gene Sequence Analysis

Sample Preparation

Venous blood samples (10 cc) were collected into EDTA tubes from all patients and controls included in the study and were transferred with a preservative to the Faculty of Medicine Department of Medical Biochemistry, where molecular analyses were performed.

DNA Extraction and PCR

DNA was extracted from peripheral blood anticoagulated with EDTA using High Pure Template Kits (Roche Diagnostics) according to the manufacturer's instructions. The 11 exons of the *EFHC1* gene were amplified by polymerase chain reaction (PCR) using intronic primers [18].

Sequencing Analysis

Direct sequencing was used to analyze the 11 exons of the *EFHC1* gene. After amplification, the samples were purified using Sephadex G-50 in a gel filtration cartridge. Cycle sequencing was performed on the purified samples using the Applied Biosystems Big Dye Terminator v3.1 Cycle Sequencing Kit according to the manufacturer's protocol. The sequencing products were analyzed in an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The 35 healthy subjects of the same ethnic background who comprised the control group were also screened by variant analysis of the *EFHC1* gene to test for the occurrence of the sequence variants identified in the JME screening sample.

Statistical Analysis

Categorical variables were expressed as numbers and percentages, whereas continuous variables were summarized as mean and standard deviation or median and minimum-maximum as appropriate. The chi-square test was used to compare categorical variables between the groups. Chi-square test was used for between-group comparisons of the genotype frequencies of single nucleotide polymorphisms (SNPs) detected in the *EFHC1* gene. Crude odds ratios and 95% confidence intervals (CI) were calculated to determine the risk of JME associated with the SNPs.

All analyses were performed using IBM SPSS Statistics Version 20.0 statistical software package. The statistical level of significance was accepted as 0.05 for all tests.

Results

The study included 72 JME patients (48 female, 24 male) with a mean age of 31.8 ± 9.9 years (range: 20-65) and 35 healthy controls (16 female, 19 male) with a mean age of 29.1 ± 7.6 years (range: 17-50).

The mean age at seizure onset in the JME group was 14.1± 3.7 years (range: 5-24). The first recognized seizure was MY in 55.6% (n = 40), GTC in 34.7% (n = 25), and AS in 9.7% (n = 7) of the patients. At the time of blood sampling for genetic screening, other seizure patterns observed during follow-up after the first seizure included GTC and MY in 59.7% (n = 43); GTC, MY, and AS in 25% (n = 18); MY and AS in 8.3% (n = 6); and MY seizures only in 6.9% (n = 5) of patients. Of the patients, 27.8% (n = 20) had parental consanguinity, 33.3% (n = 24) had a family history of epilepsy,

16.7% (n = 12) had febrile convulsion(s), and 27.8% (n = 20) had a history of head trauma (loss of consciousness was described in only 1 case, but head traumas occurred after the onset of seizures).

EEG examination performed at first admission to the clinic revealed normal findings in 16.7% (n = 12), GSW and GPSW in 54.2% (n = 39), generalized slow activity discharges in 26.4% (n = 19), and non-specified slow background activity in 2.8% (n = 2) of the patients. Radiological examinations were normal in 80.6% (n = 58) of patients and revealed non-specified gliotic foci in 4.2% (n = 3), cerebral cortical atrophy in 11.1% (n = 8), mega-cistern magna variation in 2.8% (n = 2), and cavum septum pellucidum in 1.4% (n = 1) of the patients.

Table 1. DNA variants of *EFHC1* coding regions in JME patients and normal controls

SNP No	**Location**	**NCBI dbSNP ID*[b]**	**Nucleotide Change*[a]**	**Amino acid changes**	**Function**	**Frequency among JME patients (n=72)**	**Frequency among controls (n=35)**
1	Exon 3	rs3804506	c.475 C>T	R159W	Missense	4	2
2	Exon 3	rs3804505	c.545 G>A	R182H	Missense	16	1
3	3′UTR	rs7757370	c.+121 C>A	-	-	4	26
4	Exon 11	rs17851770	c.1855 A>C	I619L	Missense	0	1

*[a] Homo sapiens EF-hand domain containing 1 (EFHC1), transcript variant A (EFHC1), mRNA from NCBI dbSNP browser Search database [ref NM_018100.4] (https://www.ncbi.nlm.nih.gov)

*[b]Variations already searched in the dbSNP database, SNPdatabase reference (http://www.ncbi.nlm.nih.gov/SNP)

At least one SNP in exon 3 and 3 prime UTR (3′UTR) was detected in 24 of the 72 patients diagnosed with JME. Of these SNPs, the most common (66.6%) was c.545 G>A in exon 3, followed by c.475 C>T in exon 3 and c.+121 C>A in 3′UTR. The SNPs detected in JME patients are shown in Figure A. At least one SNP was also detected in a total of 27 control subjects (77.1%). The majority of these subjects (26/35, 74.3%) carried the c.+121 C>A (rs7757370) polymorphism in 3′UTR of the gene, while 1 control subject carried the rs17851770 (I619L) polymorphism (Tables 1 and 3). Three cases in the control group had two SNPs together (3′UTR and R182H polymorphism in 1 and 3′UTR

and R159W polymorphism in 2 control subjects). Electropherograms of the variants are shown in Figure B.

Table 2. Comparison of the genotype frequencies of four *EFHC1* SNPs between JME patients and normal controls

SNP No	SNPs	n	Genotype, n (%)		P value
1	**c.475 C>T (rs3804506)**		**C/C**	**C/T**	
	JME	4	68 (0.94)	4 (0.05)	0.973
	Controls	2	33 (0.94)	2 (0.06)	
2	**c.545 G>A (rs3804505)**		**G/G**	**G/A**	
	JME	16	56 (0.78)	16 (0.22)	0.010
	Controls	1	34 (0.97)	1 (0.28)	
3	**c.+121 C>A (rs7757370)**		**C/C**	**C/A**	
	JME	4	68 (0.94)	4 (0.11)	<0.001
	Controls	26	9 (0.12)	26 (0.74)	
4	**c.1855 A>C (rs17851770)**		**A/A**	**A/C**	
	JME	0	72	0	0.324
	Controls	1	34	1	

SNP: Single nucleotide polymorphism, JME: Juvenile myoclonic epilepsy

Genotypically, the rs3804505 SNP was more common in JME patients and rs7757370 was more common in the control group ($p = 0.010$ and $p < 0.001$, respectively, Table 2). Of the SNPs, we determined that the presence of rs3804505 was associated with 9.7-fold higher odds of JME (95% CI: 1.232-76.580, $p = 0.031$, Table 3), while the presence of rs7757370 was associated with a 50-fold lower odds of JME development (95% CI: 13.89-166.67, $p < 0.001$, Table 3).

Of the 16 patients with an SNP (R182H), 9 (56.3%) had GTC + MY seizures, 4 (25%) had GTC + MY + AS seizures, 2 (12.5%) had MY + AS seizures, and 1 (6.3%) had only MY seizures.

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Table 3. Association of *EFHC1* SNPs with JME

SNPs	p	OR	95% CI for OR
c.475 C>T (rs3804506)	0.973	0.971	0.169-5.572
c.545 G>A (rs3804505)	0.031	9.714 *	1.232-76.580
c.+121 C>A (rs7757370)	<0.001	50 **	13.89-166.67
c.1855 A>C (rs17851770)	0.999	0	0-0

***Increased the odds of JME by 9.7 fold, **Decreased the odds of JME by 50 fold. SNP: Single nucleotide polymorphism, JME: Juvenile myoclonic epilepsy, OR: Odds ratio

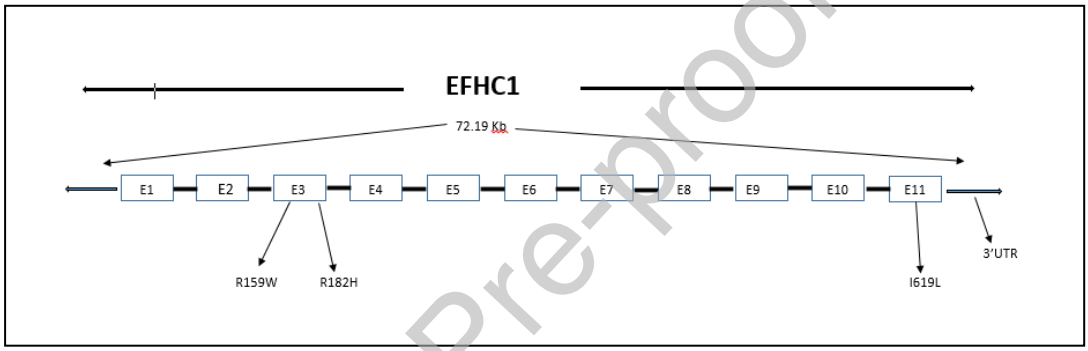


Figure A. Genomic organization of detected variants in exon 3 (E3) and exon 11 (E11) of the *EFHC1* gene in our study population.

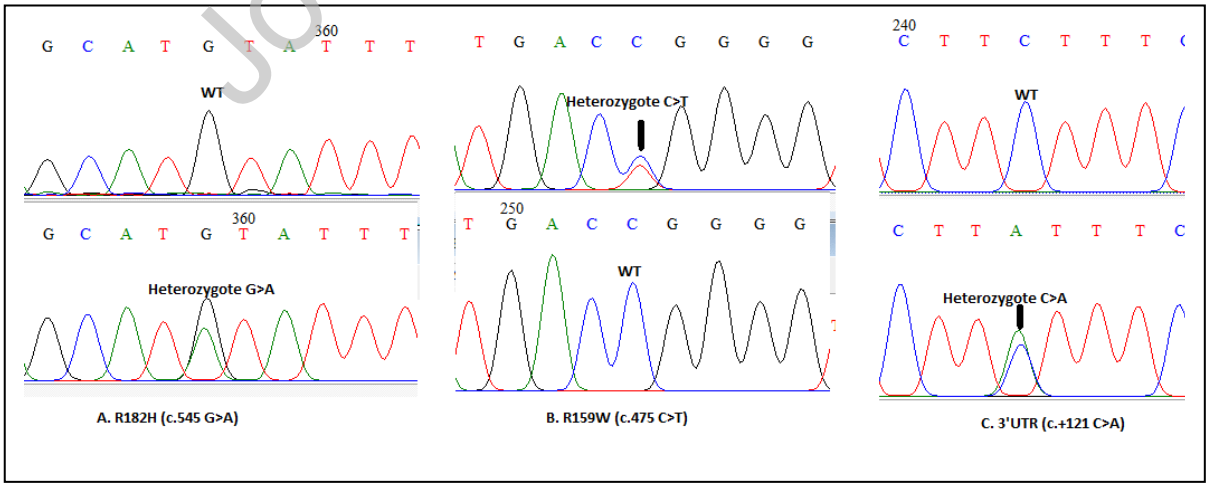


Figure B. Electropherograms of the variants wild type and heterozygote genotypes

A. R182H (c.545 G>A), **B.** R159W (c.475C>T), **C.** 3´UTR (c.+121 C>A)

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Discussion

In our *EFHC1* variant screening study in a cohort of JME patients, one-third of the patients (24/72) had at least one SNP. Two-thirds of the SNPs detected were in exon 3 and fewer were detected in exon 11 of the *EFHC1* gene. Thus, we identified two different variants, R182H (c.545 G>A) and R159W (c.475 C>T), that alter the amino acid sequence, (often c.545 G>A, less often c.475 C>T) in JME patients (Table 1 and Figure A and B). These two variants were detected together with a 3′UTR variant (c. +121 A>C) in 3 control subjects.

The two variants in exon 3 that we detected in JME patients in our study were previously identified by Suzuki et al. [11]. However, we also detected the R182H variant in one healthy control subject ($p = 0.010$). This SNP was reported in the gene segregation database (http://gnomad.broadinstitute.org) in different populations, especially Finnish and non-Finnish European populations [10, 11, 18]. The less frequently detected c.475 C>T variant is not in the Genome Aggregation Database. In the control group, the c.+121 C>A variant was statistically more frequent ($p < 0.001$). The SNPs identified in the patient group were also detected in combination with 3′UTR variant in several control subjects (R159W + 3´UTR in 2 and R182H + 3´UTR variant in 1 person). It was noteworthy that all three of these individuals were healthy and asymptomatic with no family history of epilepsy. More importantly, we determined that the presence of the c.545 G>A variant was associated with higher risk of developing JME (95% CI: 1.232-76.580, $p = 0.031$), while the presence of the rs7757370 SNP was associated with lower risk of developing JME (95% CI: 13.89-166.67, $p < 0.001$).

We detected the 3′UTR variant in only 4 JME patients (0.05%), while Stogman reported it in only 1 JME patient in their study [12]. All 4 of these patients exhibited only the coexistence of GTC + MY seizures, and EEG findings of GSW/GPSW were observed in 3 patients and generalized slow activity discharges in 1 patient. One of the other *EFHC1* gene variants identified in our study was not detected in these patients, but not all JME-associated variants reported in the literature to date were examined. Most protein-coding genes in humans contain polyadenylation [p(A)] sites in their 3´UTRs, which serve as a common regulatory layer [19]. Alternative polyadenylation (APA) leads to the creation of 3´UTR isoforms of varying lengths that potentially affect mRNA stability, translation efficiency, nuclear export, and cellular localization. Polyadenylation in the 3´UTR of some genes and

variants in adjacent RNA-cis regulatory elements have been shown to cause some Mendelian diseases [20-22]. Therefore, we do not know which APA is present in the 3´UTR isoforms identified in our patient group or which silent gene this APA caused to be expressed to reveal the phenotype, because several common SNPs that regulate APA have been implicated as the cause of complex diseases to date [21]. There are many different networks in the central nervous system. The causes of mutant gene expression are not known, and it is not clear why some of those expressed are clinically silent or dominant in a certain age range. Evaluating the APAs in the 3´UTR identified in the patient group in our study may shed light on this.

The *EFHC1* gene was first identified in Mexican families with JME [11], then in populations of different ethnic backgrounds [12-16]. There has been controversy regarding the pathological properties of identified *EFHC1* gene variants according to ethnicity [23]. It has been emphasized that JME shows autosomal dominant [6], autosomal recessive [24], digenic [24], or multifactorial [12-16] inheritance. Our study included only patients diagnosed with JME; relatives of the patients were not examined. For this reason, we could not determine how the *EFHC1* gene variants is transferred in the Turkish ethnic population. However, in this study we detected no polymorphisms other than 475C>T and 545G>A, unlike in other studies [13, 14, 16]. Annesi et al.[14] identified the 475C>T variant in 20% and the 545G>A variant in 5.8% of 82 affected patients in 27 families. Suzuki et al.[11] detected 3 coding SNPs (475C>T, 545G>A, and 1855A>C, resulting in amino acid substitutions R159W, R182H and I619L, respectively), as well as the variants 685T>C, 628G>A, and 757G>T (resulting in the amino acid substitutions F229L, D210N, and D253Y, respectively) in JME patients, and a few other less common heterozygous polymorphisms in both the patient and control group. In our study, the frequency of variants in JME patients was found to be approximately the same (22.2% for 475C>T and 5.6% for 545G>A), but with less diversity.

The *EFHC1* gene is 72 kb long and has 11 exons and 3 DM10 domains [23]. It is known to encode the 640-amino acid protein myoclonin1 [25] and include the c.545 G>A (R182H) protein sequence. R182H is found in the DM10 of the *EFHC1* span and was previous shown to be a partially benign, likely pathogenic variant identified in Mexican and Italian families [13, 14, 23]. In contrast to ethnic Turks, Bai et al.[18] reported four different new genetic variants in the *EFHC1* region of JME patients of Spanish origin instead of the c.545 G>A and c.475 C>T SNPs. In our study, we detected no different DNA variants in the *EFHC1* region.

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According to the NHGRI and ACMG guidelines for assigning disease causality, *EFHC1* variants are classified as benign [23, 26]. Suzuki et al. demonstrated that EFHC1 induces programmed cell death by increasing Ca^{2+} influx through voltage-gated channels and that mutations may prevent the elimination of unwanted neurons during central nervous system development, leading to precarious calcium hemostasis and the formation of hyperexcitable neural circuits [11]. It has been shown that in the postnatal mouse brain, *EFHC1* is mainly expressed in the cilia of the ependymal cell lining the cerebral ventricles [25]. Heterozygosity for the Phe229Leu mutation in *EFHC1* is also known to be associated with JME, which is a relatively benign form of epilepsy, whereas homozygosity for the same mutation is associated with lethal epilepsy of infancy [27]. Epileptic discharges on EEG are an important indicator of hyperexcitability. Unlike the study by Annesi et al.[14] but similar to the findings reported by Suzuki et al.[11], we observed the abnormal EEG findings typical of JME more frequently in patients carrying the rs3804505 SNP. Of 16 patients with the *EFHC1* (R182H) variant, initial EEG revealed GSW and GPSW in 8 patients and generalized slow activity discharges in 6 patients. Another interesting finding was that cerebral cortical atrophy (n = 7) and non-specific gliotic foci (n = 1) were more common in patients with the rs3804505 variant. Microstructural white matter abnormalities in the GGEs have been demonstrated with diffusion tensor imaging (DTI) [28] and nonspecific abnormal imaging findings were shown to be more common in drug-resistant JME patients in our previous study [29].

The most common SNP, rs3804505, was consistent with the seizure phenomenology of the syndrome described by the patients. All of these patients were under anti-seizure therapy. The initiation and regular use of appropriate treatment after the first observed seizure may have prevented the emergence of other seizure types seen in the syndrome. We also observed no relationship between the frequency of SNPs in JME patients and parental consanguinity (variants were detected in only 7 of 20 patients who reported consanguineous marriage and 14 of 52 patients without consanguineous marriage in the family; p=0.78) or parental history of epilepsy (variants were detected in 6 of 24 JME patients with parental history of epilepsy). In this respect, there was inconsistency between demographic characteristics and variants.

CONCLUSIONS AND FUTURE DIRECTIONS

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Genetic predisposition was determined in JME patients of Turkish origin, with at least one third of the patients having an *EFHC1* single nucleotide variant. We think that the investigation of other genetic variants in JME patients of Turkish origin and the role of 3′UTRs in these genetic variants will be important. Furthermore, determining the differentiation of APAs in the 3′UTR of SNPs in JME patients and the normal population will be informative regarding genetic variants in JME.

STRENGTHS AND LIMITATIONS

An important limitation of this study is that we did not include the other relatives of JME patients with family history. Another limitation is that different genes were not screened. Low patients and control case number is another limitation for having a conclusion about these genes. However, our analysis of potential variants in the control group is a strength of the study. The other limitation of the study was small size of the control group.

Ethics approval and consent to participate: This study was approved by the Ethics Committee for Medical Research (April 1, 2016/52) of Çukurova University Faculty of Medicine.

Consent to publish: The authors have given permission to publish.

Availability of data and materials: The data used to support the findings of this study are available from the corresponding author upon request.

Competing interests: None of the authors have any conflict of interest to disclose as it regards to this manuscript.

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