Polymorphism of ABCB1/MDR1 C3435T in Children and Adolescents with Partial Epilepsy is due to Different Criteria for Drug Resistance – Preliminary Results

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Background: The diagnosis of "drug resistance" in epilepsy can be defined and interpreted in various ways. This may be due to discrepant definitions of drug resistance to pharmacotherapy.
The aim of our study was to investigate the relationship between C3435T polymorphism of the MDR1 gene and drug resistance in epilepsy with the consideration of 4 different criteria for qualification to groups sensitive and resistant to applied pharmacotherapy.

Material/Methods: Evaluation of C3435T polymorphism of MDR1/ABCB1 gene was conducted on a group of 82 white children and young adolescents up to 18 years old. While qualifying the patients to the group of sensitive or drug resistant, the following 4 definitions of drug resistance were applied: the ILAE's, Appleton's, Siddiqui's, and Berg's.

Results: A detailed analysis of genotypes of the MDR1 gene did not show any significant discrepancies between the groups of patients resistant and sensitive to antiepileptic drugs (AEDs) in 4 consecutive comparisons taking into consideration various criteria of sensitivity and resistance to pharmacotherapy.

Conclusions: The obtained results clearly confirm the lack of a connection between the occurrence of drug-resistant epilepsy and C435T polymorphism of the MDR1 gene irrespective of the definition of drug resistance applied to the patient.

MeSH Keywords: **Adolescent • Child • Epilepsies, Partial • Genes, MDR • Patient Selection**

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Backgr000nd

Epilepsy, which is one of the most common conditions of the nervous system, is particularly important to children and youth because the symptoms occur before the age of 18 in most patients [1,2]. Almost 30% of cases are reported by 5 years of age [3,4]. Epilepsy that which responds poorly to treatment has a serious impact on the further development of the patient [5].

In recent years there has been a significant advance in the rational therapy of this condition, however, despite the introduction of many new anti-epileptic drugs (AEDs), it is assumed that 30–40% of patients have drug-resistant epilepsy [6,7].

It is unknown why there is a different efficiency of applied treatment in 2 different patients with the same type of seizures and the same clinical condition [8]. Therefore, it has been attempted to determine the predictors of drug resistance. The mechanisms behind drug-resistant epilepsy are complex and are not fully understood. The pathobiology of drug resistance in epilepsy depends on the following 4 mechanisms: pharmacokinetic, pharmacodynamic, homeostatic, and genetic [9]. It has been proven that genetic predispositions are important in etiology of the disease. Alonso-Cerezo et al. [10] confirmed an increased risk of the occurrence of epilepsy in relatives who showed resistance to anti-epileptic drugs. This indirectly confirms an assumption that genetic background predisposes a person to the disease and may also play a major role in patient response to treatment with anti-epileptic drugs.

As research suggests, various genes are involved in the incidences of drug resistance in epilepsy [11]. The latest research of Kim et al. [11] on gene-gene interaction in epilepsy indicates a relation of 4 SNPs (SNPs GABRA1, EAAT3, and GAT3) as the best model for predicting drug resistance. Nonetheless, many issues relating to this problem remain unsolved.

In predicting drug-resistant epilepsy, a key role is assigned to polymorphisms of the MDR1 gene (subfamily B of the ABC transporters, known as ABCB1) coding for P-glycoprotein (P-gp) [12]. MDR1 is expressed in many tissues, including the epithelium of the choroid plexus and in the endothelial membrane of cerebral vessels (blood-cerebrospinal fluid and blood-brain barriers [BBB]) [13]. It participates in the transport of anti-epileptic drugs such as phenytoin, phenobarbital, oxcarbamazepine, lamotrigine, felbamate, and gabapentin [14,15]. In the case of some AEDs (CBZ, VPA, TPM, and Levetiracetam) [16–18], the role of P-gp in the reduction of the neuronal pool of the drugs is controversial [19]. Its overexpression along the blood-brain barrier reduces the amount of AEDs getting into the cerebral tissues of the patients [20]. Compared to endothelial cells in normal brain tissue, endothelial cells in epileptic brain tissue showed higher P-gp expression levels [21].

C3435T polymorphism in exon 26 is one of the best known polymorphisms of the ABCB1 gene and its relation with drug resistance in epilepsies has been thoroughly researched. It is a silent polymorphism that does not lead to changes in the sequence of amino acids, but it does result in changes in P-gp expression and the pharmacokinetics of drugs [22–24, review in 17]. Siddiqui et al. [8] reported that CC genotype in the MDR1 C3435T gene may be strongly linked to a complete lack of response to anti-epileptic drug treatment. The above-mentioned results were confirmed by other research teams [24–27]. Yet some authors think that it is the 3435TT genotype of MDR1 gene that predisposes to the occurrence of drug-resistant epilepsy [28,29]. Other researchers reported no relation between C3435T MDR1 polymorphism and epilepsy. This proves that the results of research on the connection between polymorphism of the MDR1 gene and drug-resistant epilepsy are contradictory [15,20,30]. Our research also does not confirm the link between C3435T MDR1 polymorphism and epilepsy [31].

Defining drug resistance is also contentious [6,32–34]. The assumption by physicians of a particular definition often depends on the physician's personal opinion and experience [26]. The main condition is the persistence of seizures despite the application of AEDs appropriate for the type of seizures, while the number of ineffective drugs may vary. Most commonly this involves 2 [34–36] or 3 drugs [16], but there are criteria mentioning 4, 5, or even 7 AEDs [8,37]. Some criteria include the time factor, and others focus on the number, type, or frequency of epileptic seizures [8,16,32,33,37–40]. In 2010 the ILAE Commission of Therapeutic Strategies introduced the latest definition of drug-resistant epilepsy, which is still only a partial, compromise solution [26].

Considering the lack of consensus on the definition of drug resistance, it has been assumed that the discrepant results with regard to the connection of MDR1 polymorphism and epilepsy not responding to treatment with AEDs come from the various definitions of drug resistance suggested by scientists and, subsequently qualifying patients as those sensitive or resistant to treatment. Therefore, we conducted an independent, single-center analysis of the connection between the C3435T polymorphism of the MDR1 gene and a choice of drug-resistant epilepsy definition determined in accordance with 4 different criteria defining the response to pharmacotherapy.

Material and Methods

Patients

The study involved 82 white children and adolescents with epilepsy, all of them Polish (European continental origin, Silesian

Table 1. Definitions of drug resistant epilepsy.

Author	Definition	
Appleton and Gibbs [37]	Recurrence of epileptic seizures within 2 years despite the use of 3–4 AEDs in the highest tolerated doses	I
Siddiqui [8]	At least 4 epileptic seizures in the last year while taking more than three correctly selected antiepileptic drugs in the highest doses tolerated by the patient	II
Berg [33]	The occurrence of 20 complex partial seizures during 24 months with a failure of treatment of those with at least 2 AEDs used for this kind of seizures	III
ILAE [26]	The application of two appropriately selected, applied and tolerated strategies for the treatment of epilepsy (whether as mono-therapies or poly-therapies) to obtain a sustained seizure freedom, so when seizures continue and their control with further changes to pharmacological treatment is highly unlikely	IV

region), including 38 young girls and 44 young boys. The patients' ages ranged from 33 months (2 years and 9 months) to 18 years (average age of patients was 12.4±4.2 years).

The following criteria for inclusion and exclusion were considered in selecting the patients for the experiment.

The inclusion criteria: 1) Sex: male and female. 2) Age up to 18 years old. 3) Partial epilepsy diagnosed in accordance with the ILAE criteria, and clinical manifestation allowing for certain recognition of focal epilepsy and the corresponding EEG picture. 4) Recognized clinical response to the application of AED. 5) Performed neuroimaging (at least MR). 6) At least 2-year patient observation period.

The exclusion criteria: 1) Absence of unequivocal diagnosis of focal epilepsy. 2) Idiopathic partial epilepsy. 3) Progressive encephalopathy. 4) Rare forms of the disease such as *epilepsia partialis continua*, particularly Rasmussen's syndrome.

In all the patients included in the analysis at least 1 AED was used, about which there had been reports that resistance to them is related to MDR1 polymorphism or which had been subjected to research indicating that there was a connection between them and MDR1 polymorphism (phenytoin, phenobarbital, oxcarbamazepine, lamotrigine, felbamate, and gabapentin). In the majority of the patients, it concerned 1 drug (most often it was OXC or LTG), then 3 AEDs, and in a small group of patients 4 or 5 of the above-mentioned drugs.

All drugs were administered at maximal doses and they did not have unacceptable adverse effects.

The study was conducted after approval by the Bioethics Commissions of the Medical University of Silesia.

Definitions of drug resistance applied for the study group

Patients in the study group were classified as drug-resistant/drug-sensitive using the 4 different definitions of drug resistance in epilepsy. Depending on the applied criteria of drug resistance, the numbers in subgroups of epileptic patients sensitive or resistant to pharmacological treatment varied (Table 1).

Epileptic seizures were classified as cryptogenic and symptomatic. In the group of 82 patients with cryptogenic partial epilepsy, 70.5% had cryptogenic and 29.5% had symptomatic seizures.

Genotyping – identification of MDR1 polymorphism

In isolated genomic DNA obtained from peripheral blood (Blood Mini, A&A Biotechnology), the polymorphism in ABCB1 C3435T was determined using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay. Fragments of P-glycoprotein (197 bp) in exon 26 of MDR1 gene were amplified from genomic DNA with the sense primer 5'-TGTTTTCAGCTGCTTGATGG-3' and antisense 5'-AAGGCATG TATGTTGGCCTC-3'. PCR amplification was performed in a total volume of 50 μL and contained 200 ng genomic DNA, dNTP mix 10 mM/L, 5 pM/L each primers, 50 mM/L buffer with Mg^{2+} and Polymerase DNA (DyNAzyme EXT™ PCR Kit, FINNZYMES). The amplification reaction was performed using Mastercycler (Eppendorf AG, Hamburg, Germany). PCR amplification consisted of an initial denaturation for 3 min at 94°C, followed by 34 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 30 s. The terminal elongation was performed at 72°C for 7 min. After PCR amplification, product (197 bp) was digested using *Sau* 3AI restriction enzyme (Fermentas) for 16 h at 37°C generating fragments of 158 bp, 39 bp for allele 3435C; and 197 bp, 158 bp, and 39 bp for allele C3435T. For allele 3435T, no digestion products were observed. RFLP results were analyzed following electrophoresis using 3.5% agarose gel.

Table 2. Number of patients with epilepsy dependend on definition of drug resistance (n=82).

Epilepsy classification	Resistant epilepsy	Good responsive epilepsy
Appleton and Gibbs (I)	36	46
Siddiqui (II)	32	50
Berg (III)	28	54
ILAE (IV)	39	43
Acording to I classification	36	46
Acording to I and II classification	45	37
Acording to I, II and III classification	34	48
Acording to I, II, III, IV classification	31	51

Table 3. Distribution of genotypes and allels of the C3435T polymorphism of the MDR1 gene.

Genotypes/allele	Patients with epilepsy (n=82)
	n (%)
CC	10 (12.2%)
CT	49 (59.7%)
TT	23 (28.1%)
C allel	69 (42.1%)
T allel	95 (57.9%)

Statistical analysis

The distribution of genotypes and alleles in patient groups was statistically evaluated by the χ^2 test with Yates's correction. The genotype frequencies of the MDR1 polymorphism were tested for their fit to the Hardy-Weinberg equilibrium (HWE). A p value of ≤0.05 was statistically significant. The effective sample size and statistical power of association analyzes were computed using Epi Info TM 7.1.1.0 developed by the U.S. Centers for Disease Control and Prevention (CDC).

Results

Analysis of number of patients with regard to the applied criteria of drug-resistance

The number of children with epilepsy classified as sensitive and resistant to pharmacotherapy depending on 4 definitions of drug resistant epilepsy is shown in Table 2.

Genotype identification

In the investigated population, 3 genotypes of MDR 1 polymorphism were obtained: CC, CT, and TT. The distribution of MDR1 genotypes and alleles in all patients with epilepsy are listed in Table 3.

The analysis of the distribution of genotypes in the study group with epilepsy showed that the CT genotype was the most common genotype (59.7%). The CC genotype was the rarest (12.2%). The Hardy-Weinberg equilibrium was maintained for the studied group.

Analysis of genotypes with regard to the applied criteria of drug resistance

Detailed analyses of the distribution of genotypes in the group of epileptic patients with regard to applied definition of drug resistance (Table 1) did not show statistically significant differences in the frequency of the occurrence of CC, CT, or TT genotypes between the groups of patients sensitive and resistant to pharmacotherapy (Table 2).

The genotype frequency distribution for *MDR1* 3435 *loci* in the studied population, irrespective of the applied criterion of drug resistance, did not show a significant deviation from HWE (p>0.05) (Table 4).

The results of the analyses do not confirm the connection of the studied polymorphism with resistance to antiepileptic drugs. None of the genotypes (CC, CT, and TT) were significantly more common in patients with drug-resistant epilepsy, irrespective of the applied criteria classifying the patients to groups sensitive or resistant to treatment (Table 4).

Discussion

A single, detailed, and commonly accepted definition of drug resistance to antiepileptic drugs has not yet been developed. Thus, doctors and researchers apply different criteria and often claim that they study patients with drug-resistant epilepsy without presenting the detailed criteria of drug resistance they used, making the comparison of the results of clinical studies considerably more difficult [26,29,33]. The 2010 definition of drug-resistant epilepsy suggested by the ILAE Commission of Therapeutic Strategies is in reality only a suggestion and there is no agreement that it should be treated as the final definition. Therefore, in studies on predictors of drug resistance, the standardization and correct classification of patients as sensitive or resistant to treatment with AEDs is important [26].

Table 4. Heterogenity test and Hardy-Weinberg Equilibrium (HWE) MDR1 gene polymorphism C3435T depending on the epilepsy classification.

Genotyp	Epilepsy classification (n=82)							
	ILAE		Appleton		Siddiqui		Berg	
	Good responsive	Resistant	Good responsive	Resistant	Good responsive	Resistant	Good responsive	Resistant
CC	2 (4.7%)	8 (20.5%)	5 (10.9%)	5 (13.9%)	5 (10.0%)	5 (15.6%)	7 (13.0%)	3 (10.7%)
CT	28 (65.1%)	21 (53.8%)	26 (56.5%)	23 (63.9%)	29 (58.0%)	20 (62.5%)	31 (57.4%)	18 (64.3%)
TT	13 (30.2%)	10 (25.6%)	15 (32.6%)	8 (22.2%)	16 (32.0%)	7 (21.9%)	16 (29.6%)	7 (25.0%)
Hardy Weinberg test	$p=0.15$	$p=0.94$	$p=0.66$	$p=0.47$	$p=0.54$	$p=0.59$	$p=0.64$	$p=0.49$
Comparison of all genotypes*	4.81 NS ($p=0.09$)		1.11 NS ($p=0.57$)		1.29 NS ($p=0.53$)		0.36 NS ($p=0.83$)	
CC *vs*. the total of other genotypes	3.44 NS ($p=0.07$)		0.01 NS ($p=0.74$)		0.17 NS ($p=0.50$)		0.03 NS ($p=0.99$)	
CT *vs*. the total of other genotypes	1.08 NS ($p=0.30$)		0.46 NS ($p=0.49$)		0.16 NS ($p=0.68$)		0.036 NS ($p=0.54$)	
TT *vs*. the total of other genotypes	0.21 NS ($p=0.64$)		1.07 NS ($p=0.33$)		0.99 NS ($p=0.32$)		0.65 NS ($p=0.20$)	

NS – not significant; p≥0.05; * χ^2 test; p – value.

Research on the impact of P-gp on drug resistance assumes that the bioavailability of AEDs may be diminished as a result of overexpression of carrier proteins (MDR1) within the blood-brain barrier [9,41,42]. Most AEDs are such weak substrates of P-gp that the constitutional expression of this protein in the BBB cannot inhibit the flow of AEDs in a clinically significant way [43], but the congenital or acquired overexpression of P-gp in the BBB may critically reduce the penetration of AEDs into the brain and thus result in drug resistance to many AEDs [28,43,44]. Such overexpression may result from the disease itself or from the impact of AEDs on the expression of P-gp or ABCB1 polymorphism. P-gp is coded by the ABCB1 (*MDR1*) gene and its expression and function are linked to ABCB1 C3435T polymorphism, thus the SNP ABCB1 is the object of interest.

In healthy individuals, P glycoprotein is detected in vessel endothelial cells of the BBB, while in epileptic patients the expression of P-glycoprotein in the endothelium is intensified and additionally detected in neurons and astrocytes [45]. In patients with temporal lobe epilepsy, *MDR1* overexpression is reported in the hippocampus and surrounding area. Chronic seizures may induce overexpression of P-gp and other carrier proteins in this location [46], which may explain clinical observations of patients with drug-resistant epilepsy who are usually resistant to a wide spectrum of AEDs with varied mechanisms of action, which has been reported for different AEDs, including OXC, PHT, and PB [17,47]. Hoffmeyer et al. [22] concluded that CC genotype of *MDR1* gene causes the overproduction of P-gp in the intestinal mucosa and Siddiqui et al.

[8] found the same correlation within the BBB. These findings have stimulated scientists to further study the *MDR1* gene polymorphism. The confirmation of Siddiqui's findings was obtained in non-Caucasian patients, which may indicate that racial factors play a role [25,27,48].

Results of studies of the relation of polymorphism of the *MDR1* gene and epilepsy so far are contradictory. A meta-analysis of 22 studies conducted in between 2003 and 2009, concerning the connection of C3435T polymorphism of the *MDR1* gene with drug resistance of epileptic patients, confirmed the link between drug resistance and the CC genotype [20]. Some researchers indicate that the TT genotype predisposes a person to the occurrence of drug-resistance in epilepsy. However, the latest studies by Nurmohamed et al. [30], as well as a meta-analysis by Bournissen et al. [15] of 11 case-control studies involving 3371 patients with drug-resistant epilepsy and 1725 controls, did not identify a significant association between AED resistance and ABCB1 polymorphism, including 3435 SNP. The lack of a connection between C3435T polymorphism and epilepsy in children was confirmed by Chen et al. [49] and von Stülpnagel [50]. However, Alpman et al. [7] did not confirm a connection of the *MDR1* gene with epilepsy in children, but they did report that the coexistence of CC3435/ GG2677 polymorphisms in the *MDR1* gene may be related to drug resistance to AED.

Our research conducted on children and adolescents up to 18 years old also did not find a relationship between the

polymorphism of the *MDR1* gene and drug resistance [31]. The most common genotype in the study group was the CT genotype (59.7%), followed by TT (28.1%) and CC (12.2%). The distribution of genotypes was similar to that observed by Alpman et al. [7] in children with epilepsy (CT 52.6%, TT 31.6%, and CC 15.8%). The analysis in the group of patients and in the study group showed a similar distribution of alleles to that study of Alpman et al. [7].

There is an indication that research on predictors of drug resistance in epilepsy treatment should be carried out in different age groups and upon consideration of various etiologies of the disease. This may help to discover real connections between the polymorphisms of genes, candidates for predictors of drug resistance, and drug resistance in epilepsy. Takano et al. [51], while conducting an examination of healthy volunteers using the PET method, did not find a correlation between the brain distribution of verapamil (a commonly known inhibitor of P-gp) and the ABCB1 haplotype. However, the results obtained by other authors are clearer.

Sanchez et al. [52], based on the analysis of ABCB1, concluded that the obtained results depend on the age of the patient and the etiology of epilepsy. Epileptic adults with the TT genotype were at a lower risk of drug-resistant epilepsy than those with the CC or GG genotypes. Patients with symptomatic epilepsy and the CT or TT genotypes were at lower risk of developing epilepsy than those with the CC genotype. In the results of examinations performed on children, no statistical significance was found in any of the subgroups.

In the case of children and adolescents, changes in the expression of the P-glycoprotein in ontogenesis must be considered. It has been reported that there is an increase in the *MDR1* expression during ontogenesis in mice and rats, reaching its highest level in adult animals [53]. Therefore, a hypothesis can be put forward that the changes in *MDR1* expression may explain the varying metabolism of AEDs in children compared to adults, so the examinations of gene expressions in adults should not be used with reference to children [50].

In the current literature there are no studies on the connection between the polymorphisms of genes involved in the occurrence of drug resistance with reference to various criteria of defining drug-resistant epilepsy in the same group of patients. Studies of selected gene polymorphisms did not provide solid data for solving the problem of a drug resistance pathomechanism for epilepsy. One mechanism under consideration is the transporter, target, and metabolic concept. The hypothesis of disturbed transport as a causative factor for drug-resistant epilepsy has been investigated mostly in the ABC-transporter. Still under consideration is the non-ABC transporter, RLIP76, and its influence on drug resistance [54].

As our analyses shows, a patient who was initially classified as responding poorly to pharmacotherapy as defined by the ILAE, when applying the definition of Appleton, is classified as drug-sensitive. The analysis indicated that, depending on the use of different definitions of drug resistance, a change in the number of children classified as sensitive or resistant to pharmacotherapy can be observed. In a large meta-analysis of studies concerning MDR1 C3435T polymorphism and resistance to AEDs, Bournissen et al. [15] found no statistically significant associations. One should consider that in this huge analysis, various criteria of drug-resistant/drug-sensitive patients were applied. The authors of this meta-analysis confirm that there might be some "borderline-resistant" patients. We believe that this problem is underestimated. Lack of uniform criteria for eligible patients, especially children, as resistant or sensitive to pharmacotherapy, can cause discrepancies in qualification of epilepsy patients (Table 2). These discrepancies can result in contradictory results regarding the relationship of C3435T polymorphism of the MDR 1 gene with absence of drug resistance in epilepsy obtained by various authors. We are the first to investigate whether the choice of the definition of drug resistance could influence the results of analysis of connections between MDR1 C3435T polymorphism and drug resistance in epilepsy. Our analysis was a single-center pilot study. Due to the nature of a pilot study, a number of patients who could be included is limited.

Because of the small number of patients enrolled in our study and the fact that the χ^2 test with Yates's correction was used, we evaluated the statistical test power, which revealed that the overall sample had 55% power to detect a 5-fold increase in observed differences in the CC genotype frequency between groups (ILAE classification). Results of remaining models were shown not to be involved in determining any drug resistance in children. There were no statistically significant differences between drug-resistant and drug-sensitive patients. Considering that this was a single-center study, our results should be treated as preliminary due to the weaker statistical power, which reached 55% (at 95% CI). Even a 2-fold increase in study group size would result in an 80%increased test power. Thus, further studies with more patients are necessary.

Although our single-center study did not confirm a link between the polymorphism of the *MDR1* gene with drug resistance, and showed that the adaptation of different definitions of drug resistance does not affect the results of the analysis of the *MDR1* gene for the presence or lack of a connection between drug resistance and a particular polymorphism of a gene, it cannot be excluded that this would be important for other molecular markers. Our study shows that in analysis of possible connections between other polymorphisms and drug resistance, the possible influence of choice of drug resistance definition should be considered.

Conclusions

We conclude that there is no link between the C3435T polymorphism of the *MDR1* gene with drug-resistant epilepsy, irrespective of the applied definition of drug resistance. Because the results of studies of other genetic predictors of drug resistance conducted on various groups of patients are contradictory, it would be interesting to examine the influence of choice of applied definition of drug resistance on the results of these studies.

Conflict of interest

The authors confirm that they have no conflicts of interest regarding this study.

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