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Association analysis of intractable epilepsy with C3435T and G2677T/A *ABCB1* gene polymorphisms in Iranian patients

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ABSTRACT – *Objective*. The results from studies investigating a possible association between *ABCB1* polymorphism and drug-resistant epilepsy are so far inconsistent. Moreover, recent meta-analyses studies do not confirm any link between *ABCB1* C3435T polymorphism and drug resistance. Yet, if patients with comparable clinical status (same type of epilepsy, antiepileptic drugs, epilepsy onset and gender) are evaluated, the link between *ABCB1* polymorphisms and drug resistance may be unmasked. We studied the association between C3435T and G2677T/A *ABCB1* gene polymorphisms and drug resistance in Iranian epilepsy patients. *Methods*. Two hundred healthy subjects and 332 epilepsy patients (200 drug-responsive and 132 drug-resistant) were selected. Genotypes were determined by polymerase chain reaction followed by restriction fragment length polymorphism or the amplification refractory mutation system. *Results*. The risk of drug resistance was higher in patients with a C/T genotype than in those with C/C or T/T genotypes at position 3435 in patients with cryptogenic epilepsy (p=0.01). A higher risk of drug resistance was observed in adult patients with a C/C genotype than in those with a T/T genotype at position 3435 (25.8% *vs* 15.8%, p=0.01). The risk of drug resistance was also higher in female patients with a C/C genotype than in those with a T/T genotype at position 3435 (26.8% *vs* 16.3%, p=0.04). No significant association was found between G2677T/A polymorphism and epilepsy drug resistance in the

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different subgroups of patients. *Conclusion*. Iranian adult female patients with a C/C genotype at position 3435 of the *ABCB1* gene have a higher risk of resistance to antiepileptic drugs. Replication studies with large sample sizes are needed to confirm the results.

Key words: *ABCB1*, drug-resistant epilepsy, Iranian, single nucleotide polymorphism

Epilepsy is the second most common neurological disorder after stroke (Porter and Meldrum, 2001). Although new antiepileptic drugs (AEDs) have been available since the late 1980s, resistance to treatment is still an important issue in epilepsy care. Only two thirds of patients are seizure-free under pharmacological treatment (Kwan and Brodie, 2000).

Mechanisms responsible for drug-resistant epilepsy are complex and not completely understood (Beck, 2007). Many factors ranging from acquired to genetic are involved in resistance to AEDs which affect AED pharmacokinetics or pharmacodynamics. One of the factors affecting the pharmacokinetics of AEDs which reduces the accumulation of AEDs in the seizure foci is over-expression of efflux drug transporters at the blood-brain barrier (Sisodiya, 2003; Loscher and Potschka, 2005). P-glycoprotein (P-gp) is an energy-dependent efflux pump that expels several AEDs (Potschka *et al*., 2001; Potschka and Loscher, 2001; Loscher and Potschka, 2002; Sisodiya, 2003). This protein is the product of an ATP-binding cassette subfamily b member 1 (*ABCB1*), also known as the multi-drug resistance 1 (*MDR1*) gene (Sisodiya, 2003). It has been suggested that increased brain expression of efflux transporters, such as P-gp, could be a result of genetic factors, such as polymorphisms in the *ABCB1* gene (Loscher and Delanty, 2009). Single nucleotide polymorphisms (SNPs) in the *ABCB1* gene have been shown to be associated with refractory epilepsy by many researchers at different nucleotide positions and such polymorphisms include T129C and T1236C in exon 12, G2677T in exon 21 and C3435T in exon 27 (Siddiqui *et al*., 2003; Tan *et al*., 2004; Zimprich *et al*., 2004; Sills *et al*.,2005; Hung *et al*., 2005, Hung *et al*., 2007; Seo *et al*., 2006; Kim *et al*., 2006a, Kim *et al*., 2006b; Leschziner *et al*., 2006, Leschziner *et al*., 2007; Shahwan *et al*., 2007; Ebid *et al*., 2007; Kwan *et al*., 2007; Ozgon *et al*., 2007; Dericioglu *et al*., 2008; Lakhan *et al*., 2009; Kim *et al*., 2009). However, the results are not consistent and have yet to confirm an association. A correlation between *ABCB1* gene polymorphism and antiepileptic drug responses was also not identified in recent meta-analyses studies (Bournissen *et al*., 2009; Nurmohamed *et al*., 2010; Haerian *et al*., 2010).

To better understand drug resistance in epilepsy, multiple aspects including clinical factors (aetiology, early age at seizure onset, type of epileptic syndrome and seizure, and structural brain abnormalities or lesions) should be considered (Regesta and Tanganelli, 1999; Kwan and Brodie, 2002; Loscher, 2005; French, 2007). In most studies of *ABCB1* polymorphism and drug-resistant epilepsy, in addition to variation in phenotype definition (definition of resistance and response to AEDs), patients with multiple types of epilepsy taking different types of AEDs were enrolled in the studies. The multiplicity of factors involved may affect the results and lead to distorted and/or varied findings (Loscher *et al*., 2009). Hypothetically, classification of drug-resistant epilepsy patients into subgroups based on clinical, and non-clinical specifications and analysis of association between polymorphisms and drug-resistant epilepsy in each subgroup, may lead to more accurate results.

According to a report from the Iranian Epilepsy Association, there were about 80,000 registered epilepsy patients in Iran until the end of 2007 (Iranian Epilepsy Association, 2008) of whom 25,000 patients were resistant to drug therapy. The possible association between *ABCB1* polymorphism and drug-resistant epilepsy in the Iranian population has not yet been studied. If an association exists, it may help the early diagnosis of drug-resistant epileptic patients, increasing the success of therapy and reducing the cost imposed on patients and the health care system. We have recently identified that Iranian female patients with AED-resistant epilepsy are more likely to have a C/C genotype than T/T genotype at position 1236 of the *ABCB1* gene (Maleki *et al*., 2010). In the study presented here, we investigated a possible association between two other widely investigated SNPs of the *ABCB1* gene,

Abbreviations

ABCB1: ATP-binding cassette subfamily B member 1
AEDs: antiepileptic drugs
AED: antiepileptic drug
ARMS: amplification refractory mutation system
CI: confidence interval
DNA: deoxyribonucleic acid
OR: odds ratios
P-gp: P-glycoprotein
PCR: polymerase chain reaction
RFLP: restriction fragment length polymorphism
SNP: single nucleotide polymorphism

C3435T (rs1045642) and G2677T/A (rs2032581), and drug-resistant epilepsy in Iranian epileptic patients. Possible associations were investigated using patients stratified by age, gender and aetiology of epilepsy.

Methods

Subjects

The study was approved by the ethics committee of the Pasteur Institute of Iran and conforms to the declaration of Helsinki. A total of 132 patients with drug-resistant seizures and 200 patients with drug-responsive seizures were enrolled in the study. All Iranian subjects who had been receiving antiepileptic drug treatment for at least one year were recruited from the epilepsy clinic of Loghman hospital, Shahid Beheshti University of Medical Sciences. Control subjects were recruited from the Pasture Institute of Iran. All subjects participated in this study voluntarily. Written informed consent was obtained from all subjects following a complete description of the study. A 5-mL venous blood sample was taken for DNA extraction and genotyping. Subject information and genotype data were identified by a code to ensure that the genotyping was performed blind.

Phenotyping

Three groups were defined: drug-responsive epileptics, drug-resistant epileptics and normal non-epileptic subjects. Patients who had not experienced any seizure for at least one year up to the date of enrolment, and received a stable dose of an AED, were considered drug-responsive (Kwan and Brodie, 2000). Patients who had at least one seizure per month or 10 seizures over the previous year, despite treatment with two or more antiepileptic drugs at the maximally tolerated doses and therapeutic serum drug concentrations, were considered to be drug-resistant (Hung *et al*., 2005; Kwan *et al*., 2007). Subjects without epilepsy or any history of epilepsy were considered as normal.

Genotyping for C3435T and 2677A polymorphisms

Genomic DNA was extracted from peripheral blood leukocytes using the standard salting out extraction method (Miller *et al*., 1988) and diluted to a final concentration of 20 ng/μL with 1×TE buffer (pH 7.5). Polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP) analysis was used to obtain the genotypes of the two groups. 200 ng of genomic DNA were amplified in a 25 μl reaction containing 10 pmol of forward and reverse primers (listed in *table 1*), 1x PCR buffer (10 mM Tris hydrochloride pH 8.5, 50 mM potassium chloride), 0.2 mM deoxynucleotide triphosphates, 1.5 mM magnesium chloride, and 1 U of SmarTaq DNA polymerase (Cinnagen, Iran). The PCR conditions were as follows: an initial denaturation step at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 60 s, annealing for 30 s at 58°C, an extension step at 72°C for 60 s, and a final extension step at 72°C for 5 min. PCR products were digested with respective restriction endonucleases at 37°C for 8 h in enzyme buffers. Digested PCR products were run on 2.5% agarose gel and the bands were visualized under ultraviolet light after staining with ethidium bromide. The RFLP genotyping methods were verified using a 100% concordance rate after sequencing eight PCR products of each genotype. The SNP genotyping method including primer sequences, PCR product length, restriction endonucleases and genotype determination is summarized in *table 1*.

Genotyping for G2677T polymorphism

The amplification of exon 22 of the *ABCB1* gene was carried out via the Amplification Refractory Mutation System (ARMS) using four primers. PCR was carried out in a total volume of 25 μl using about 50 ng of genomic DNA, 10 pmol of each forward and reverse primer, 2.5 mM dNTP, 10x Buffer and 2 U of SmarTaq™DNA polymerase (Invitrogen). The PCR conditions were as follows: an initial denaturation at 95°C for 5 min followed by 27 cycles of denaturation at 95°C for 30 s, annealing at 68°C for 40 s, and synthesis at 72°C for 60 s. The final synthesis was carried out for 5 min at 72°C. The products were separated on a 1.5% agarose gel. The ARMS-PCR method was verified using a 100% concordance rate after sequencing eight PCR products of each genotype. The SNP genotyping method including primer sequences, PCR product length, and genotype determination is summarized in *table 2*.

Data analysis

To evaluate the influence of patient age, patients were divided into child (≤12 years) and adolescent/adult (>12 years) subgroups. To evaluate the influence of aetiology of epilepsy, patients were divided into idiopathic, cryptogenic and symptomatic epilepsy subgroups. Seizures were classified as generalised tonic-clonic, partial, and complex partial. The SPSS for Windows version 11.5 software was used for statistical analysis. The Hardy-Weinberg equilibrium for genotype frequency distributions was verified using the chi-square goodness-of-fit test. The differences in genotype, sex and age frequencies between drug-responsive and drug-resistant patients were tested

Table 1. Primers and restriction endonucleases used for SNP genotyping.

SNP	Primer sequences	PCR product	Restriction endonucleases	Genotype determination
C3435T (rs1045642)	Forward: 5′ TTG ATG GCA AAG AAA TAA AGC 3′ Reverse: 5′ CTT ACA TTA GGC AGT GAC TCG 3′	207bp	MboI	TT: 207bp TC: 207bp/145bp/62bp CC: 145bp/62bp
2677A (rs2032581)	Forward: 5′ CCA TCA TTG CAA TAG CAG GA 3′ Reverse: 5′ AAG AAT GCT TTG AGG AAT GGT 3′	216bp	RsaI	A/A: 136bp/80bp A/X: 216bp/136bp/80bp

by binary logistic regression. The Unpaired Student's t-test was used to compare the age in the two groups. The level of significance for all statistical tests was 0.05.

Results

Demographic data

Demographic characteristics of the patients are presented in *table 3*. There was no significant difference between drug-responsive and drug-resistant patients regarding age. There was a higher proportion of male patients in the drug-resistant group (59.8%) compared with the drug-responsive group (48%, p=0.04). A significant difference between drug-resistant and drug-responsive patients was found with regards to epilepsy (p<0.001). There was a larger proportion of patients with localisation-related (cryptogenic or symptomatic) epilepsies in the drug-resistant group (92.5%) compared with the drug-responsive group (61.5%) (p<0.001). The AEDs administered to patients were phenytoin, phenobarbital, primidone, carbamazepine, valproate, oxcarbazepine, levetiracetam, lamotrigine, clonazepam and topiramate. Drug-resistant patients received two to four of the above-mentioned AEDs at the maximum tolerated doses and only one patient was treated with five AEDs.

Analysis of genotype frequencies

Our results indicate that both SNPs are polymorphic in the Iranian population *(tables 4, 5)*. The genotype distributions of both SNPs for both normal subjects and epileptic patients were consistent with the Hardy-Weinberg equilibrium. Genotype success rate was 100% for SNPs. The PCR-RFLP method identified wild-type heterozygous or homozygous variation at the two polymorphic sites.

Both Iranian epilepsy patients and normal subjects were more likely to have the T allele than the C allele at position 3435 of the *ABCB1* gene. The frequencies

Table 2. Primers used for ARMS-PCR genotyping.

SNP	Primer sequences	PCR product	Genotype determination
G2677 (rs2032581)	Forward: 5′ CAC TGA AAG ATA AGA AAG AAC TAG AAG GTG 3′ Reverse: 5′ GGA AAG TGG GGA GGA AGG AAG AAC 3′	811bp/559bp/309bp	G/G: 811bp/309bp G/T:811bp/559bp/309bp
T2677 (rs2032581)	Forward: 5′ ATT CCT AGT TTG TCA GAC TCC TTT ATC TTG 3′ Reverse: 5′ CAT ATT TAG TTT GAC TCA CCT TCC CAG A 3′	811bp/559bp/309bp	T/T: 811bp/559bp G/T: 811bp/559bp/309bp

Table 3. Demographic characteristics of epileptic patients.

Subgroup	Category	Drug-responsive patients	Drug-resistant patients	OR (95% CI)	P
Patient age in years (Mean ± SD)		27±13	28.8±11	-	0.19
Patient age groups	<12 years >12 years	10 (5%) 190 (95%)	4 (3%) 128 (97%)	1.68 (0.52-5.48) 1	0.39
Gender	Male Female	96 (48%) 104 (52%)	79 (59.8%) 53 (40.2%)	1.62 (1.03-2.52) 1	0.04
Type of seizure	Complex partial Generalised tonic-clonic Generalised tonic-clonic + complex partial Generalised tonic-clonic + partial	1 (0.5%) 199 (99.5%) 0 0	0 0 118 (89.4%) 14 (10.6%)		
Type of epilepsy	Complex partial Generalised tonic-clonic Juvenile myoclonic	122 (61%) 76 (38%) 2 (1%)	126 (95.5%) 6 (4.5%) 0	13.08 (5.49-31.16) 1	<0.001
Aetiology of epilepsy	Idiopathic Cryptogenic Symptomatic	77 (38.5%) 120 (60%) 3 (1.5%)	10 (7.5%) 118 (89.5%) 4 (3%)	1 0.13 (0.07-0.27) 1.36 (0.30-6.19	<0.001 0.69
Number of administered antiepileptic drugs	1 2 3 4 5	191 (99.5%) 9 (0.5%) 0 0 0	0 18 (13.6%) 88 (66.6%) 25 (18.9%) 1 (0.8%)		

Table 4. Genotype and allele frequencies of C3435T and G2677T/A in the *ABCB1* gene in normal non-epileptic subjects.

Nucleotide position	Genotype frequency	Allele frequency
C3435T (rs1045642)	C/C 47 (23.5%) C/T 90 (45%) T/T 63 (31.5%)	C 184 (46%) T 216 (54%)
G2677T/A (rs2032581)	T/T 73 (36.5%) G/G 45 (22.5%) G/T 72 (36%) A/T 7 (3.5%) A/G 3 (1.5%)	T 225 (56.2%) G 165 (41.3%) A 3 (1.5%)

Table 5. Genotype frequencies and drug resistance odds ratios for C3435T and G2677T/A *ABCB1* gene polymorphisms in Iranian epileptic patients.

Subgroup of age	Genotype	Drug-responsive patients	Drug-resistant patients	OR (95% CI)	P
C3435T (n=332)	C/C	32 (16.0%)	34 (25.7%)	2.17 (1.18-3.98)	0.01
	C/T	80 (40.0%)	55 (41.6%)	1.41 (0.85-2.32)	0.18
	T/T	88 (44.0%)	43 (32.6%)	1	
Adults (n=318)	C/C	30 (15.8%)	33 (25.8%)	2.20 (1.19-4.08)	0.01
	C/T	76 (40.0%)	53 (41.4%)	1.40 (0.84-2.32)	0.20
	T/T	84 (44.2%)	42 (32.8%)	1	
Children (n=14)	C/C	2 (20.0%)	1 (25.0%)	Small sample size	
	C/T	4 (40.0%)	2 (50.0%)		
	T/T	4 (40.0%)	1 (25.0%)		
G2677T/A (n=332)	G/G	36 (18.0%)	31 (23.5%)	1	
	G/T	97 (48.5%)	60 (45.5%)	0.72 (0.40-1.28)	0.26
	T/T	61 (30.5%)	37 (28.0%)	0.70 (0.37-1.32)	0.28
	A/G	2 (1%)	2 (1.5%)	1.16 (0.15-8.75)	0.89
	A/T	4 (2%)	2 (1.5%)	0.54 (0.01-3.39)	0.55
Adults (n=318)	G/G	34 (17.9%)	31 (24.2%)	1	
	G/T	93 (48.9%)	58 (45.3%)	0.68 (0.38-1.23)	0.21
	T/T	58 (30.5%)	35 (27.3%)	0.66 (0.35-1.26)	0.21
	A/G	4 (2.1%)	2 (1.6%)	0.55(0.09-3.21)	0.51
	A/T	1 (0.5%)	2 (1.6%)	2.19(0.19-25.40)	0.53
Children (n=14)	G/G	2 (20%)	0	Small sample size	
	G/T	4 (40%)	2 (50%)		
	T/T	3 (30%)	2 (50%)		
	A/G	1 (10%)	0		
	A/T	0	0		

of C/C and T/T genotypes in the control population were similar to those in drug-resistant patients *(tables 4, 5)*. The frequency of the C/C genotype in drug-resistant patients was significantly greater than that in drug-responsive patients. Both Iranian epilepsy patients and normal subjects were more likely to have the T allele than the G allele at position 2677 of the *ABCB1* gene *(tables 4, 5)*. The frequency of T/T, T/G or G/G genotypes at position 2677 did not differ significantly between drug-responsive and drug-resistant patients.

When patients were stratified by patient age, a significant association was observed between C3435T polymorphism and drug resistance in adults *(table 5)*. The odds ratios indicated a higher risk of drug resistance in patients with the genotype C/C than in those with the genotype T/T. In children, sample size was too small to enable us to analyze the genotype frequencies and determine the relationship between patient age and drug resistance.

With regards to aetiology of epilepsy, no significant association was found between C3435T polymorphism of the *ABCB1* gene and drug resistance in patients with idiopathic epilepsy *(table 6)*. Although there was a trend towards a higher risk of drug resistance in patients with cryptogenic epilepsy who had the C/C genotype, this was not statistically significant (p=0.05). The odds ratios indicated a higher risk of drug resistance in patients with the genotype C/T than in those with the genotype T/T or C/C. However, the association between aetiology of epilepsy and resistance or response to antiepileptic drugs was not significant.

When patients were stratified by gender, a significant association was observed between C3435T polymorphism and drug resistance in female (p=0.04) but not male patients *(table 7)*. The odds ratios indicated a higher risk of drug resistance in the female patients with the genotype C/C than in those with the genotype T/T.

Table 6. Genotype frequencies of C/T at position 3435 and G/T at position 2677 in the ABCB1 gene with odds ratios in patients with idiopathic or symptomatic epilepsy.

Aetiology of epilepsy	Genotype	Drug-responsive patients	Drug-resistant patients	OR (95% CI)	P
C/T at 3435 (n=332)					
Idiopathic (n=87)	C/C	7 (9%)	1 (10%)	1.07 (0.10-11.13)	0.95
	C/T	40 (52%)	5 (50%)	0.94 (0.23-3.79)	0.93
	T/T	30 (39%)	4 (40%)	1	
Cryptogenic (n=238)					
	C/C	24 (20%)	30 (25.4%)	1.96 (0.99-3.86)	0.05
	C/T	38 (31.6%)	51 (43.2%)	2.10 (1.16-3.79)	0.01
	T/T	58 (48.4%)	37 (31.3%)	1	
Interaction					0.57
Symptomatic (n=7)					
	C/C	1 (33.3%)	3 (75%)	Small sample	
	C/T	2 (66.7%)	0	size	
	T/T	0	1 (25%)		
G/T at 2677 (n=332)					
Idiopathic (n=87)	T/T	25 (32.5%)	4 (40%)	1	
	G/G	11 (14.3%)	0	0.46 (0-4.03)	0.52
	G/T	40 (52%)	5 (50%)	0.78 (0.15-4.3)	1.00
	A/T	1 (1.2%)	1 (10%)	5.7 (0.06-509.49)	0.60
	A/G	0	0		
Cryptogenic (n=238)	G/G	25 (20.8%)	29 (24.6%)	1	
	G/T	56 (46.7%)	54 (45.8%)	0.83 (0.41-1.68)	0.70
	T/T	36 (30%)	32 (27%)	0.77 (0.35-1.67)	0.58
	A/T	3 (2.5%)	1 (0.8%)	0.29 (0.005-3.92)	0.56
	A/G	0	2 (1.7%)	1.99 (0.15-+infinity)	0.60
Symptomatic (n=7)	G/G	0	2 (50%)	Small sample	
	G/T	1 (33.3%)	1 (25%)	size	
	T/T	0	1 (25%)		
	A/T	1 (33.3%)	0		
	A/G	1 (33.3%)	0		

Discussion

Results of the present study demonstrated that by analyzing all patients as a whole, genotype and allele frequencies of G2677T/A polymorphism of the *ABCB1* gene did not differ between drug-responsive and drug-resistant epilepsy patients. However, a higher risk of drug resistance was observed in patients with the C allele than in those with the T allele at position 3435. The T/T genotype at position 3435 was previously shown to be associated with decreased P-gp activity in European Caucasians (Hoffmeyer *et al*., 2000). Therefore, drug-resistant epilepsy in Iranians with the C allele might be caused by greater P-gp activity, extruding AEDs from the brain and leading to drug-resistant epilepsy.

There are plenty of studies which have investigated an association between C3435T polymorphism of the *ABCB1* gene and drug-resistant epilepsy, however, there is disagreement amongst the results (Loscher and Delanty, 2009). After the initial report indicating the more prevalent C/C genotype at position 3435 in drug-resistant epilepsy patients (Siddiqui *et al*., 2003), several studies of subjects with different ethnicities

Table 7. Genotype frequencies of C3435T and G2677T/A *ABCB1* gene polymorphisms with odds ratios in male and female epileptic patients.

Subgroup of age	Genotype	Drug-responsive patients	Drug-resistant patients	OR (95% CI)	P
C3435T					
Male (n=172)	T/T	40 (41.7%)	26 (34.2%)	1	
	C/T	41 (42.7%)	31 (40.8%)	1.16 (0.59-2.29)	0.66
	C/C	15 (15.6%)	19 (25.0%)	1.95 (0.84-4.50)	0.12
Female (n=160)	T/T	48 (46.2%)	17 (30.4%)	1	
	C/T	39 (37.5%)	24 (42.9%)	1.74 (0.82-3.68)	0.15
	C/C	17 (16.3%)	15 (26.8%)	2.49 (1.03-6.05)	0.04
G2677T/A					
Male (n=172)	T/T	30 (31.2%)	23 (30.3%)	1	
	G/T	47 (49.0%)	30 (39.5%)	0.83 (0.41-1.69)	0.61
	G/G	18 (18.8%)	19 (25.0%)	1.38 (0.59-3.20)	0.46
	A/G	0 (0%)	2 (2.6%)	Small sample size	
	A/T	1 (1.0%)	2 (2.6%)	Small sample size	
Female (n=160)	T/T	31 (29.8%)	14 (25.0%)	1	
	G/T	50 (48.1%)	30 (53.6%)	1.33 (0.61-2.89)	0.47
	G/G	18 (17.3%)	12 (21.4%)	1.48 (0.56-3.88)	0.43
	A/G	1 (1.0%)	0 (0%)	Small sample size	
	A/T	4 (3.8%)	0 (0%)	Small sample size	

have confirmed an association between C3435T and G2677T/A polymorphism and drug-resistant epilepsy (Hung *et al.*, 2007; Zimprich *et al.*, 2004; Hung *et al.*, 2005; Ebid *et al.*, 2007). In contrast, two studies of non-Caucasian epilepsy patients showed the opposite association of a high frequency of T/T genotype in drug-resistant compared to drug-responsive epilepsy patients (Seo *et al.*, 2006; Kwan *et al.*, 2007). Other studies in either Caucasian or non-Caucasian epilepsy patients were not able to demonstrate an association between *ABCB1* polymorphism and resistance to antiepileptic drugs (Tan *et al.*, 2004; Sills *et al.*, 2005; Kim *et al.*, 2006a, Kim *et al.*, 2006b; Leschziner *et al.*, 2006; Ozgon *et al.*, 2007; Shahwan *et al.*, 2007; Dericioglu *et al.*, 2008; Kim *et a*l., 2009; Lakhan *et al.*, 2009; Vahab *et al.*, 2009; Ufer *et al.*, 2009). Differences in results between studies have been mostly attributed to phenotype definition, small sample size, overlap in substrate specificity between P-glycoprotein and other drug efflux transporters, as well as to inclusion of AEDs that may not be P-glycoprotein substrates (Loscher *et al.*, 2009; Loscher and Delanty, 2009). In the present study, drug resistance was defined as failure of two or more AEDs with a seizure frequency of at least 10 per year. This is similar to criteria for drug resistance used by some other researchers (Zimprich *et al.*, 2004; Hung *et al.*, 2005; Hung *et al.*, 2007; Seo *et al.*, 2006; Kwan *et al.*, 2007). In all the studies that used this criterion, a positive association was found between C3435T polymorphism and drug-resistant epilepsy (Zimprich *et al.*, 2004; Hung *et al.*, 2005; Hung *et al.*, 2007; Seo *et al.*, 2006; Kwan *et al.*, 2007). However, when different criteria were used, some researchers identified an association (Siddiqui *et al.*, 2003; Ebid *et al.*, 2007), while some others did not (Tan *et al.*, 2004; Sills *et al.*, 2005; Kim *et al.*, 2006a; Kim *et al.*, 2006b; Leschziner *et al.*, 2006; Lakhan *et al.*, 2009; Dericioglu *et al.*, 2008). This was also the case for G2677T/A polymorphism and AED resistance. In this study, we did not find any differences in G2677T/A genotype frequencies between drug-resistant and drug-responsive Iranian patients with epilepsy. However, whereas some researchers, who used the same criteria for drug resistance as those used in this study, found an association between G2677T/A polymorphism and drug-resistant epilepsy (Zimprich *et al.*, 2004; Hung *et al.*, 2005; Hung *et al.*, 2007; Seo *et al.*, 2006), this was not the case for others who used a different definition for intractability (Kim *et al.*, 2006b; Lakhan *et al.*, 2009). There would therefore seem to be other important factors that influence drug-resistant epilepsy which give rise to a degree of variability between different studies leading to contradictory results. To determine drug resistance in epilepsy patients, multiple aspects including clinical factors (aetiology, early age at seizure onset, type of epilepsy syndrome and seizure, and structural

brain abnormalities or lesions) should be considered (Regesta and Tanganelli, 1999; Kwan and Brodie, 2002; Loscher, 2005; French, 2007). In most studies performed on *ABCB1* polymorphism and drug-resistant epilepsy, in addition to the use of different definitions of phenotype, patients with multiple types of epilepsy taking multiple AEDs were enrolled. These factors may affect the results and lead to variation in findings (Loscher *et al*., 2009). Thus, the meta-analyses studies that included the data of the above-mentioned studies have not found any association between *ABCB1* polymorphism and AED resistance (Bournissen *et al*., 2009; Nurmohamed *et al*., 2010; Haerian *et al*., 2010).

In order to unmask the effect of some of the variable factors, we stratified the patients according to age, gender or aetiology of epilepsy and analyzed the association between polymorphisms and drug resistance in the subgroups. Regarding C3435T *ABCB1* gene polymorphism and patient age, drug resistance in adult was more frequent for the patients with the genotype C/C than in those with the genotype T/T. With regards to G2677T/A polymorphism, no significant association was found between genotype and drug resistance in adult patients. The results for adults were similar to those for all patients analysed as a whole, since the majority of subjects in the study were adults and the number of children was low. With regards to C3435T polymorphism and patient gender, a lower risk of drug resistance was observed in female patients with the T allele than in those with C allele. This finding is inconsistent with a recent study (Kwan *et al*., 2009) which reported that C3435T is associated with drug resistance in male but not female patients. The authors explained this finding by the evidence indicating that female sex hormones at physiological levels down-regulate P-gp levels in the cells (Mutoh *et al*., 2006) and also inhibit P-gp activity (Ichikawa-Haraguchi *et al*., 1993; Frohlich *et al*., 2004). The discrepancy of our results with this study may be due to the different ethnicity of the subjects studied (Iranians versus Han Chinese), giving rise to the different pattern of linkage disequilibrium and resulting in a contradictory association between C3435T polymorphism and gender. We have recently found that the risk of drug resistance is lower in Iranian female patients with a T allele than in those with a C allele at position 1236 of the *ABCB1* gene (Maleki *et al*., 2010). The subjects of the present study and those of a previous study (Maleki *et al*., 2010) were the same and genotyping and statistical analyses were performed at the same time for both studies. In population-based studies bias may occur. In these two studies, *ABCB1* polymorphisms were in Hardy-Weinberg equilibrium, suggesting that Mendelian randomisation was present. These studies were performed in a double-blind manner. Neither the statistician nor the genotyping staff were aware of the demographic characteristics of the patients. However, a replication study with large sample size is required to confirm the findings. With regards to G2677T/A polymorphism, no significant association was found between genotype and drug-resistant epilepsy, neither in male nor female patients. Regarding aetiology of epilepsy, the risk of drug resistance was higher in patients with a C/T genotype than with C/C or T/T genotypes at position 3435 in patients with cryptogenic epilepsy but not in those with idiopathic epilepsy. Symptomatic epilepsy is reported to be more drug-resistant than idiopathic epilepsy (Kwan and Brodie, 2000; Kwan *et al*., 2007; Schiller and Najjar, 2008) as was also found in our study since 92.5% of drug-resistant patients had localisation-related (symptomatic or cryptogenic) epilepsies while 61.5% of drug-responsive patients had localisation-related epilepsies. In a recent report similar to this study, the association between *ABCB1* polymorphism and drug-resistant epilepsy was compared between Caucasian children and adolescents/adults (Blanca Sanchez *et al*., 2010). These investigators found that adults with a T/T genotype at position 3435 or 2677 had a lower risk of drug resistance than those with C/C or G/G genotypes. Furthermore, patients with symptomatic epilepsy with C/T or T/T genotypes at position 3435 had a lower risk of drug resistance than those with a C/C genotype. However, in two other studies in Turkish patients, a significant association between C3435T polymorphism and drug resistance was not found in a subgroup of patients with hippocampal sclerosis (Ozgon *et al*., 2007) or in patients who required resective brain surgery (Dericioglu *et al*., 2008).

It is well known that not all AEDs are substrates of human P-gp (Loscher *et al*., 2009). Studies using *in vitro* models have indicated that phenytoin, phenobarbital, lamotrigine and levetiracetam, but not valproate and carbamazepine, are transported by human P-gp (Cucullo *et al*., 2007; Luna-Tortos *et al*., 2008). Therefore, it is suggested that inclusion of patients treated with drugs that are not a substrate for P-gp is likely to reduce any association between *ABCB1* polymorphism and drug-resistant epilepsy (Loscher and Delanty, 2009). In our study, from 132 patients with drug-resistant epilepsy who received several AEDs such as phenytoin, phenobarbital, primidone, carbamazepine, valproate, oxcarbazepine, levetiracetam, lamotrigine, clonazepam and topiramate, just 10 patients took carbamazepine and valproate. Exclusion of the patients treated with carbamazepine and valproate did not affect the final result regarding the association between C3435T and G2677T/A polymorphisms and AED resistance. This is in line with the common clinical observation that many patients with drug-resistant epilepsy fail to respond to many

different drugs, including those AEDs that are (such as phenytoin and lamotrigine) and those that are not (*e.g.*, carbamazepine) substrates of P-gp transport (Loscher and Delanty, 2009).

Conclusion

Our results indicate a higher risk of drug resistance in Iranian epilepsy patients with a C/C genotype than in those with a T/T genotype at position 3435 of the *ABCB1* gene. Moreover, Iranian female epilepsy patients with a C/C genotype at position 3435 have a higher risk of drug resistance compared to those with a T/T genotype at position 3435. With regards to G2677T/A polymorphism, no significant association was found between genotypes and epilepsy drug resistance when patients were stratified by age, gender, and aetiology of epilepsy. A replication study with large sample size is required to confirm the present findings. Our results indicate that stratification of drug-resistant epilepsy patients into subgroups with comparable clinical status (same type of epilepsy, AEDs, epilepsy onset and other factors that influence drug resistance) and subgroup association analysis of polymorphisms and drug-resistant epilepsy may result in more consistent and replicable results. This point should also be borne in mind when the link between genetic polymorphism and risk of drug resistance is assessed by meta-analysis. □

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