KCC2 rs2297201 Gene Polymorphism Might be a Predictive Genetic Marker of Febrile Seizures

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Abstract
Introduction: Febrile seizures (FS) are the most common neurological disease in childhood. The etiology of FS is the subject of numerous studies including studies regarding genetic predisposition. Aim: The aim of the study was to analyze the association of TRPV1 rs222747 and KCC2 rs2297201 gene polymorphisms with the occurrence of FS. Materials and Methods: The study included 112 patients diagnosed with FS classified as simple febrile seizures (SFS) or complex febrile seizures (CFS). We analyzed selected polymorphisms of KCC2 and TRPV1 genes using the Real-time PCR method. Results: The CT and TT genotypes of the rs2297201 polymorphism of the KCC2 gene are significantly more common in the group of children with FS than the control group (p = .002) as well as the allele T of this polymorphism (p = .045). Additionally, genotypes CT and TT of the rs2297201 polymorphism of the KCC2 gene were more frequent in the group of children with CFS compared to the control group (p < .001). Different genotypes and alleles of the rs222747 TRPV1 gene polymorphism were not associated with the occurrence of febrile seizures or epilepsy, nor were associated with the occurrence of a particular type of febrile seizure (p = .252). Conclusion: These results indicate that the CT and TT genotypes, as well as the T allele of rs2297201 polymorphism of the KCC2 gene, could be a predisposing factor for the FS, as well as the occurrence of CFS.

Keywords
febrile seizure, gene polymorphism, KCC2 gene, TRPV1 gene

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Introduction
Febrile seizures (FS) are the most common neurological disease in childhood. Due to a high incidence of the disease, as well as the age that enables the repetition, this disease represents a particular challenge in pediatric practice (Xixis et al., 2021).

The International League Against Epilepsy (ILAE) defines FS as seizures occurring in children older than 6 months of age, in an association with a febrile illness which is not caused by a central nervous system (CNS) infection, without prior neonatal or afebrile seizures, and which do not meet the criteria for other acute symptomatic attacks (Capovilla et al., 2009).

The diagnosis of FS is based on the patient’s anamnesis and clinical examination (Xixis et al., 2021), but it is very important to identify the underlying disease that caused the fever. Based on the clinical picture, FS can be further divided into:

Simple Febrile Seizures (SFS) are characterized as primarily generalized seizures that last less than 15 min and do not recur within 24 h. They occur at the age span of 6 months to 5 years, in children without neurological deficit (children

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with normal psychomotor development and without the previous occurrence of afebrile seizures (Capovilla et al., 2009).

Complex Febrile Seizures (CFS) are manifested by focal seizures or status epilepticus (equal to or longer than 15 min) at a body temperature lower than 38°C. They occur outside the typical age span and recur within 24 h (they can occur three or more times). The EEG test results during or between seizures may be altered. The group of complex febrile seizures includes febrile seizures in children with previous neurological deficits and seizures with postictal neurological abnormalities (Capovilla et al., 2009).

The etiology of FS is complex and it is still the subject of numerous studies. The topic is extremely demanding due to the extreme complexity of this disease, which is caused by both genetic and environmental factors. Increased brain temperature is known to directly affect the sensitivity of ion channels, which might be sufficient to generate febrile seizures. (Xixix et al., 2021) Therefore, in the studies of the genes potentially involved in the development of FS, special attention is paid to genes encoding ion channels that play an important role in the excitability of the immature brain, including the KCC2 and TRPV1 genes (Buttila et al., 2018; Hao et al., 2020; Kong et al., 2019; Raol et al., 2019).

Current knowledge about the possible functions of the KCC2 channel is primarily based on studies conducted on animal models (Côme et al., 2020; Raol et al., 2019). The KCC2 channel is responsible for the regulation of the gradient of chloride ions in neurons, i.e. maintains low concentrations of Cl− within the cell (Côme et al., 2019, 2020; Raol et al., 2019). It is a key mediator of synaptic inhibition (Ben-Ari et al., 2012; Côme et al., 2019, 2020). During brain maturation, the level of KCC2 expression increases, resulting in a small amount of Cl− in the mature neuron. The studies on animals have shown that mice without the KCC2 gene have frequent generalized seizures and die soon after birth (Hübner et al., 2001; Woo et al., 2002), while those with a heterozygous deletion of the KCC2 gene had a lowered threshold for seizures (Woo et al., 2002). Mutations in this gene have been found in the human population in children with previous neurological deficits and seizures with postictal neurological abnormalities (Capovilla et al., 2009).

Materials and Methods
This study included 112 patients diagnosed with FS, aged 1 to 14 years, hospitalized or treated at the University Children’s Clinic in Belgrade. The research was approved by the Ethics Committee of the Faculty of Medicine, University of Belgrade. The study was planned in accordance with the ethical standards given in the Declaration of Helsinki (revised version from 1983). For each patient, the FS was diagnosed based on the ILAE definition (Capovilla et al., 2009). The main criterion for inclusion in the study was the occurrence of FS without the previous onset of afebrile seizures. The control group consisted of 109 children older than 5 years. The control group was selected from preschool and school dispensaries in the territory of Belgrade by the method of random numbers. The subjects were selected from the group of children who did not have the occurrence of febrile and afebrile seizures in their anamnesis. They were all of the similar gender distribution, from the same background, and had similar socio-epidemiological characteristics as the patients who were included in the study. Parents of examinees gave written consent for participation in the study. All children with the disease and individuals from the control group are members of the same population (Serbian). All relevant anamnestic data were used from patient histories or ambulance records. After a neurological examination (performed by a neurologist with over 5 years of experience), a blood sample was taken from the subjects with the aim to isolate DNA. We did not have related individuals among FS patients.

The patients were divided into groups, based on the type of FS and according to the definition of ILEA (Capovilla et al., 2009):

1. The group with simple febrile seizure (SFS) (N = 68)
2. The group with complex febrile seizure (CFS) (N = 44)

In addition to the FS, there was also a diagnosis of epilepsy in some patients (EFS) (N = 20).
The diagnosis of epilepsy was made based on the occurrence of at least two afebrile seizures (excluding the occurrence of one afebrile seizure and neonatal seizures) followed by specific EEG epileptiform changes. Patients with diagnosed intracranial infection or metabolic imbalance, as well as patients with incomplete data, were not included in the study. In the group of children with FS, 78 were younger than 5 years, 14 had FS after typical age, and 20 had epilepsy. Clinical and demographical characteristics of patients and controls are presented in Table 1.

DNA was isolated from peripheral blood leukocytes using the salting out-method, according to standard protocol (Miller et al., 1988). Detection of selected KCC2 and TRPV1 gene polymorphism genotypes was performed by real-time PCR method, using commercial TaqMan SNP genotyping assays (Thermo Fisher Scientific, Walthman, MA, USA) and Real-Time PCR machine AB7500 (Applied biosystems, Foster, CA, USA). This research is registered on the ClinicalTrials.gov ID NCT04368936.

**Statistical Analysis**

In order to analyze differences in genotypes and alleles frequencies between patients’ groups and between patients’ and control, we have used the chi-square test ($\chi^2$) or, when it was appropriate, Fisher’s exact test. Statistical analyses were performed in IBM SPSS 20 program. We were not able to perform a real-time PCR reaction for the same number of people for both polymorphisms studied.

**Results**

**The Analysis of the rs2297201 KCC2 (SLC12A5) Gene Polymorphism**

Out of the total number of children in whom the genotypes of the rs2297201 KCC2 gene polymorphism were successfully analyzed, 112 had FS, and 107 were healthy subjects. Among children with FS, the most common was CC genotype (78 children (69.64%)). The CT (32 child (28.6%)) or TT genotypes (2 children (1.8%)) were present in 34 children (30.36%). In the control group, CC genotype was the most common (93 children (86.92%)), while 14 children (13.08%) had the CT genotype, and the TT genotype was not detected. (Table 2).

All analyzed genotypes in children with FS and control group are in Hardy-Weinberg equilibrium. The frequencies of genotypes and alleles of the rs2297201 polymorphism and their differences between the FS group and the control group are presented in Tables 2 and 3. The T allele was statistically significantly more prevalent in children with FS (16.07%) compared to the control group (6.54%), ($\chi^2 = 4.017\; df = 1\; p = .045$) (Table 3).

The genotypes CT and TT of the rs2297201 polymorphism of the KCC2 gene are significantly more common in children with FS (30.36%) in comparison to the control group (13.08%) ($\chi^2 = 9.54;\; p = .002$) (Table 2).

The genotypes CT and TT of the rs2297201 polymorphism of the KCC2 gene were detected in 6 children (30.0%) in the group of children with epilepsy (EFS) and 14 children (13.08%) in the control group. These difference did not reach statistical significance ($\chi^2 = 2.471;\; p = .116$) (Table 2). There was a statistically significant difference in the frequency of the genetic variants of the rs2297201 KCC2 gene polymorphism in the group of children with CFS and the control group. The genotypes CT and TT were significantly more common in the group of children with CFS (40.9%) compared to the control group (13.08%) ($\chi^2 = 14.45;\; p < .001$) (Table 2).

No significant difference in the frequency of rs2297201 genotype of the KCC2 gene polymorphism was observed between the children with SFS and the control group ($\chi^2 = 3.194;\; p = .074$) (Table 2).

Of the total number of children with FS in whom the rs2297201 KCC2 gene polymorphism was analyzed, 44 (39.3%) children had CFS and 68 (60.7%) children had SFS. The CC genotype of polymorphism rs2297201 of the KCC2 gene was present in 26 (59.1%) children with CFS and 52 (76.5%) children with SFS. There is no significant difference in the frequency of the examined polymorphism between children with SFS and CFS ($\chi^2 = 3.817;\; p = .051$) (Table 2).

**The Analysis of Frequency of rs222747 TRPV1 Gene Polymorphism**

The rs222747 genotypes of the TRPV1 gene polymorphism were determined in 111 children from the FS group and 109 children from the control group. Among children with FS, the CC genotype was detected in 10 subjects (9.0%), the
CG genotype in 37 (33.3%), and 64 (57.7%) children had the GG genotype. In the control group GG genotype was detected in 64 children (58.7%), CG in 41 (37.6%), and 4 children (3.7%) had the CC genotype (Table 4).

The allele C was detected in 25.68% of children with FS and 22.48% of children in the control group. There is no statistically significant difference in TRPV1 gene rs222747 polymorphism alleles distribution between these group ($\chi^2 = 0.161$ df = 1 p = .688) (Table 5).

There was no statistically significant difference in the frequencies of genotypes of the rs222747 polymorphism of TRPV1 gene between the group of children with FS and the control group ($\chi^2 = 2.759$; $p = .252$) (Table 4).

Out of 20 children with epilepsy, 3 (15.0%) had the CC genotype, 8 (40.0%) had the CG genotype and 9 children (45.0%) had the GG genotype (Table 4). No statistically significant difference in the frequencies of rs222747 genotypes of the TRPV1 gene between the group of children with epilepsy (EFS) and the control group of children (CN) could be found ($\chi^2 = 4.585$; $p = .101$) (Table 4).

Out of the 111 children with FS successfully genotyped for the rs222747 polymorphism of the TRPV1 gene, 44 (39.6%) had CFS and 67 had SFS (60.4%). Four (9.1%) children with CFS and six (9.0%) children with SFS had CC genotype (Table 4). Thirty-seven children with FS had the CG genotype, out of which 12 (27.3%) experienced CFS and 25 (37.3%) had SFS. Of all tested children, 28 (63.6%) with CFS and 36 (53.7%) children with SFS had a GG genotype. There is no statistically significant difference in the frequencies of the genotypes of rs222747 polymorphism of the TRPV1 gene between SFS and CFS types of febrile seizures ($\chi^2 = 1.256$; $p = .534$) (Table 4).

There is no statistically significant difference in the frequencies of genotypes of the rs222747 TRPV1 gene polymorphism between the group of children with CFS and SFS and the control group ($\chi^2 = 2.856$; $p = .240$) ($\chi^2 = 2.223$; $p = .329$) (Table 4).

### Discussion

There is an age-specific response of neurons after binding of GABA to receptors in the CNS. This difference between a mature and an immature neuron is reflected in the response of the GABAA receptor after GABA neurotransmitter binding. After stimulation of GABAA receptors, in an immature neuron occurs depolarization and consequent neuronal excitation, while mature neuron’s response to the stimulation of these receptors is repolarization. The cause of this difference is increased KCC2 expression during the neurodevelopmental period, which is an important factor for the functional development of the brain (Liu et al., 2020; Rakhade and Jensen, 2009). Depolarization in immature neurons mediated by GABA transmitter is the result of altered expression of the KCC2 transporter at an early age. It has been shown that depolarization of neurons after receptor stimulation by GABA neurotransmitters increases the likelihood of excitatory-inhibitory balance disorders and leads to a reduced convulsive threshold in children (Liu et al., 2020; Rakhade and Jensen, 2009). As FS are age-specific and appear up to 5 years of age, the role of $KCC2$ genes in the development of epilepsy and FS has been analyzed in recent years (Duy et al., 2019; Puskarjov et al., 2014; Silayeva et al., 2015). Although the $KCC2$ gene is among the most intolerant genes in the human population in terms of variations, which is the reason why polymorphisms in this gene could contribute to the phenotypic expression of FS (http://www.chgv.org/GenicIntolerance), the data regarding the role of functional polymorphisms in the $KCC2$ gene in the FS development are very few. For example, there is a $KCC2$-dependent insulin release and two studies found an association of the rs2297201 polymorphism of the $KCC2$ gene and diabetes mellitus (Bento et al., 2008; Lewis et al., 2010).

Additionally, polymorphisms in the sodium channel genes $SCN1A$ (rs6432860) and $SCN2A$ (rs3769955) have already been associated with febrile seizures that were a serious adverse event following measles, mumps, and rubella.
(MMR) vaccination in a single GWAS study (Feenstra et al., 2014). Previous research have found several changes in the KCC2 gene in patients with FS. There is a description of a rare variant of the KCC2 gene (KCC2-R952H) linked to FS in one Australian family. The in vitro functional studies showed a significantly reduced surface expression of the KCC2 mutated protein (61% wild-type), as well as a reduced Cl⁻ release from neurons. Thus, the mutated protein is associated with insufficient Cl⁻ output from neurons, which causes reduced hyperpolarization, and could be a trigger for the occurrence of FS (Puskarjov et al., 2014). Kahle et al. (2014) identified the same variant of the KCC2 gene (R952H) in a population of people of French-Canadian descent who had idiopathic generalized epilepsy. Additionally, these authors described a heterozygous R1049C variant of the KCC2 gene (Kahle et al., 2014) and showed that in vitro this variant affects the exit capacities of Cl⁻ ions from neurons, which could lead to impaired KCC2 channel function and contributes to the development of epilepsy (Kahle et al., 2014).

Our results show that there is a significant difference in the genotype frequencies of the KCC2 gene rs2297201 polymorphism (χ² = 9.54; p = .002) between the group of children with FS and the control group of children (Table 2). The CT and TT genotypes are significantly more common in children with FS compared to the control group of children. The T allele was statistically significantly more prevalent in children with FS compared to the control group (χ² = 4.017 df = 1 p = .045) (Table 3). The obtained results indicate that the CT and TT genotypes, as well as the T allele of rs2297201 polymorphism of the KCC2 gene, could be a predisposing factor for the FS, but for final conclusions, further research should be conducted on a larger sample, as well as on different populations.

In a minority of cases, FS precede later development of epilepsy. The cause of mesial temporal lobe epilepsy with hippocampal sclerosis is unknown, but there is an association with childhood febrile seizures (Beker-Acay et al., 2017). A meta-analysis by Kasperaviciute D. revealed a genome-wide significant association of mesial temporal lobe epilepsy with hippocampal sclerosis with febrile seizures and rs7587026 within an intron of the SCN1A gene (Kasperaviciute et al., 2013). This result was confirmed by Skotte L. et al. (Skotte et al., 2022). Furthermore, a genome-wide mega-analysis that included 15,212 individuals with epilepsy and 29,677 controls revealed the 21 most likely epilepsy associated genes, including genes coding for ion-channel subunits (The
International League Against Epilepsy Consortium on Complex Epilepsies, 2018).

However, there is no significant difference in frequencies of the analyzed genotypes of the KCC2 gene between the group of children with epilepsy and the control group in our sample ($\chi^2 = 2.471; p = .116$) (Table 2). As KCC2 channel dysfunction affects brain excitability, we expected differences between these groups, especially regarding the literature data that describes a mutation in this gene in children with early infantile epileptic encephalopathy from two unrelated families (Stodberg et al., 2015). As the KCC2 channel has decreased expression during brain development, which correlates with age-specific FS occurrence, we hypothesize that even small changes in protein function may cause a reduced convulsive threshold at this age. But, in respect to our results, we can assume that the examined polymorphism of the KCC2 gene has no role in the development of epilepsy, i.e. it is the evidence of a different mechanism of the occurrence of FS and epilepsy. It is important to note that the increase in body temperature affects the reduced function of this channel (Hartmann and Nothwang, 2011), so maybe fever in combination with this polymorphism could have a cumulative effect on the occurrence of FS. Additionally, we should not ignore that small number of children with EFS in our study could affected results.

The results of previous research indicate that different genetic factors influence the possible CFS or SFS manifestation (Eckhaus et al., 2013). We have found that the subgroup of children with CFS shows highly significant difference in the frequencies of the rs222747 KCC2 genotypes compared to the control group ($\chi^2 = 14.454; p < .001$) (Table 2). The difference between the control group and SFS was noticed but was not significant ($\chi^2 = 3.194; p = .074$) (Table 2). Based on our results, there could be a positive association of CT and TT genotypes of this polymorphism with CFS in children, and maybe the occurrence of a specific type of FS could depend on the interaction of different genes and environmental risk factors.

The TRPV1 gene encodes the TRPV1 channel which is predominantly present in the brain. These channels control various neuronal and glial functions, such as thermoregulation and synaptic transmission (Garami et al., 2020; Storozhuk et al., 2019). Thermal hyperpnea, an increase in tidal volume and breathing frequency, is associated with hyperthermia in immature rats and results in respiratory alkalosis that triggers FS. TRPV1 plays an important role in the respiratory response to hyperthermia, and its activation has already been associated with the increased susceptibility to FS in immature rats (Barrett et al., 2018).

The TRPV1 channel is a ligand-dependent nonselective cation channel. It is composed of 6 transmembrane domains with intracellular amino- and a carboxyl terminus. There is a segment at the amino-terminus with at least three ankyrin repeating segments that play an important role in channel function (Luti and Rosenbaum, 2019). SNP rs222747 is localized in the region coding for the ankyrin repeat domain and the GG variant is associated with a higher maximal response to TRPV1 channel agonists (Xu et al., 2007). So far, rs222747 polymorphism of this gene has been associated with susceptibility to type 1 diabetes mellitus (Sadeh et al., 2013), cold hypaesthesia in subgroup of patients with chronic pain (Binder et al., 2011), and dyspepsia (Triantafyllou et al., 2017).

Previous studies in the human population have shown that homozygotes for the GG genotype of this polymorphism play a role in increased synaptic transmission of glutamate and affect neuronal excitability (Mori et al., 2012), and we expected a higher frequency of G allele in people with FS, especially in the group of children with FS who have developed epilepsy. No significant difference was observed in the frequencies of genotypes of rs222747 TRPV1 gene polymorphism between the group of children with FS and the control group ($\chi^2 = 2.759; p = .252$) (Table 4). There is also no significant difference in the frequencies of genotypes of the rs222747 TRPV1 gene polymorphism ($\chi^2 = 4.585; p = .101$) between the group of children with epilepsy (EFS) and the control group (Table 4). The difference in the frequencies of genotypes of rs222747 TRPV1 gene polymorphism between the group of children with CFS and the control group ($\chi^2 = 2.856; p = .240$), as well as between children with SFS and the control group ($\chi^2 = 2.223; p = .329$) is not statistically significant (Table 4).

The difference in the frequencies of genotypes and alleles of the examined TRPV1 gene polymorphism among children with SFS and CFS was also not significant ($p > .05$) (Tables 4 and 5). These results could support the assumption that this polymorphism does not represent a risk factor for the development of FS, nor for the manifestation of a certain type of FS. However, these results can possibly be affected by the small sample included in the study, which is why they should be considered with caution.

Conclusion

The obtained results indicate that the CT and especially TT genotypes, as well as the T allele of rs2297201 polymorphism of the KCC2 gene, could be a predisposing factor for the FS. The CT and TT genotypes could be associated with the appearance of CFS, until the connection between this polymorphism and epilepsy was detected. However, the different genotypes and alleles of the rs222747 TRPV1 gene polymorphism are not associated with the occurrence of febrile seizures or epilepsy, nor are they associated with the occurrence of a particular type of febrile seizure.

Data Availability

The data used to support the findings of this study are included within the article.
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Declaration of Conflicting Interests
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