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The influence of C3435T polymorphism of ABCB1 gene on penetration of phenobarbital across the blood—brain barrier in patients with generalized epilepsy

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Summary

Background: Epilepsy is refractory to medical treatment in about one-third of the patients. The exact pathological mechanism of epilepsy pharmacoresistance is still unclear, but a decreased antiepileptic drug (AED) uptake into the brain is suspected to play a role. P-glycoprotein (Pgp), a transmembrane transporter encoded by ABCB1 gene and located at the endothelial cells of the blood—brain barrier (BBB), has been associated with epilepsy pharmacoresistance.
Objective: To analyze the effect of two ABCB1 gene polymorphisms, C3435T and G2677T/A, on phenobarbital (PB) concentrations in the cerebrospinal fluid (CSF) and serum (S) and to assess the relationship of ABCB1 polymorphisms to phenobarbital penetration across BBB *in vivo* and seizure frequency.
Methods: CSF PB and S PB concentrations were measured in 60 patients with idiopathic primary generalized epilepsy receiving phenobarbital monotherapy. CSF/S PB concentration ratio was calculated as an index of phenobarbital penetration across BBB. The patients were genotyped for the ABCB1 gene C3435T and G2677T/A polymorphisms. Seizure frequency was recorded during the 6-month phenobarbital monotherapy.
Results: Patients with different C3435T polymorphism had significantly different CSF PB concentrations and CSF/S PB concentration ratio. In comparison with CT

Abbreviations: AED(s), antiepileptic drug(s); Pgp, P-glycoprotein; BBB, blood—brain barrier; CSF, cerebrospinal fluid; S, serum; PB, phenobarbital.

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heterozygotes and TT homozygotes, CC homozygotes had a significantly lower CSF PB concentration ($p = 0.006$) and CSF/PB concentration ratio ($p < 0.001$). G2677T/A polymorphism showed no such effect ($p = 0.466$). CC genotype and low CSF/S PB concentration ratio correlated with increased seizure frequency.
Conclusions: C3435T polymorphism of ABCB1 gene was demonstrated *in vivo* to significantly influence the CSF/S PB concentration ratio and seizure frequency.
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Introduction

Epilepsy is one of the most frequent neurologic disorders, affecting approximately 1–2% of the world population.[1,2] In around 20–30% of the patients, the condition is refractory to medical treatment.[3–8] Clinically, epilepsy is considered pharmacoresistant if seizures continue to occur even though the patient is treated with two to three first-line antiepileptic drugs (AEDs) usually used for the treatment of given epilepsy.[9–14] Characterized by high morbidity and mortality, pharmacoresistant forms of epilepsy remain a major health problem despite advances in antiepileptic pharmacotherapy and new AEDs developed in the last two decades.[1,2,15]

Most patients who are resistant to one major AED are also refractory to other AEDs, although the drugs act by different mechanisms.[5,15] The real pathological mechanism of drug resistance remains obscure in spite of numerous studies conducted.[10–14] Neither the reported associations of pharmacoresistance with early onset of the disease, multiple seizure types, high frequency of seizures before treatment, a history of febrile seizures, structural brain lesions, and malformations of cortical development have illuminated the mechanism of this phenomenon.[10–14,16–18] Furthermore, drug resistance is not associated with the same type of epilepsy. The same type of the disorder may be drug resistant in one patient and drug responsive in another.[19] The fact that most patients with refractory epilepsy are resistant to most AEDs, although the drugs act by different mechanisms, points to a nonspecific mechanism such as decreased drug uptake into the brain as a major cause of pharmacoresistance.[5,15,7,8,20,21]

During the last 12 years, reports on P-glycoprotein 750 (Pgp) involvement in epilepsy pharmacoresistance have appeared in the literature.[4,6–9,14,15] Pgp is a large transmembrane protein expressed in endothelial cells of the blood–brain barrier (BBB) and functions as a drug-transport pump transporting a variety of drugs from the brain back into the blood and reducing drug accumulation in the brain.[22–28] Accumulated research evidence from animal and in vitro studies suggests that some AEDs are Pgp substrates.[9,13,15,21,29–34] Pgp is encoded by ABCB1 gene and its expression and function are associated with the ABCB1 C3435T polymorphism.[6,8,15,35] Based on these data, some authors report on a possible connection between the C3435T polymorphism of ABCB1 gene and pharmacoresistance in epilepsy patients.[19,36,37] On the other hand, there are studies in which no association was found between the C3435T polymorphism of ABCB1 gene and Pgp expression and the pharmacoresistance of epilepsies.[38,39]

So far, there has been no conclusive evidence that the altered Pgp function is associated with pharmacoresistance. No *in vivo* human studies have been performed to investigate if C3435T mutation of ABCB1 gene influences the brain uptake of AEDs, which is one of the basic presumptions of the mechanism of pharmacoresistance.

The aim of this study was to investigate the relationship between C3435T and G2677T/A polymorphisms of ABCB1 gene and the brain uptake of phenobarbital (PB) in patients with primary generalized epilepsy.

Methods

Genotyping was performed in 60 unrelated patients (35 men and 25 women; mean age 37 ± 9 years) who suffered from idiopathic primary generalized epilepsy (PGE) with tonic–clonic seizures. All patients were diagnosed with PGE on the basis of anamnesis, heteroanamnesis, and video-electroencephalographic recording. Symptomatic epilepsy was excluded by magnetic resonance imaging (MRI) at 1.5 or 2 T. The patients were randomly selected from the database of the Reference Center for Epilepsy of the Ministry of Health and Social Welfare of Croatia at the Zagreb University Hospital Center. All patients received phenobarbital for 6 months and did not take any other AED or other drugs known to be a Pgp substrate. They were also asked to keep records on the number of seizures during the 6-month period of phenobarbital monotherapy. After at least 3 months of phenobarbital monotherapy, the patients underwent lumbar puncture. Cerebrospinal fluid (CSF) and serum (S) sampling were

Table 1 PB concentration in CSF and S and CSF/S PB ratio with respect to C3435T genotype of 60 patients with primary generalized epilepsy

Parameter	C3435T polymorphism (mean ± S.D.)			p[a]
	CC (n = 16)	CT (n = 31)	TT (n = 13)	
PB doses/mg (range)	550.0 ± 81.7 (400–700)	532.3 ± 116.6 (200–800)	530.8 ± 125.1 (300–800)	0.836
PB S (μmol/L)	102.2 ± 15.0	102.9 ± 25.3	103.8 ± 13.6	0.947
PB CSF (μmol/L)	44.1 ± 12.9	52.5 ± 13.5	64.4 ± 14.4	0.006
CSF/S PB	0.4 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	<0.001

[a] Kruskal–Wallis test, H = 18.52738.

performed at the same time at 8.00 a.m., before the morning dose of phenobarbital. PB concentration was measured in both cerebrospinal fluid (CSF PB) and serum (S PB) by fluorescence polarization immunoassay (FPIA) on TDx analyzers (Abbott Laboratories, Abbott Park, IL, USA).[40] The CSF/S PB concentration ratio was calculated as an index of phenobarbital crossing the blood–brain barrier.

All patients were genotyped for C3435T (CC, CT, and TT genotypes) and G2677T/A (GG, GT, and TT genotypes) polymorphisms of ABCB1 gene. Five milliliters of blood with Na-EDTA were collected for genotyping procedure. Genomic DNA was extracted from peripheral lymphocytes using salting out procedure.[41] Analysis of 2677G/T/A polymorphisms in exon 21 was performed according to the method described by Cascorbi et al,[42] whereas 3435C/T polymorphism in exon 26 was analyzed by the method described by Sakaeda et al.[43] Substitution G2677T/A in exon 21 was detected by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method with NdeII restriction endonuclease. The G and T alleles were represented by 193 bp and 144 bp fragments, respectively. PCR-RFLP method with BanI restriction endonuclease was used to detect MDR1–C3435T substitution. The C and T alleles were represented by 198 bp and 224 bp fragments, respectively.

The study was approved by the local ethics committee and all patients gave a written informed consent before entering the study.

Data were presented as mean values ± standard deviation (S.D.). Since the C3435T genotypes did not follow a Gaussian distribution, Kruskal–Wallis test was used to compare C3435T genotype groups for phenobarbital concentration in the cerebrospinal fluid and serum and CSF/S PB concentration ratio. G2677T/A genotype groups were compared for the same parameters using Kruskal–Wallis test. The relationship between CSF/S PB concentration ratio and seizure frequency over the 6-month period was analyzed by ANOVA–MANOVA one-way test followed by a Tukey test. Statistical analysis was performed with SPSS 10.0 for Windows (SPSS Inc., Chicago, IL, USA), and the level of significance was set at $p \leq 0.05$.

Results

We found differences in both CSF PB concentration and CSF/S PB concentration ratio among patients with different C3435T genotypes (Table 1). The S PB concentration did not differ among patients with CC, CT, or TT genotype, but the penetration of phenobarbital into the brain was reduced in CC homozygotes, who had a significantly lower relative concentration of phenobarbital in the cerebrospinal fluid than did CT heterozygotes and TT homozygotes.

In the same patients, no differences in CSF PB concentration and CSF/S PB concentration ratio were found with respect to the G2677T/A genotype (Table 2). Not a single variant A allele was detected in our patients, and age and sex did not correlate with differences in CSF/S PB ratio (data not shown).

Seizure frequency correlated with CSF/S PB concentration ratio (Table 3). Patients with lower CSF/S

Table 2 PB concentration in CSF and S and CSF/S PB ratio with respect to G2677T/A genotype of 60 patients with primary generalized epilepsy

Parameter	Polymorphism (mean ± S.D.)			p[a]
	GG (n = 14)	GT (n = 29)	TT (n = 17)	
PB doses/mg (range)	521.4 ± 152.8	548.3 ± 82.8	529.4 ± 84.89	0.5353
PB S (μmol/L)	104.03 ± 11.32	102.7 ± 25.73	102.36 ± 16.96	0.8274
PB CSF (μmol/L)	50.75 ± 14.06	53.38 ± 13.45	53.69 ± 18.87	0.4323
CSF/S PB	0.49 ± 0.11	0.53 ± 0.11	0.52 ± 0.14	0.466

[a] Kruskal–Wallis test; H = 1.527117.

Table 3 PB concentration in CSF and S, CSF/S PB ratio, and C3435T and G2677T/A polymorphisms in 60 patients with primary generalized epilepsy with respect to the number of seizures over a 6-month period

Parameter	No. of seizures									p[a]
	0 (n = 10)	1 (n = 10)	2 (n = 10)	3 (n = 14)	4 (n = 4)	5 (n = 4)	6 (n = 2)	7 (n = 3)	8 (n = 2)	9 (n = 1)	
PB S (μmol/L)	104.4 ± 9.7	106.4 ± 18.6	93.2 ± 13.2	104.0 ± 34.7	98.8 ± 18.3	108.9 ± 5.6	106.5 ± 19.1	109.7 ± 9.0	93.5 ± 7.8	120.0	0.077
PB CSF (μmol/L)	65.8 ± 12.4	61.2 ± 15.4	46.9 ± 8.6	51.5 ± 15.8	47.2 ± 10.4	47.2 ± 10.3	43.1 ± 7.4	46.9 ± 4.0	32.7 ± 2.6	33.6	0.001
CSF/S PB	0.6 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.03	0.4 ± 0.1	0.4 ± 0.01	0.28	0.001
C3435T polymorphism											
CC (n = 16)	0	1	2	3	1	2	2	3	1	1	<0.001
CT (n = 31)	4	3	8	10	3	2	0	0	1	0	
TT (n = 13)	6	6	0	1	0	0	0	0	0	0	
G2677T polymorphism											
GG (n = 14)	4	2	2	2	1	1	0	1	1	0	0.538
GT (n = 29)	2	2	5	12	2	3	1	2	0	0	
TT (n = 17)	4	6	3	0	1	0	1	0	1	1	

[a] ANOVA–MANOVA test followed by a Tukey test.

PB concentration ratio had a higher incidence of seizures during the 6-month period (ANOVA–MANOVA, $p = 0.001$). Seizure frequency correlated with CSF PB concentration in same manner it did with CSF/S PB concentration ratio, while S PB concentration showed no influence on seizure frequency; S PB concentration was within therapeutic limits in all patients.

C3435T polymorphism showed a significant correlation with seizure frequency (Table 3). Patients with CC genotype had a higher seizure frequency than those with CT or TT genotype (ANOVA–MANOVA, $p < 0.001$). There were more patients with CT and TT genotypes among those with lower number of seizures, and more patients with CC genotype among those with higher number of seizures. In the same patients, G2677T/A polymorphism showed no correlation with seizure frequency ($p = 0.538$).

Discussion

We found that both phenobarbital concentration in the cerebrospinal fluid and CSF/S PB concentration ratio was significantly lower in patients with the CC genotype than in patients with CT or TT genotypes of the ABCB1 C3435T polymorphism. The seizure frequency was also higher in CC homozygotes in our study.

A hypothesis of pharmacoresistance currently favored by many authors is decreased drug uptake into the brain and its restricted access to the site of action, caused by overexpression of the multidrug transporters, such as Pgp, at the blood–brain barrier.[5,7,8,14,15,20,21,30–34] As the C3435T polymorphism of ABCB1 gene has been associated with the expression and function of Pgp in humans, some authors suggest that this polymorphism is associated with the response to some AEDs.[19,36,37] Our results showed a correlation between C3435T polymorphism, CSF/S PB concentration ratio, and frequency of seizures, implying that Pgp may play a role in AED pharmacoresistance.

Results of many previous studies, mainly animal and *in vitro*, suggested the possible link between Pgp and clinical response to AEDs.[7,15,42,29–33] Histopathologic analysis after neurosurgical operations in epilepsy patients also suggested the same association.[4,6,8,44] However, connection between C3435T polymorphism and AEDs uptake into the brain has never been tested in humans *in vivo*. The hypothesis is that patients with hyperexpression of Pgp at the blood–brain barrier have reduced penetration of AED into the brain, resulting in poor therapeutic efficacy. Some studies have demonstrated the interaction of AEDs with human Pgp.[45–47] Several AEDs

have been reported to induce Pgp or inhibit its function,[15,22–27,42] including the recent study by Schuetz et al.[48] who showed that phenobarbital induces Pgp. However, it is still unclear if some AEDs, including phenobarbital, could be substrates for human Pgp. Crowe and Teoh[49] tested a variety of AEDs for their ability to be transported by Pgp through Caco-2 monolayers and found only one, acetazolamide, to be a weak substrate of human Pgp. On the other hand, Pgp efflux ratios determined by *in vitro* high-throughput screening tests, where the transport conditions such as pH gradient and concentration are fixed, cannot be routinely used to predict a possible limited brain penetration *in vivo*.[50] Our results show that C3435T polymorphism of the ABCB1 gene, which encodes Pgp, influences the brain uptake of phenobarbital in patients with epilepsy. Whether this finding implies that phenobarbital is a human Pgp substrate remains to be confirmed.

There are also studies that argue against the influence of the ABCB1 gene C3435T polymorphism on epilepsy pharmacoresistance.[38,39] These studies differed in inclusion criteria and involved a large number of patients with symptomatic epilepsy (caused by hippocampal sclerosis, cortical dysplasia, stroke, or other reasons). In these patients Pgp hyperexpression could have resulted from the action of other local factors in the altered tissue such as a release of excitotoxic metabolites during frequent seizures, i.e. irrespective of the ABCB1 gene C3434T polymorphism. In addition, these studies also included patients regardless of the type of AED therapy they received (monotherapy or polytherapy, substrates or non-substrates of Pgp). Thus, inclusion of patients taking valproate could have confounded the results, because valproate has not been shown to be a Pgp substrate.[21,51,52] Furthermore, competitive inhibition in case of AED polytherapy and failure to exclude patients taking other drugs that are potential substrates or inhibitors of PGP could also have biased the results of these studies. To avoid these possible influences, we included only patients with idiopathic generalized epilepsy taking phenobarbital monotherapy.

To the best of our knowledge, the present study is the first to show involvement of ABCB1 C3435T polymorphism in the brain uptake of an AED in humans *in vivo*. The seizure frequency was found to correlate with CSF/S PB concentration ratio, which fits the "decreased drug uptake" theory of pharmacoresistance modulated by Pgp. On the other hand, the sample size in our study was small and the results should be interpreted with caution and confirmed in a larger number of patients. Although evidence from the literature suggests that at least some AEDs are Pgp substrates, the exact influence of C3435T polymorphism on different AEDs uptake into the brain, especially in humans, remains to be determined. More *in vivo* human studies including large groups of patients could provide better insight into the role of Pgp and ABCB1 polymorphism in epilepsy pharmacoresistance. Also, attention should be paid to other factors that may play important roles in the multifactorial phenomenon of pharmacoresistance, including pharmacodynamic and pharmacokinetic mechanisms, polytherapy, and other protein transporters at the blood–brain barrier.

Conclusion

C3435T polymorphism of ABCB1 gene influences the penetration of phenobarbital through the blood–brain barrier. Correlation between C3435T polymorphism of ABCB1 gene, CSF/S phenobarbital concentration ratio, and seizure frequency also suggest involvement of ABCB1 gene in pharmacoresistance of idiopathic primary generalized epilepsies due to reduced drug uptake into the brain. Larger *in vivo* human studies are needed to confirm these results.

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