

ORIGINAL ARTICLE

# Association of Multidrug Resistance in Epilepsy with a Polymorphism in the Drug-Transporter Gene ABCB1

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ABSTRACT

**BACKGROUND**

One third of patients with epilepsy have drug-resistant epilepsy, which is associated with an increased risk of death and debilitating psychosocial consequences. Because this form is resistant to multiple antiepileptic drugs, the mode of resistance must be non-specific, involving drug-efflux transporters such as ATP-binding cassette sub-family B member 1 (ABCB1, also known as MDR1 and P-glycoprotein 170). We hypothesized that the CC genotype at the ABCB1 C3435T polymorphism, which is associated with increased expression of the protein, influences the response to antiepileptic-drug treatment.

**METHODS**

ABCB1 3435 was genotyped in 315 patients with epilepsy, classified as drug-resistant in 200 and drug-responsive in 115, and 200 control subjects without epilepsy. Recently devised methods were used to control for population stratification, and linkage disequilibrium was calculated across the gene.

**RESULTS**

As compared with patients with drug-responsive epilepsy, patients with drug-resistant epilepsy were more likely to have the CC genotype at ABCB1 3435 than the TT genotype (odds ratio, 2.66; 95 percent confidence interval, 1.32 to 5.38;  $P=0.006$ ). There was no genetic stratification between the two groups of patients. The polymorphism fell within an extensive block of linkage disequilibrium spanning much or all of the gene, implying that the polymorphism may not itself be causal but rather may be linked with the causal variant.

**CONCLUSIONS**

These pharmacogenomic results identify a genetic factor associated with resistance to antiepileptic drugs.

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**T**HE CONTINUED OCCURRENCE OF SEIZURES despite the use of several antiepileptic drugs, even as polytherapy at maximal tolerated doses, is a major health problem and increases the risk of death from epilepsy. None of the reported associations with clinical drug resistance, such as remote symptomatic causes, early onset, multiple seizure types, and a high frequency of seizures before the initiation of treatment, provide a mechanistic explanation of the phenomenon.<sup>1-3</sup> In addition, it is not clear why the same type of epilepsy may be drug-resistant in one person and drug-responsive in another and whether this is a pharmacogenomic phenomenon.

Drug resistance in epilepsy constitutes a true multidrug-resistance phenotype, since antiepileptic drugs act through diverse mechanisms. Most antiepileptic drugs are planar lipophilic agents and thus theoretical substrates for the ATP-binding cassette subfamily B member 1 (ABCB1) transporter (also known as MDR1 and P-glycoprotein 170), the up-regulation of which causes a multidrug-resistance phenotype in cancer cells.<sup>4</sup> ABCB1 can transport at least four antiepileptic drugs.<sup>5</sup> In patients with epilepsy, the multidrug-resistance phenotype is associated with increased lesional ABCB1 levels in resected brain tissue.<sup>6-11</sup> Thus, the ABCB1 gene is an important candidate gene potentially influencing the response to antiepileptic drugs. To evaluate its involvement in drug resistance in epilepsy and explore its pharmacogenomics, we typed the ABCB1 polymorphism (C to T) at position 3435 in 315 patients with epilepsy classified as either drug-resistant or drug-responsive. The CC genotype is associated with both increased levels and increased activity of ABCB1 protein.<sup>12</sup>

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## METHODS

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### SUBJECTS

The study was approved by the joint research ethics committee of the National Hospital for Neurology and Neurosurgery and the University College London Institute of Neurology in London. All patients gave written informed consent. All patients were recruited through the same epilepsy clinic at the National Hospital for Neurology and Neurosurgery and were consecutive patients with an established clinical diagnosis of epilepsy (defined by the occurrence of two or more unprovoked seizures). Patients were eligible if they had either drug-resistant or drug-responsive epilepsy according to the following

definitions and criteria. Drug resistance was defined as the occurrence of at least four seizures over the year before recruitment with trials of more than three appropriate antiepileptic drugs at maximal tolerated doses, which were established on the basis of the occurrence of clinical side effects at supra-maximal doses. Where it was possible, only the recognized, more specific drugs for a syndrome were considered appropriate — for example, failure of a case of primary generalized epilepsy to respond to carbamazepine was not considered a failure for the purposes of this study.

Drug responsiveness was defined as complete freedom from seizures for at least a year, up to the date of the last follow-up visit, in patients with epilepsy treated with antiepileptic drugs. Of the patients who were invited to participate, six declined (less than 2 percent). Control subjects were 200 consecutive white patients with neurologic conditions without epilepsy who were identified from a DNA repository at the Institute of Neurology. Patients who had undergone surgical treatment for their drug-resistant epilepsy were classified as having drug-resistant disease irrespective of the outcome of surgery. All DNA was analyzed and all studies were conducted in a blinded fashion.

### GENOTYPING AT ABCB1 3435

DNA was extracted from blood samples according to standard methods. Samples from the first 262 patients and 90 controls were genotyped by Epidauros by an automated method on purified genomic DNA by a TaqMan assay specifically devised to distinguish the C3435T polymorphism of the human ABCB1 gene. The C3435T polymorphism was analyzed in samples from the remaining patients and controls in the United Kingdom by direct sequencing with use of information supplied by Epidauros. Amplified polymerase-chain-reaction (PCR) products were sequenced with the use of forward and reverse primers, the Big Dye Terminator cycle sequencing kit (Perkin Elmer), and an automated sequencer (model 377, ABI). The sequences were analyzed with use of the Sequence Navigator program (Perkin Elmer).

### GENOMIC CONTROL

To address the possibility that any observed differences in allele frequency between drug-resistant and drug-responsive populations might be due to an unsuspected population structure, we used a set of genetic markers with intermediate allele frequencies

that were not linked to ABCB1. The markers used — polymorphic Alu insertion markers — code for variable lengths of amino acid residue repeats.

Nine polymorphic Alu insertion markers were typed on the basis of their heterozygosity and location in the human genome.<sup>13,14</sup> Alu insertion polymorphisms were amplified by PCR in all 315 patients with epilepsy. In a 50- $\mu$ l reaction, 50 to 100 ng of genomic DNA was used with 40 pmol of each primer, 2.5 mM of deoxynucleoside triphosphate, Promega Taq DNA polymerase (0.2 U), and 5  $\mu$ l of Taq DNA polymerase buffer (containing 1.2 mM magnesium chloride). Samples were amplified with the following cycles: initial denaturation of 4 minutes at 95°C, then 1 minute of denaturation (95°C), 1 minute at the annealing temperature, and 1 minute of extension (72°C), repeated for 35 cycles and followed by another 10-minute period of extension (72°C). PCR products were run on agarose gels with ethidium bromide and analyzed under ultraviolet light. Two Alu markers showed significant deviation from the expected population distribution (Hardy–Weinberg equilibrium), owing to genotyping errors. Results from these markers were therefore not included in the analysis: their inclusion, however, did not change the overall results.

#### CALCULATION OF LINKAGE DISEQUILIBRIUM

Resequencing was carried out on 32 Centre d'Etude du Polymorphisme Humain trios (mother, father, and child) to genotype single-nucleotide polymorphisms in intron 2 (single-nucleotide polymorphism identity rs2888599; available at <http://www.ncbi.nlm.nih.gov/SNP/>), exon 12 (C1236T and

C+44T),<sup>12,15</sup> exon 21 (G2677A),<sup>16</sup> and exon 26 (C3435T).<sup>12</sup> PCR conditions were as follows: 15 minutes of initial denaturation at 95°C, then 35 cycles of denaturation (94°C), annealing (57°C), and extension (72°C), each for 30 seconds, and a final 10-minute period of extension (72°C). PCR was carried out with 50 ng of DNA, 2.5 mM magnesium chloride, 0.2 mM of each deoxynucleoside triphosphate, 0.5  $\mu$ M of each primer, and 0.25 U of HotStarTaq polymerase (Qiagen), in a total volume of 10  $\mu$ l. PCR products were sequenced in the forward direction with the Big Dye Terminator cycle sequencing kit, cleaned by standard isopropanol precipitation and analyzed with an ABI Prism automated sequencer (model 3700, Applied Biosystems).

#### STATISTICAL ANALYSIS

Allele frequencies were compared between populations with use of the chi-square test. Observed and expected allele frequencies within populations were compared by means of the Hardy–Weinberg test. Correction for genetic stratification was undertaken with use of the method of Reich and Goldstein.<sup>17</sup> All reported P values are two-sided; a P value of less than 0.05 was considered to indicate statistical significance.

## RESULTS

As compared with the 115 patients with drug-responsive epilepsy, the 200 patients with drug-resistant epilepsy were more likely to have the CC than the TT genotype (odds ratio, 2.66; 95 percent confidence interval, 1.32 to 5.38;  $P=0.006$ ) (Table 1). The frequency of the CT genotype did not differ significantly between the two groups. Thus, the C polymorphism was overrepresented among patients with drug-resistant epilepsy as compared with those with drug-responsive epilepsy ( $P=0.008$ ). As compared with the 200 control subjects without epilepsy, patients with drug-resistant epilepsy had a higher frequency of the CC genotype and a lower frequency of the TT genotype ( $P=0.05$ ). The frequencies in the drug-responsive group did not differ significantly from those of ethnically matched control subjects or published reports.<sup>15</sup> No significant deviations from Hardy–Weinberg equilibrium were observed in either group of patients ( $P=0.39$  for the drug-resistant group and  $P=0.26$  for the drug-responsive group).

Unexpected differences in genetic backgrounds

Phenotype	Total No.	ABCB1 3435 Genotype		
		CC	CT	TT
		no. (%)		
Drug-resistant epilepsy	200	55 (27.5)	106 (53.0)	39 (19.5)
Drug-responsive epilepsy	115	18 (15.7)	63 (54.8)	34 (29.6)
Control	200	37 (18.5)	116 (58.0)	47 (23.5)

\* For all 315 patients with epilepsy, patients with drug-resistant epilepsy were more likely than those with drug-responsive epilepsy to have the CC genotype than the TT genotype ( $\chi^2=7.65$ ,  $P=0.006$ ). The results were similar when the analysis was restricted to the 297 white patients ( $\chi^2=6.66$ ;  $P=0.01$ ; odds ratio, 2.58; 95 percent confidence interval, 1.25 to 5.36).

between study groups — or population stratification — may result in spurious associations. To minimize overt population stratification, all subjects were from the same hospital clinic. In the drug-resistant and drug-responsive groups, 187 and 110 patients were white, respectively, and 13 and 5 patients were Asian, respectively ( $P=0.43$ ). The association persisted when the analysis was confined to white patients (Table 1). There was no significant difference between those with drug-resistant epilepsy and those with drug-responsive epilepsy with respect to proportions of partial, generalized, or unclassified epilepsy syndromes (data not shown).

To determine whether drug-resistant and drug-responsive groups might be covertly stratified, we analyzed unlinked genomic-DNA markers (Alu insertion markers) in disparate positions across the genome. There were no significant deviations from Hardy–Weinberg equilibrium for the seven markers within either the drug-resistant or drug-responsive group (Table 2). The mean  $\chi^2$  statistic for the comparison of allele frequencies between the drug-resistant and drug-responsive groups was 0.94, implying that the two cohorts were not significantly stratified. As an additional correction for stratification,<sup>17</sup> we divided the observed  $\chi^2$  by the maximal  $\chi^2$  across unlinked sites<sup>17</sup> consistent with the observed mean value of 0.96 and found that the ABCB1 3435 C allele remained significantly overrepresented among patients with drug-resistant epilepsy as compared with those with drug-responsive epilepsy ( $P<0.05$ ).

Genotyping in 32 European trios (mother, father, and child) of ABCB1 polymorphisms in intron 2 and exons 12a, 12b, 21, and 26 showed a high degree of linkage disequilibrium spanning at least between 12a and 26 (Table 3). The linkage disequilibrium may also extend upstream of exon 12, but the marker upstream of exon 12 was not sufficiently polymorphic to estimate the level of association. A simple pattern of groupings of single-nucleotide polymorphisms, or haplotype, was observed, in which five common haplotypes accounted for 88.5 percent of parental chromosomes for which complete phase information was available (haplotypes A, B, C, D, and E) (Table 4).

## DISCUSSION

A role for ABCB1 in resistance to treatment of epilepsy has biologic plausibility. ABCB1 contributes to drug resistance in other diseases, including many

**Table 2. Distribution of Alu Insertion Polymorphisms among the Patients with Drug-Resistant and Drug-Responsive Epilepsy.**

Alu Insertion Polymorphism and Group	Chromosome No.	<i>no. of patients</i>			P Value*
		+/+ Genotype	+/- Genotype	-/- Genotype	
YCIRG68	7				0.17
Drug-resistant		15	56	129	
Drug-responsive		6	27	82	
YB8NBC65	6				0.57
Drug-resistant		21	78	101	
Drug-responsive		13	48	54	
YA5NBC102	17				0.16
Drug-resistant		14	76	110	
Drug-responsive		5	37	73	
YA5NBC27	11				0.19
Drug-resistant		20	64	116	
Drug-responsive		8	33	74	
YA5NBC51	3				0.88
Drug-resistant		41	86	73	
Drug-responsive		26	46	43	
YA5NBC5	2				0.90
Drug-resistant†		12	57	129	
Drug-responsive		7	34	74	
YA5NBC109	22				0.73
Drug-resistant		40	110	50	
Drug-responsive		23	60	32	

\* The P values relate to the results of  $\chi^2$  tests with one degree of freedom for differences in allele frequencies between patients with drug-resistant epilepsy and those with drug-responsive epilepsy. A P value of less than 0.05 corresponds to a  $\chi^2$  value of more than 3.84.

† Two patients could not be genotyped.

forms of cancer,<sup>19</sup> rheumatoid arthritis,<sup>20</sup> and inflammatory bowel disease.<sup>21</sup> The postulated role of a gene product in disease pathophysiology may be supported by the demonstration that polymorphisms in the encoding gene can influence that role. In whites, the ABCB1 3435 TT genotype is associated with low levels of ABCB1 in enterocytes,<sup>12</sup> CD56+ natural killer cells,<sup>22</sup> and peripheral-blood mononuclear cells.<sup>23</sup> In comparison to the CC genotype, the TT genotype is also associated with functional restriction, being associated with higher plas-

**Table 3. Pairwise Comparisons for Linkage Disequilibrium among Five Single-Nucleotide–Polymorphism Loci in ABCB1.\***

Locus of Single-Nucleotide Polymorphism	Locus of Single-Nucleotide Polymorphism				
	Intron 2	Exon 12a	Exon 12b	Exon 21	Exon 26
Intron 2	—	P=1.00	P=1.00	P=1.00	P=0.39
Exon 12a	r <sup>2</sup> =0.01	—	P=0.29	P<0.001	P<0.001
Exon 12b	r <sup>2</sup> =0.002	r <sup>2</sup> =0.07	—	P=0.06	P=0.006
Exon 21	r <sup>2</sup> =0.01	r <sup>2</sup> =0.61	r <sup>2</sup> =0.06	—	P<0.001
Exon 26	r <sup>2</sup> =0.02	r <sup>2</sup> =0.20	r <sup>2</sup> =0.12	r <sup>2</sup> =0.40	—

\* The r<sup>2</sup> values were obtained according to the method of Weir.<sup>18</sup> The P values were obtained with Fisher's exact test. Genotyping was carried out on 32 white European trios (mother, father, and child).

**Table 4. Haplotypes of Five Single-Nucleotide–Polymorphism Loci in ABCB1.**

Haplotype Identification Code	Haplotype*					Frequency†
	Intron 2	Exon 12a	Exon 12b	Exon 21	Exon 26	
						%
A	1	0	1	0	1	34.6
B	1	1	1	1	0	23.1
C	1	1	1	1	1	15.4
D	1	1	1	0	1	7.7
E	1	1	0	1	0	7.7
F	1	0	1	1	0	5.8
G	1	0	1	0	0	3.8
H	0	1	1	1	0	1.9

\* The number 1 denotes a major allele, and 0 a minor allele.

† The frequency was calculated with the use of 52 parental haplotypes from 32 white European trios (mother, father, and child) for which complete phase information was available.

polymorphism in ABCB1 is associated with drug resistance in epilepsy, irrespective of the type of epilepsy or antiepileptic drug used.

Although the association with variation in the ABCB1 gene is strong, recent evidence that the human genome is structured in blocks of linkage disequilibrium<sup>26</sup> and the fact that this well-known C3435T polymorphism is silent raise the possibility that the polymorphism is not itself causal but rather in linkage disequilibrium with the causal variant. Our data show evidence of linkage disequilibrium extending from at least exon 26 to exon 12, and possibly farther. Consistent with other recent evidence, we also showed that the studied region of the ABCB1 gene has a simple haplotype structure, involving five common haplotypes. These common haplotypes formed a consistent network compatible with a process of mutation without recombination (although the rare haplotypes displayed inconsistencies that could have arisen from rare recombination events). Thus, the low r<sup>2</sup> values between some single-nucleotide polymorphisms reported in Table 3 do not indicate increased levels of recombination but rather different mutational clades, or groups. This implies that there could be many polymorphisms included within this block of linkage disequilibrium that have high r<sup>2</sup> with the C3435T polymorphism, if they occurred in a similar part of the genealogy as the C3435T polymorphism. Thus, it may be possible to obtain a reliable clinical prediction of drug resistance without identifying the causal variant, but the possibility that there may be different patterns of association in different populations means that care may be necessary in applying such predictors to heterogeneous populations.

In fact, the extensive linkage disequilibrium across ABCB1 illustrates an analytic disadvantage of the block structure of linkage disequilibrium in the human genome<sup>26</sup>: association data may not provide precise information about the location of the causal variant.<sup>27</sup> The final identification of the causal site may require detailed functional analyses. We note, however, that ABCB1 may be unusual in this regard, since it has an intermediate phenotype of high prevalence that could help pinpoint the causal variant. For example, although there is a high level of linkage disequilibrium across the gene, some of the rare haplotypes involving C3435T appear to have been generated by recombination. Thus, if association is incomplete, assessment of ABCB1 levels in persons with rare recombinant haplotypes may help pinpoint the variant responsible for altered expres-

ma levels of the substrate digoxin.<sup>12</sup> Results from other studies are conflicting: one suggested that the TT genotype is associated with increased ABCB1 levels and decreased plasma levels of the substrate fexofenadine in white American and black American subjects,<sup>24</sup> whereas no difference in plasma fexofenadine levels was seen among white German subjects.<sup>25</sup> We have demonstrated that the C3435T

sion. Such an assessment could be undertaken in resected brain tissue from patients with epilepsy.

There is mechanistic plausibility for the observed association between ABCB1 and drug-resistant epilepsy. The fluorescence of rhodamine allows quantitative determination of alterations in its intracellular retention caused by changes in the activity of ABCB1, for which it is a substrate. By comparing the intracellular retention of rhodamine under various controlled conditions, Hitzl et al. determined the influence of the C3435T polymorphism on ABCB1 transport.<sup>22</sup> In peripheral-blood CD56+ leukocytes from volunteers, they demonstrated reduced rhodamine retention, a marker of increased ABCB1 drug transport, in leukocytes from subjects with the CC genotype, as compared with subjects with the TT genotype. Leukocytes from subjects with the CT genotype had intermediate function, providing evidence of the existence of a nucleotide-sequence-dependent level of ABCB1 function in these subjects. These findings were subsequently confirmed.<sup>24</sup> Leukocyte levels of ABCB1 messenger RNA showed a similar trend; the highest levels were in subjects with the CC genotype and the lowest levels in subjects with the TT genotype. Rhodamine transport is a robust marker of the drug-transport capacity of ABCB1.<sup>28</sup> These findings show that the C3435T polymorphism is truly linked to the drug-transport capacity of ABCB1 and provide an underlying mechanism.

The site of action of the observed association is likely to be within brain parenchyma. ABCB1 is present in the blood-brain barrier, and ABCB1-knockout mice have higher brain levels of ABCB1 substrates than do wild-type mice.<sup>29</sup> Although the CC genotype was weakly associated with reduced serum levels of phenytoin, an ABCB1-substrate antiepileptic drug, in Turkish subjects,<sup>30</sup> all our patients with drug-resistant epilepsy had received their own individual maximal tolerated doses of antiepileptic drugs. Thus, it would seem unlikely that polymorphisms in genes encoding drug-metabolizing proteins could explain our results; no such polymorphisms have yet been correlated strongly with drug-resistant epilepsy. In addition, overexpression of ABCB1 by parenchymal glial cells in the brain,

which effectively constitutes a second blood-brain barrier, has been identified in resected specimens from subjects with drug-resistant epilepsy and is confined to the epileptogenic region.<sup>9</sup> The majority (80 percent) of such surgically treated subjects become seizure-free after lesional resection, even with reduced antiepileptic-drug dosage, suggesting that localized overexpression within the epileptogenic region of the brain is more important than expression elsewhere, such as the duodenum. In mice, systemic pharmacologic inhibition of ABCB1 leads to increased levels of the ABCB1 substrate nelfinavir in brain, with only small changes in plasma levels.<sup>31</sup>

Our data show that the drug-resistant phenotype in epilepsy is associated with the ABCB1 3435 polymorphism. Although the patients with drug-resistant epilepsy and those with drug-responsive epilepsy were not stratified, all were seen at one referral center and thus were not random patients. Therefore, we cannot infer from these data the probability of resistance related to ABCB1 genotype in the general population of patients with epilepsy. Within the patient base studied, however, the probability of resistance is 75.3 percent for the CC genotype and 53.4 percent for the TT genotype. Therefore, genetic factors other than ABCB1 or environmental factors (or both) must also contribute to the response. Probably not all antiepileptic drugs are ABCB1 substrates, since some, such as vigabatrin, are not lipophilic planar molecules. Indeed, drug resistance in epilepsy is most likely multifactorial, compounded by unidentified mechanisms.

Our results suggest new avenues for early molecular prediction of drug resistance in patients with epilepsy. Use of antiepileptic drugs that are not ABCB1 substrates, inhibition of ABCB1,<sup>32</sup> or the development of drugs that can evade ABCB1 might improve the efficacy of treatment in some patients with drug-resistant epilepsy.

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