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Research Article | Araştırma Makalesi

INVESTIGATION OF *IFIT3* AND *KCNS3* GENE EXPRESSION PATTERNS IN THE PERIPHERAL BLOOD OF PATIENTS WITH CRYPTOGENIC EPILEPSY

KRİPTOJENİK EPİLEPSİ HASTALARININ PERİFERİK KANINDA IFIT3 VE KCNS3 GEN EKSPRESYON PATERNLERİNİN İNCELENMESİ

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ABSTRACT

Objective: Cryptogenic epilepsy is a subtype of epilepsy marked by the absence of identifiable structural or metabolic causes, making both diagnosis and treatment particularly challenging. Molecular approaches, such as gene expression profiling, may offer insights into underlying mechanisms and help refine clinical strategies. This study aimed to evaluate the peripheral blood expression profiles of *IFIT3* and *KCNS3* genes in individuals diagnosed with cryptogenic epilepsy.

Methods: Peripheral blood samples were obtained from 20 patients with cryptogenic epilepsy and 20 age- and sex-matched healthy individuals. Total RNA was extracted and subsequently reverse-transcribed into complementary DNA (cDNA). Quantitative real-time PCR (RT-qPCR) was utilized to determine the relative expression levels of *IFIT3* and *KCNS3*, with *ACTB* serving as the internal control gene.

Results: Analysis revealed a significant upregulation of *KCNS3* expression in the patient group compared to healthy controls ($p < 0.0001$). In contrast, no statistically significant difference was observed in *IFIT3* expression between the two groups

Conclusion: The elevated expression of *KCNS3,* which encodes a voltage-gated potassium channel subunit, suggests a potential involvement of ion channel dysregulation in cryptogenic epilepsy pathophysiology. The lack of significant change in *IFIT3,* an interferon-stimulated gene, may imply that immune-related pathways are less central in this context, reinforcing the hypothesis that channelopathy plays a key role in this patient population.

Keywords: Cryptogenic epilepsy, channelopathy, gene expression, protein interaction

ÖZ

Amaç: Kriptojenik epilepsi, yapısal veya metabolik olarak tanımlanabilir bir nedenin bulunmadığı epilepsi alt türlerinden biridir ve bu durum hem tanı hem de tedavi sürecini zorlaştırmaktadır. Gen ekspresyonu gibi moleküler yaklaşımlar, hastalığın altında yatan mekanizmaları aydınlatma ve klinik stratejileri geliştirme potansiyeli taşımaktadır. Bu çalışmada, kriptojenik epilepsi tanısı almış bireylerin periferik kan örneklerinde *IFIT3* ve *KCNS3* genlerinin ekspresyon düzeyleri incelenmiştir.

Yöntem: Çalışmaya kriptojenik epilepsili 20 hasta ve yaş ve cinsiyet açısından eşleştirilmiş 20 sağlıklı birey dahil edilmiştir. Katılımcıların periferik kan örneklerinden toplam RNA izole edilerek tamamlayıcı DNA (cDNA) sentezlenmiştir. *IFIT3* ve *KCNS3* genlerinin göreli ekspresyon düzeylerini belirlemek amacıyla Gerçek Zamanlı Kantitatif PCR (RT-qPCR) uygulanmıştır. *ACTB* geni ise referans (housekeeping) gen olarak kullanılmıştır.

Bulgular: *KCNS3* geninin ekspresyon düzeyinin hasta grubunda sağlıklı kontrollere kıyasla anlamlı düzeyde yüksek olduğu bulunmuştur ($p < 0.0001$). *IFIT3* gen ekspresyonu açısından ise gruplar arasında istatistiksel olarak anlamlı bir fark gözlenmemiştir.

Sonuç: Potasyum kanalı alt birimini kodlayan *KCNS3* geninin artmış ekspresyonu, kriptojenik epilepside iyon kanalı bozukluklarının (kanalopatilerin) patofizyolojide rol oynayabileceğini düşündürmektedir. *IFIT3* geninde anlamlı bir değişiklik olmaması ise bağışıklıkla ilişkili yolların bu hasta grubunda daha az etkili olabileceğini ve kanalopati hipotezinin daha ön planda olduğunu desteklemektedir.

Anahtar Kelimeler: Kriptojenik epilepsi, kanalopati, gen ekspresyonu, protein etkileşimi

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Introduction

Epilepsy is a chronic neurological disease with heterogeneous presentations, the most common of which are recurrent seizures. Even with improved health care and reduced mortality, it remains a significant burden, affecting millions of people of all ages[1]. Diagnosis of epilepsy consists of evaluating the patient's medical history, including a report from an eyewitness, electroencephalography (EEG), neuroimaging studies, and laboratory tests. The lack of a distinct element suggesting the disease and the broad spectrum of manifestations both create a more challenging process[2].

Identifying the underlying cause of epilepsy is crucial in clinical settings, as it significantly impacts treatment, prognosis, and the disease's progression[3]. The International League Against Epilepsy (ILAE) has categorized epilepsies into six etiological groups: 1) genetic, 2) structural, 3) infectious, 4) metabolic, 5) immune, and 6) unknown causes[4]. Cryptogenic epilepsy is typically classified under the group of unknown etiologies in the most recent ILAE system. However, in earlier classifications, it referred to cases where there were no signs of previous brain injury or clear etiology[5]. While presumed to be symptomatic, the actual cause remains elusive. Cryptogenic epilepsy accounts for approximately 40% of adult-onset epilepsy cases, making it a significant contributor[6].

A deeper understanding of the disease's underlying mechanisms is crucial for improving diagnosis and management. Recent research has explored various contributing factors such as neurodegeneration, inflammation, brain trauma, and channelopathies[7]. Genetic studies on patients with epilepsy can enhance clinical decision-making by facilitating more accurate diagnoses, identifying risk factors, and informing prognoses. These studies can also offer valuable insights into the biological mechanisms of epilepsy[8].

The aim of this study was to establish whether cryptogenic epilepsy patients have a difference in terms of genetic expression profiles in comparison to healthy individuals. Through this approach, we targeted for a better understanding of the underlying causes of the disease and a better sight into the mechanisms leading to the disease. In our study, we investigated genes involved in pathways (channelopathy and inflammation) that play significant roles in the molecular pathogenesis of epilepsy, particularly cryptogenic epilepsy[8-10]. To explore the potential etiology of channelopathy, we studied the *KCNS3* gene, which encodes a subunit of the Potassium Voltage-Gated Channel Modifier Subfamily S Member 3. Potassium channels play a critical role in regulating neuronal excitability, and their dysfunction has been associated with various forms of epilepsy, including cryptogenic epilepsy [11]. As for the inflammation pathway, we studied the *IFIT3* gene, encoding Interferon-Induced Protein with Tetratricopeptide Repeats 3. Protein interactions of *IFIT3* and *KCNS3* gene products obtained from the STRING database are demonstrated in Figure 1.

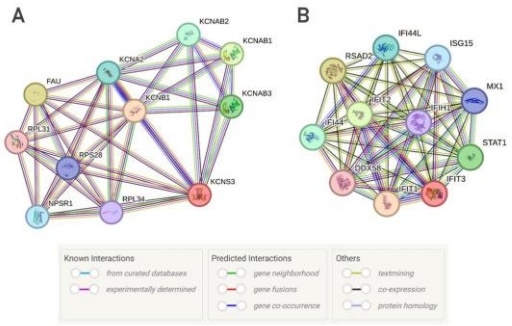

Figure 1. Protein interactions of A. KCNS3, B. IFIT3 obtained from STRING database (v12.0)

Methods

Patient Recruitment

The study consists of epilepsy patients without obvious etiology, who are long-term patients of Bezmialem Vakıf University, Faculty of Medicine, Neurology Department outpatient clinics, and healthy controls. The patients were screened in terms of; age, gender, time of onset of seizures, types of seizures, patient history, neurological examination, family history, 1.5 Tesla cranial MRI (Siemens Avanto, Erlangen, Germany), EEG findings, and the number of antiepileptics used. Patients over the age of eighteen, who were followed up by a clinician for at least a year, with no defined underlying cause for epilepsy, were included in the study. Pediatric patients (<18 years), patients with a known underlying factor leading to epilepsy (tumor, stroke, etc.), and patients with another systemic or neurological disease (diabetes, hypertension, neurodegenerative diseases, etc.) were excluded. The control group consisted of 20 healthy volunteers (11 males and 9 females) without any known neurological or systemic disorders. They were selected to be age- and sex-matched to the patient group to minimize demographic bias. Participants were informed about the study, and those who had given verbal and written consent were included in the study. According to power analysis, the optimal numbers of participants for patients (n1) and healthy controls (n2) were calculated as n1=n2=20 with a total of n=40, to obtain odds ratios of 1 and 2.16, with a power of 80%, a significance level of 0.05 and a 95% confidence interval.

RNA Isolation & Complementary DNA (cDNA) Conversion

From patients and controls who agreed to participate in the study, ten milliliters of peripheral blood samples were drawn into EDTA tubes. The collected samples were then transported to the laboratory on ice and were studied immediately. Total RNAs from the blood samples were isolated using the QIAamp RNA Blood Mini Kit (QIAGEN, Helden, Germany) according to the manufacturer's protocol. The quantitative assessment of

RNA purity was carried out using the Multiskan GO (Thermo Fisher Scientific, Boston, MA, USA) device. The RNA samples achieving an A260/A280 absorbance ratio between 1.9-2.1 were confirmed and stored at -80 °C until the next step. RNA samples were reverse transcribed to complementary DNA (cDNA), using the SensiFAST™ cDNA Synthesis Kit (Meridian Bioscience Inc., Cincinnati, OH, USA). cDNA samples were stored at -20 °C until further processing.

Quantitative Real-Time Polymerase Chain Reaction (qRT- PCR)

For the evaluation of messenger RNA (mRNA) expressions, the *ACTB* gene was designated as the housekeeping gene by the GeNorm program. Primers for *ACTB*, *KCNS3* and *IFIT3* genes were designed with the Primer3 v4.1.0 program. Primers for the *ACTB* (NM_001101.5) gene (Pf: CATCCGCAAAGACCTGTACG,Pr:CCTGCTTGCTGATCCACATC), *IFIT3* (NM_001549.6) gene (Pf:CACTTGGGCAGACTCTCAGA,Pr:AAACACACCTTCGCCCTTTC), and *KCNS3* (NM_002252) gene (Pf:AATCGCTACCAGGAACGCAA,Pr:CGATCTCCACTCCTTCCAGC). Following the optimization of annealing temperatures, RT- PCR was conducted for three genes (*ACTB, IFIT3, KCNS3*) for both patient samples and healthy controls. All samples were studied in duplicates. The SensiFAST™ SYBR No-ROX Kit P (Meridian Bioscience Inc., Cincinnati, OH, USA) was used and the experiments were conducted according to the manufacturer's instructors.

Statistical Analysis

All statistical analysis was performed using the Ct data retained from the qRT-PCR. The changes in expression levels were calculated using the delta-delta Ct method. Normalized expression levels ($2^{-\Delta Ct}$) were compared between the patient and control groups using appropriate statistical tests. Fold-change analysis using the $2^{-\Delta\Delta Ct}$ method was not applied in this study[12]. The data distribution was tested for normality with the Shapiro-Wilk test. Subsequently, a two-tailed Mann-Whitney U test was performed on independent samples to compare the expression levels between the patient and healthy control groups, using GraphPad Prism 8.0 (GraphPad Software, Inc., CA, USA). Results with a p-value of < 0.05 were considered statistically significant. Receiver operating characteristic (ROC) curves were demonstrated and area under the curve (AUC) values were calculated in order to evaluate the diagnostic potential of target genes.

Results

Clinical Assessments

The study consisted of 20 cryptogenic epilepsy patients and 20 healthy patients, who were matched to the sex distribution of patients (p <0.05). The patients who participated were between the ages of 19 to 62, with a mean of 29.2 ±10.14. Eleven were male and nine were female. Time of onset of seizures ranged from 2 to 40 years, with a mean of 18.25 ±10.45. Eighteen patients were suffering from focal seizures and two patients had focal seizures with secondary generalization. There were no findings in any of the patients' personal medical history, including their birth history or neurodevelopmental history. Two patients had consanguineous parents, but none of them had a family history of epilepsy, including those two patients. None of the patients had any findings on their 1.5 Tesla Cranial MRI (data not shown). Fourteen patients were using only one medication to control the seizure. The EEG recordings were normal in eleven of the patients. The recordings demonstrated unilateral temporal epileptiform abnormalities in five patients (Figure 2A), unilateral frontotemporal epileptiform abnormalities in two patients, unilateral frontal epileptiform abnormality in one patient and multifocal abnormalities (bilateral temporal and frontal abnormalities) in one patient (Figure 2B). Clinical characteristics of cryptogenic epilepsy patients included in the study are shown in Table 1.

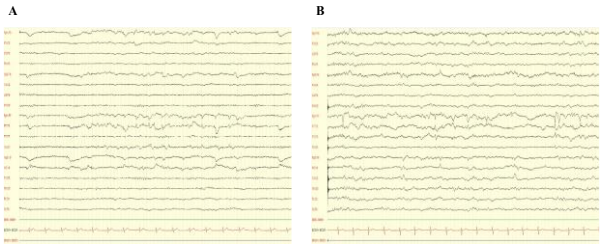

Figure 2. Demonstrated EEG findings in patients A. left temporal epileptiform anomaly, B. bilateral temporal and right frontal (multifocal) epileptiform anomaly

mRNA Expression Levels

The mean values of the cycle threshold (Ct) data obtained by the qRT-PCR study are presented in Table 2. Accordingly, the mRNA expression levels of the *KCNS3* gene were statistically higher in the patient group (p<0.0001) (Figure 3). However, levels of the *IFIT3* mRNA expression didn't show a statistical difference (Figure 4). The AUC value of *KCNS3* gene expression was determined as 0.9569 at the 95% confidence level by a ROC curve analysis (p<0.0001, Figure 5).

Table 2. The average expression levels of *KCNS3* and *IFIT3* genes

	***KCNS3* Ct**	***ACTB* Ct**	**ΔCt**	**$2^{-\Delta Ct}$**
Patients	34.25	21.68	12.57	0.00016
Controls	34.6	20.45	14.15	0.00005
	***IFIT3* Ct**	***ACTB* Ct**	**ΔCt**	**$2^{-\Delta Ct}$**
Patients	32.85	21.68	11.17	0.00062
Controls	31.63	20.45	11.18	0.00058

ΔCt values were calculated using *ACTB* as the reference gene. *KCNS3* expression was higher in patients, whereas *IFIT3* levels were comparable between groups.

Table 1. Patient Characteristics

Characteristic	Value
Age	
Range	19-62 years
Mean (SD)	29.2 (10.14)
Sex *(n of patients)*	
Male	11
Female	9
Time of onset of seizures *(average± SD)*	
Range	2-40 years
Mean (SD)	18.25 (10.45)
Types of seizures *(n of patients)*	
Focal Seizures	15
Tonic-Clonic Seizures	2
Unclassified	3
Patient history	
Medical history	No findings
Birth history	No findings
Neurodevelopmental history	No findings
Family history	
History of epilepsy	No findings
Consanguineous parents	2
Number of antiepileptics used *(n of patients)*	
One medication	14
Multiple medications	6
Cranial MRI findings (1.5 Tesla)	No findings
EEG findings *(n of patients)*	
Normal EEG	11
Unilateral temporal epileptic abnormalities	5
Unilateral frontotemporal epileptic abnormalities	2
Unilateral frontal epileptic abnormalities	1
Multifocal epileptic abnormalities	1 (bilateral temporal + unilateral frontal findings)

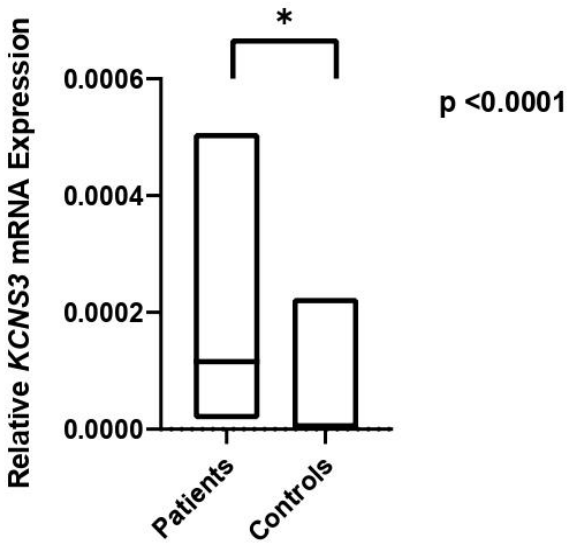

Figure 3. Comparison of relative mRNA expression levels of target gene *KCNS3*

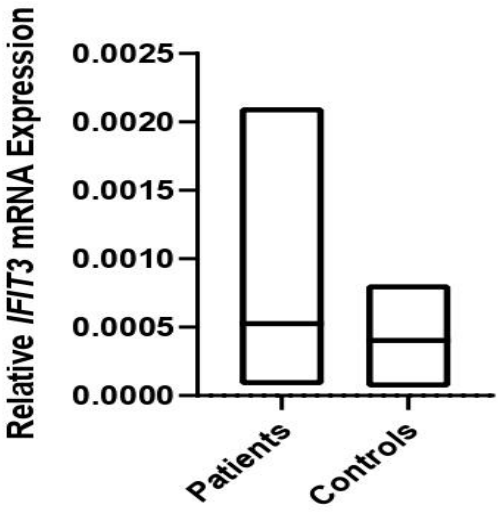

Figure 4. Comparison of relative mRNA expression levels of target gene *IFIT3*

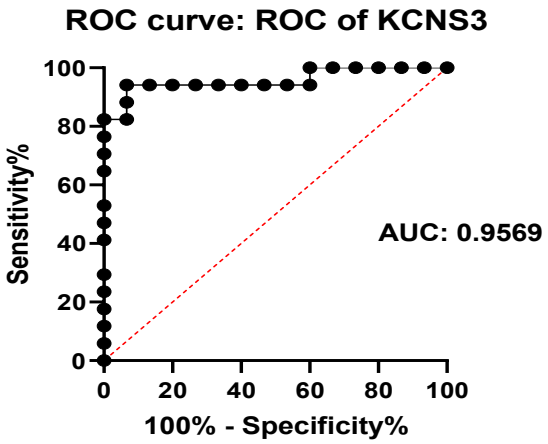

Figure 5. ROC curve analysis results reveal the diagnostic performance of *KCNS3* gene

Discussion

Epilepsy is the second most prevalent neurological disorder, following stroke[13]. It is estimated that around 0.8% of the population experiences some form of epilepsy. In approximately 30% of cases, epilepsy is linked to a specific brain injury that has caused the condition, known as symptomatic epilepsy[14]. Another 30% of patients are considered to have presumed symptomatic epilepsy, termed 'cryptogenic epilepsy,' where it is suspected that an underlying brain pathology exists but has not yet been detected with current methods[15].

Epileptogenesis refers to the sequence of molecular and cellular alterations triggered by an initial brain injury, eventually leading to spontaneous seizures. These changes include neurodegeneration, neurogenesis (the creation of new neurons), axonal growth and damage, dendritic restructuring, gliosis, the infiltration of inflammatory cells, angiogenesis (new blood vessel formation), modifications to the extracellular matrix, and the development of acquired channelopathies[16,17]. Various ion channels play a role in the regulation of current flow. For example, voltage-gated ion channels undergo conformational shifts, alternating between open and closed states. These shifts are driven by changes in membrane electrical potentials, controlling the selective ion movement across membranes. Therefore, it is evident that dysfunction in these ion channels can result in brain hyperexcitability[18,19]. Mutations in genes such as *SCN1A, SCN2A, SCN8A* (which encode voltage-gated sodium channels), and *CACNA1A* (which encodes a voltage-gated calcium channel) are associated with various forms of epilepsy. For example, loss-of-function mutations in *SCN1A* can lead to hyperexcitability in conditions like Dravet syndrome, while pathogenic variants in *CACNA1A* have been linked to childhood absence epilepsy. These genetic alterations highlight the role of channelopathies in the pathogenesis

of epilepsy[20,21]. Voltage gated potassium channels (Kv) are activated by membrane depolarization, allowing potassium to exit the cell, which helps return the membrane to its resting state[22]. These channels, found in axons, play a crucial role in delaying axonal action potentials[23]. The *KCNT1* gene encodes the KCa4.1 subunit, a sodium-activated potassium channel, associated with up to 50% of cases of epilepsy of infancy with migrating focal seizures (EIMFS)[24]. *De novo* pathogenic variants in the *KCNQ2* gene are associated with self-limited neonatal epilepsy (SeLNE) and may also be inherited in an autosomal dominant manner, leading to self-limited familial neonatal epilepsy (SeLFNE)[25].

Among these voltage-gated potassium channels, the epilepsy-related gene we examined in this study is *KCNS3*, which encodes the Potassium Voltage-Gated Channel Modifier Subfamily S Member 3. *KCNS3* operates within the same network as the *CACNA1* calcium channel protein gene and the *KCNB1* gene. While KCNS3 does not form functional channels on its own, it can create functional heterotetrameric channels by pairing with KCNB1. This interaction modulates the activation and deactivation rates of the delayed rectifier voltage-gated potassium channel, KCNB1[26]. Additionally, according to KEGG pathway analysis, the gene is associated with the serotonergic pathway via *SLC6A4* and has been reported in the literature to influence neuronal excitability through serotonin signaling[27]. A wide range of mechanisms have been studied to identify the causes of epilepsy. Among the previously explored mechanisms, our study aimed to further investigate potential genetic disturbances in these patients, with a particular focus on inflammation and ion channel disorders. Accordingly, our study found that *KCNS3* gene expression is elevated in the cryptogenic epilepsy group compared to the control group. This suggests that the altered expression of *KCNS3* could influence neuronal excitability through the regulation of potassium channel proteins and may play a role in the pathogenesis of cryptogenic epilepsy by impacting the integration of energy metabolism. Additionally, the change in *KCNS3* expression might affect its interacting partners, *KCNB1* and *SLC6A4*, which are involved in potassium channel functionality and serotonin signaling, respectively. Therefore, we propose that the altered expression of *KCNS3* may contribute to epilepsy through these mechanisms.

To this end, we also examined the levels of the *IFIT3* gene in patients with cryptogenic epilepsy and in controls. *IFIT3*, known as the interferon-inducible protein with tetratricopeptide repeats 3, is a key member of both the IFIT family and the interferon-stimulated genes family. It shares typical features of the IFIT family in terms of gene and protein structures and can be activated through the classical PRRs-IFN-JAK/STAT pathway[28]. According to the literature, via the JAK-STAT signaling pathway, *IFIT3* gene expression is found to be elevated in viral infections[29]. In a previous study comparing the differential genetic expression profiles of epilepsies with different origins, *IFIT3* expression levels were found to be higher in cryptogenic epilepsy patients compared to those with symptomatic or idiopathic epilepsies[30]. However, in terms of differentiating these patients from those of the healthy population, despite the observed difference of distribution between patients and healthy individuals, a statistically significant distinction of *IFIT3* expression levels could not be identified.

Additionally, in our study investigating the contribution of inflammation and channelopathy to the etiology of epilepsy, we found that the levels of the *KCNS3* gene are elevated in patients with cryptogenic epilepsy. Various K-channel Various potassium channel (K^+ channel) subunit genes have been reported to be altered in epileptic patients in the literature[31]. However, to our knowledge, no prior studies have examined the levels of the *KCNS3* gene, which we identified as being higher in the cryptogenic epilepsy group. We suggest that this gene may play a role in the pathogenesis of the disease and could serve as a potential biomarker for cryptogenic epilepsy in future research. As known, ROC curve analysis calculates the sensitivity and specificity of target molecules and is a useful graphical tool for assessing the diagnostic value of biomarkers[32]. The current study examined *KCNS3* as a diagnostic biomarker by calculating the AUC value using ROC curve analysis. Consequently, *KCNS3* may be a helpful diagnostic biomarker for cryptogenic epilepsy.

Moreover, our findings suggest that potassium (K^+) related channelopathy may be a more plausible underlying mechanism in these patients, rather than an inflammatory process. A larger cohort study is needed to better elucidate this result though.

Conclusion

In conclusion, our study highlights the potential role of the *KCNS3* gene in the pathogenesis of cryptogenic epilepsy, suggesting that alterations in potassium channel function, rather than inflammatory processes, may contribute to the disease mechanism. The observed elevation in *KCNS3* gene expression in cryptogenic epilepsy patients underscores its potential as a biomarker, warranting further investigation in larger cohorts. While the *IFIT3* gene showed differential expression patterns, its role in distinguishing cryptogenic epilepsy from other forms of epilepsy and from healthy individuals remains inconclusive. Future studies focusing on the *KCNS3* gene and its network, as well as additional research into the genetic profiles of cryptogenic epilepsy patients, will be essential to better understand the underlying mechanisms and to develop targeted therapeutic strategies.

Ethical Approval

All procedures involving human participants were conducted in accordance with the ethical principles outlined in the Declaration of Helsinki (1964) and its subsequent amendments, as well as the relevant guidelines set forth by national and institutional research

ethics boards. The study protocol was reviewed and approved by the Ethics Committee of Bezmialem Vakıf University (Approval No: 9/3, Date: 05/11/2022).

Conflict of Interest

The authors have no conflict of interest to declare.

Author Contributions

E.Y. conceptualized the research idea, designed the study, conducted data analysis, and interpreted the findings. G.O. and F.I.U. were responsible for data acquisition. The initial draft of the manuscript was prepared by G.O. and N.D.K. Critical revision for important intellectual content was carried out by G.O., N.D.K., S.S., and E.Y. All authors reviewed and approved the final version of the manuscript to be published.

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