ABCB1 gene variants as predictors of multidrug-resistant epilepsy in Croatian population

D. Sporš, S. Bašić, N. Božina¹, T. Babić², S. Hajnšek³, J. Sertić¹, I. Šušak, I. Marković

ABSTRACT - P-glycoprotein (Pgp) is a drug efflux transporter and is the encoded product of the human multidrug resistance gene ABCB1 (MDR1). Pgp is expressed in the blood-brain barrier (BBB) and blood-cerebrospinal fluid (CSF) barrier, and reduces the brain penetration of antiepileptic drugs (AEDs). Functional polymorphism or overexpression of Pgp in the BBB of patients with epilepsy may play a role in pharmacoresistance. The aim of this study was to investigate the possible association of ABCB1 gene polymorphisms C1236T in exon 12, G2677T in exon 21, and C3435T in exon 26 with the development of resistance to antiepileptic therapy. All patients enrolled in the study had an established diagnosis of partial complex epilepsy with or without secondary generalisation and have been suffering from it for more than two years. Patients were divided into two groups. The first group comprised 57 patients refractory to the current therapy, while group 2 consisted of 48 patients with well-controlled seizures. Results of our study showed statistically significant difference in the allele and genotype frequency of ABCB1 G2677T between resistant and nonresistant patients. Haplotype G2677/C3435/C1236 was overrepresented among resistant patients. According to our results, ABCB1 variants C1236T, G2677T and C3435T might be associated with therapeutic response to AED in patients with partial epilepsy with or without secondary generalization and represent a possible predictive factor for pharmacoresistance.

Key words: ABCB1 gene, epilepsy, multi-drug resistance, polymorphism

INTRODUCTION

Epilepsy is characterized by recurrent spontaneous seizures and is one of the most common neurological disorders (1). Despite advances in antiepileptic drug (AED) therapy, about one-third of patients with epilepsy are resistant to drug treatment. Most

¹Dubrava University Hospital, Department of Neurology, Zagreb, Croatia
²Zagreb University Hospital Center, Center for Functional Genomics and Clinical Institute of Laboratory Diagnosis, Zagreb, Croatia
³Research, CNS Scientific Affairs, Maidenhead, UK
⁴Zagreb University Hospital Center, University Department of Neurology, Zagreb, Croatia
patients with refractory epilepsy are resistant to several, if not all, AEDs despite the fact that these drugs act by different mechanisms (2). Epilepsy was the first central nervous system (CNS) disorder for which drug resistance was associated with enhanced expression of multidrug transporters in the brain (3). In addition to intrinsic and acquired overexpression of multidrug transporters in the blood brain barrier (BBB) of patients with epilepsy, functional polymorphisms of these transporters might contribute to pharmacoresistance (4).

P-glycoprotein (Pgp), the encoded product of the human multidrug-resistance gene ABCB1, is a drug efflux transporter of particular clinical relevance because many drugs are substrates and/or inhibitors, among which there are several major AEDs, including phenytoin, phenobarbital, carbamazepine, lamotrigine, topiramate and gabapentin (5–10).

P-glycoprotein is widely localized in normal tissues including the apical membrane of the gastrointestinal tract, blood cells, the biliary canicular membrane of hepatocytes, and the luminal membranes of proximal tubular epithelial cells in the kidney, and thus limits the cellular uptake of xenobiotics by excreting these compounds into the bile, urine and intestinal lumen (11–13). Pgp is expressed in the BBB or blood-cerebrospinal fluid (CSF) barrier, combining them to reduce the brain penetration of AEDs.

More than 50 single nucleotide polymorphisms (SNPs) and insertion/deletion polymorphisms in the large ABCB1 gene have been reported (14), and mutations at positions 2677 and 3435 have been associated with alteration of Pgp expression and/or function (14–16). A silent C to T transition in exon 26 of ABCB1 (3435C>T) has been associated with differences in Pgp levels and activity (17). The polymorphism has also been associated with Pgp function, and with clinical conditions such as drug-resistant epilepsy (18). However, available data suggest that this polymorphism may not directly cause altered Pgp transport activity but may be associated with one or more causal variants in the stretch of linkage disequilibrium surrounding it (19). In a study from Austria (20), in which functional variants in ABCB1 were genotyped in patients with temporal lobe epilepsy, a common ABCB1 gene haplotype CGC/CGC was identified, which was significantly associated with the risk of pharmacoresistance. A few other studies confirmed the association of polymorphism, linkage disequilibrium and specific haplotype combinations with pharmacoresistance in patients with epilepsy (21–24). However, other studies failed to replicate an association of polymorphism in ABCB1 gene with multidrug-resistant epilepsy (25–32).

The aim of this study was to investigate the possible association of ABCB1 gene polymorphisms in exon 21 G2677T, exon 26 C3435T and exon 12 C1236T with the development of antiepileptic drug resistance in Croatian population.

PATIENTS AND METHODS

Patients

This cross-sectional study is part of the ongoing pharmacogenomic study of epilepsy in Croatian population and has been approved by the Ethics Committee of the Zagreb University Hospital Center. Patients were consecutively recruited through the Referral Center for Epilepsy, University Department of Neurology, and laboratory methods were done at the Center for Functional Genomics and Clinical Institute of Laboratory Diagnosis. All patients enrolled in the study had an established diagnosis of partial complex epilepsy with or without secondary generalization and had been suffering from it for more than two years. They were divided into two groups. Group 1 consisted of 57 patients refractory to current therapy, while group 2 included 48 patients with well-controlled seizures. Refractory epilepsy was defined as one or more seizures per month during the past year, while on therapy with two or more established AEDs at the maximally tolerated doses. The patients with well-controlled seizures were free from seizures in the same period. Compliance was determined by measuring serum concentration of anticonvulsants (33,34). Seizure frequency was recorded from the patient medical records, seizure diaries and patient interview. Patients in-between these two groups were excluded from the study. Patients with a history of CNS infection, head trauma, brain tumor, cerebrovascular disease, neurodegenerative and psychiatric diseases, or pseudoattacks were excluded from the study. All patients signed an informed consent to participate in the study. In all patients, the allele, genotype and haplotype frequencies were determined as well as the number of AEDs and MDR1 substrates, age at onset, duration of epilepsy and interictal electroencephalogram (EEG). Association with ABCB1 gene polymorphisms was compared between the two groups.
The mean patient age was 42.64 years (SD 13.91). Non-responders took a significantly higher mean number of medications ($\chi^2=55.322; \text{df}=4; P<0.001$), as compared with responders (2.9 vs. 1.9 antiepileptics). Furthermore, the mean number of AEDs, substrates of MDR1 transporter, was statistically significantly higher in the group of non-responders as compared with the group of responders (2.6 vs. 1.6). In the non-responder group, most patients were taking three (49%) or two (47%) AEDs, MDR1 substrates. In the responder group, 48% were taking one AED, MDR1 substrate, and 46% were taking two AEDs, MDR1 substrates. Carbamazepine was the most frequently administered AED (n=95, 88%), either as monotherapy or in combination with other drugs. Lamotrigine was used by 54 (50.5%), phenobarbital by 46 (43%), valproate by 23 (21%) and topiramate by 18 (16.8%) patients. Gabapentin and phenytoin were used by 7/6 patients.

### Genotyping procedures

Genotyping of 1236C/T, 2677G/T/A and 3435C/T variants of the ABCB1 gene was performed by the PCR-RFLP and real-time PCR methods according to the previously described procedures (35–40).

### Statistical analysis

A test for Hardy-Weinberg equilibrium using Markov chain method (41) and the linkage-disequilibrium likelihood-ratio test between loci whose gametic phase is unknown (42) were performed, as implemented in Arlequin ver. 3.01 (43). Haplotype frequencies were estimated using Expectation-Maximization algorithm implemented in the same program, leading to maximum likelihood estimates of haplotype frequency. The $\chi^2$-test was used for pair-wise comparisons of allele frequencies between the groups. Log likelihood ratio tests were performed to compare distributions of the estimated haplotypes between the groups, as well as comparisons of genotype frequencies. $P$ values less than 0.05 were considered statistically significant. The $\chi^2$-test or $t$-test was performed for comparison of age at onset, duration of epilepsy, interictal electroencephalogram, antiepileptic drugs and MDR1 substrates between genotypes and in resistant vs. non-resistant patients. All statistical analyses were carried out using the SPSS 11.5 (SPSS Inc., Chicago, IL, USA) statistical software package. The pattern was calculated with Altman's algorithm and power of 80%.

## RESULTS

All samples were successfully genotyped. We found no case of 2677A variant. Genotype frequencies of the ABCB1 2677GG, 2677GT and 2677TT in the sample were 35, 48 and 22; of the 3435CC, 3435CT and 3435TT 26, 44, 37; and of the 1236CC, 1236CT and 1236TT 33, 41 and 25, respectively.

No significant deviations from the expected Hardy-Weinberg proportions were observed in the total sample and at the loci (ABCB1 G2677T: $P=0.43$; C3435T: $P=0.08$; C1236T: $P=0.516$). Test result for linkage disequilibrium (LD) between the loci was significant among all loci. LD between exon 21 G2677T and exon 26 C3435T: $\chi^2=94.97, P<0.001$, ss=1; LD between exon 21 G2677T and exon 12 C1236T: $\chi^2=58.72, P<0.001$, ss=2; and LD between exon 26 C3435T and exon 12 C1236T: $\chi^2=40.49, P<0.001$, ss=2.

Pair-wise comparisons of the allele frequency between resistant and non-resistant patients revealed a statistically significant difference for exon 21 G2677T ($P=0.041, \chi^2=4.19, \text{df}=1$), while in exons 26 and 12 there was no statistically significant difference (Table 1). Patients with allele G of G2677T had 85% odds ratio (31% risk ratio) for resistance recorded in patients with T allele. Analysis of various C3435T alleles in exon 26 did not yield statistical difference ($P=0.124$) according to antiepileptic therapeutic efficacy. Analysis of different C1236T alleles in exon 12 yielded no statistical difference ($P=0.821$) according to antiepileptic therapeutic efficacy (Table 1).

A statistically significant difference was found in genotype based analysis of exon 21 G2677T ($P=0.016$, likelihood ratio G= 8.214, ss=2), but not in exon 26 C3435T and exon 12 C1236T (Table 1). Patients with G/T allele had a statistically significantly lower chance for pharmacoresistance as compared with patients with G/G allele. Patients with T/T allele did not show statistical difference according to resistance. C3435T polymorphism in exon 26 did not show significant correlation with pharmacoresistance, although patients with C/T allele showed a statistically significantly lower chance for resistance as compared with patients with C/C allele. C1236T polymorphism in exon 12 did not show statistical difference according to antiepileptic therapeutic efficacy (Table 2).

 Likewise, statistically significant differences were found in the distribution of the estimated haplotypes between the groups. Haplotype G2677/C3435/C1236 was overrepresented among resist-
ant patients (likelihood ratio $G=9.641; ss=3; P=0.023$) (Table 2); in the analysis of genotype combination frequencies of ABCB1 exon 21, 26 and 12, genotype combination GG/CC/CC was statistically significantly overrepresented among resistant patients (likelihood ratio $G=9.641; ss=3; P=0.023$) (Table 3).

There were significant differences between resistant and non-resistant patients in the age at disease onset (mean age $\pm$ SD: 11.8 $\pm$ SD 6.37 vs. 23.4 $\pm$ SD 11.57, $t=8.448, ss=105, P<0.001$) and duration of illness (mean value $\pm$ SD: 30.4 $\pm$ SD 11.97 vs. 19.9 $\pm$ SD 11.59, $t=4.495, ss=105, P<0.001$). Furthermore, in the resistant group of patients we found significantly higher focal altered EEG findings ($\chi^2=11.545, ss=1, P<0.001$).

**DISCUSSION**

As indicated by the data presented, ABCB1 variants seem to be associated with AED response in patients with partial complex epilepsy.

Many studies aimed to highlight the role of intestinal Pgp in drug interactions, of P-glycoprotein expressed in the BBB for drug penetration into the CNS, the role of pharmacological inhibition of Pgp function to increase drug concentrations in sanc-
tuary sites (e.g., for the HI virus), and for the potential role of MDR1 polymorphisms for Pgp expression, drug disposition, adverse drug reactions and disease risk (44). Active drug transport is now considered an important additional mechanism limiting drug accumulation in multiple tissues, including the CNS. Despite the magnitude of extensive research, many conflicting data still need to be clarified.

AED resistance could be either the result of each single polymorphic variant of ABCB1 gene, or of many different polymorphic variants in the wide spectrum of linkage disequilibrium.

An important characteristic of refractory epilepsy is that most patients are resistant to most, and often to all AEDs (2). This argues against epilepsy-induced alterations in specific drug targets as a major cause of pharmacoresistant epilepsy. It points out other mechanisms such as decreased drug uptake into the brain by seizure-induced overexpression of multidrug transporters in the BBB (45). Our results are in accordance with the findings reported by Hung et al., Zimprich et al. and Kwan et al. (20,22,46), confirming the possible role of ABCB1 gene variants in pharmacoresistance. The results of the presented study confirmed the existence of linkage disequilibrium of 12, 21 and 26 exon of ABCB1 gene that could be responsible for altered function of drug transporter and as a consequence for refractory epilepsy. Association between combinations of GG/CC/CC genotypes and pharmacoresistance in patients with partial complex epilepsy has been reported. Furthermore, the analysis showed that carriers of GG2677 genotype had more often diagnosis of refractory epilepsy. Single GCC haplotypes in the resistant group showed a statistically significantly difference in comparison with single TTT and TTC haplotypes in the nonresistant group. Siddiqui et al. (18) were the first to report on the association between single nucleotide polymorphisms in the ABCB1 gene and refractory epilepsy. That study led to a hypothesis that drug resistance in epilepsy might be genetically determined. Patients with AED-resistant epilepsy were more likely to carry 3435CC genotype, which is associated with increased Pgp expression in comparison to 3435TT genotype.

Soranzo et al. (19) suggest that C3435T polymorphism might not be the only cause of the altered Pgp activity. They identified three intron polymorphisms that are associated with 3435C>T polymorphism. Their study showed the possible importance of linkage disequilibrium for the Pgp function. Our results are in part consistent with the data reported by Hung et al. (22). They demonstrated association between C3435T, C1236T and G2677T polymorphisms with AED resistance. They emphasize that the CGC, TGC and TTT haplotypes and haplotype combinations CGC/CGC, CGC/TGC, CGC/TTT and TGC/TTT were found more often in the resistant group in comparison with nonresistant group. Zimprich et al. (20) performed genotyping for functional ABCB1 variants in patients with temporal lobe epilepsy and found the 2677GG/3435CC genotype to be significantly associated with resistance. Kwan et al. found that in 464 Chinese epilepsy patients, the ABCB1 intronic polymorphism rs3789243 and the coding polymorphism 2677, and haplotypes containing them might be associated with drug resistance (46). Contrary to our results and the studies mentioned above, many other studies did not confirm positive association between ABCB1 polymorphisms and multidrug resistance in epilepsy patients (25–32).

Table 3. Distribution of haplotype frequencies of of ABCB1 exon 21 G2677T, exon 26 C3435T and exon 12 C1236T between subjects who are resistant and non resistant to treatment.

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>G2677T</th>
<th>C3435T</th>
<th>C1236T</th>
<th>Resistant n=51</th>
<th>Non resistant n=44</th>
<th>OR (95%C.I.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1</td>
<td>G</td>
<td>C</td>
<td>C</td>
<td>50,47</td>
<td>22,41</td>
<td>1</td>
</tr>
<tr>
<td>H2</td>
<td>G</td>
<td>C</td>
<td>T</td>
<td>6,54</td>
<td>8,30</td>
<td>0,35 (0,11-1,14)</td>
</tr>
<tr>
<td>H3</td>
<td>T</td>
<td>C</td>
<td>T</td>
<td>12,26</td>
<td>6,78</td>
<td>0,80 (0,26-2,52)</td>
</tr>
<tr>
<td>H4</td>
<td>G</td>
<td>T</td>
<td>T</td>
<td>2,74</td>
<td>4,51</td>
<td>0,27 (0,06-1,22)</td>
</tr>
<tr>
<td>H5</td>
<td>T</td>
<td>C</td>
<td>C</td>
<td>0</td>
<td>2,89</td>
<td>x</td>
</tr>
<tr>
<td>H6</td>
<td>T</td>
<td>T</td>
<td>T</td>
<td>4,27</td>
<td>8,52</td>
<td>0,22 (0,06-0,80)</td>
</tr>
<tr>
<td>H7</td>
<td>T</td>
<td>T</td>
<td>C</td>
<td>33,73</td>
<td>33,19</td>
<td>0,48 (0,24-0,96)</td>
</tr>
</tbody>
</table>

Likelihood ratio G=12,99; ss=3; p=0,043.
Some clinical features could be associated with drug resistance, in particular early onset of seizures, high seizure frequency prior to treatment initiation, a history of febrile seizures, type of seizures (especially partial seizures) or epilepsy, structural brain lesions, and various malformations of cortical development (47). The present study revealed differences in main characteristics between the resistant and nonresistant group. Earlier onset and longer duration of the disease were recorded in the former, and earlier epilepsy progression from partial seizure to secondary generalized form of seizure in the latter. Contrary to the nonresistant group, patients in the resistant group had significantly higher focally altered EEG findings. Aikia et al. (48) report that focal epileptic EEG activity could be one of the predictive factors for pharmacoresistance, which is confirmed by our results.

An important fact is the compelling evidence that candidate gene strategies are dependent on ethnic stratification, and divergent results could be due to ethnic and racial differences in frequency distribution of polymorphic alleles. Significant interethnic variability was documented for frequencies of MDR1 variants. The frequency of exon 26, C3435 allele is 43%-54% in Caucasians, 46%-61% in Asians and 73%-90% in Africans (17,39,40,49). Documented frequency of exon 21, G2677 allele is 57% in Caucasians, 43% in Japanese and 34% in Indian population (50). Some studies point out that the high frequency of C3435 allele in Africans could be connected to the high frequency of more aggressive and drug resistant tumors, like breast carcinoma in subjects of African origin (51,52).

In conclusion, according to our results, ABCB1 variants C1236T, G2677T and C3435T might be associated with therapeutic response to AED in patients with partial epilepsy with or without secondary generalization and represent the possible predictive factor for pharmacoresistance.

REFERENCES


Address for Correspondence: Davor Sporiš, MD, Department of Neurology, Dubrava University Hospital. Av. Gojka Šuška 6, 10000 Zagreb, Croatia; E-mail: davor.sporis@kbd.hr