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ORIGINAL ARTICLE

RLIP76 GENE VARIANTS ARE NOT ASSOCIATED WITH DRUG RESPONSE IN TURKISH EPILEPSY PATIENTS

Manguoğlu E[1,*], Akdeniz S[1], Dündar NO[2], Duman Ö[2],
Aktekin B[3], Haspolat Ş[2], Bilge U[4], Özel D[4], Lüleci G[1]

***Corresponding Author:** Esra Manguoğlu, Department of Medical Biology and Genetics, Faculty of Medicine, Akdeniz University, Antalya, Turkey;
Tel.: +90-242-249-6977; Fax: +90-242-249-6906; E-mail: emanguoglu@akdeniz.edu.tr

ABSTRACT

Approximately 30% of epileptic patients remain untreated, in spite of trials with maximum tolerable doses of more than one drug. The RalA binding protein 1 (RALBP1/RLIP76), a multifunctional, anti-apoptotic, multidrug transporter protein, has been proposed as being responsible for the drug resistance mechanism in epilepsy. We have investigated polymorphic differences in the coding regions and exon-intron boundaries of the *RLIP76* gene, between 146 refractory and 155 non refractory epileptic patients in Turkey, using denaturing high performance liquid chromatography (HPLC) and sequencing analysis techniques. We have detected the following sequence variants: c.160-4G>A, c.187C>G, c.1562-38G>A, c.1670+107G>A, c.1670+93G>A, c.1670+96G>A, c.1670+100C>T, c.1670+130C>T, c.1670+131G>C, c.1670+140 G>C, and found no statistically significant correlation between allele frequencies and drug response status. We conclude that sequence variants of this gene are not involved in drug resistance in epilepsy.

Keywords: Epilepsy; Neuropharmacology; Pharmacogenetics; Polymorphism; *RLIP76* gene

INTRODUCTION

Drug resistance in epilepsy is one of major obstacles in clinical medicine. In a 37-year follow-up study, 67% of 144 patients were reported to be in remission at the end of follow-up, while 14% of the patients relapsed after a period of remission and did not re-enter remission, and remaining 19% of the patients never entered remission during the whole period of follow-up [1]. Optimum doses of anti-epileptic drugs (AEDs), prognosis and treatment outcomes showed considerable variation in the patients and could mostly be attributed to genetic variation between individuals [2]. Genetic variants of some proteins that are responsible for drug absorption, distribution, metabolism and elimination, have been extensively studied [3].

The RalA binding protein 1 (RLIP76 or RALBP1) is a protein that has been proposed to be associated with drug resistance in epilepsy [4]. It binds ras-related v-ral simian leukemia viral oncogene homolog A (RalA) and modulates the Rho pathway through the Ras pathway [5]. It is also involved in the transport of glutathione (GSH) conjugates and xenobiotics and in modulating drug sensitivity in cancer cells [6]. It

[1] Department of Medical Biology, Faculty of Medicine, Akdeniz University, Antalya, Turkey
[2] Department of Pediatric Nuerology, Faculty of Medicine, Akdeniz University, Antalya, Turkey
[3] Department of Neurology, Yeditepe University Hospital, Istanbul, Turkey
[4] Department of Biostatistics and Medical Informatics, Faculty of Medicine, Akdeniz University, Antalya, Turkey

was shown that RLIP76 is the major ATP-dependent transporter of GSH conjugates as well as doxorubicin in human erythrocytes [7]. More recently, Rossé *et al.* [8], proposed that RLIP76 is required for Epsin phosphorylation to switch endoyctosis off. RLIP76 was shown to be required as an effector for PKCα that is protein kinase transmitting downstream signals in response to stressors such as chemotherapeutic agents [9]. Moreover, RLIP76 is induced by oxidative stress and has been suggested to play a role in insulin resistance under increased oxidative stress [10].

RLIP76 is expressed in normal human breast, heart, liver, erythrocytes, and, to a lesser extent in colon and brain parenchymal tissues [4]. Blood vessels from refractory and non refractory epileptic brain tissues showed higher levels of expression, predominantly in the endothelium. It was also demonstrated that RLIP76 is an important carbamazepine and phenytoin transporter in human epileptic brain tissue. As a result, investigators proposed that RLIP76 might be involved in a drug-resistant epilepsy mechanism [4]. On the contrary, immunohistochemical analysis revealed that RLIP76 was co-localized with a neuronal, but not epithelial marker, in normal and epileptic brain tissues. In addition, no association was found between six common single nucleotide polymorphisms (SNPs) of the *RLIP76* gene and 262 drug-responsive and 107 drug-resistant patients. Also, no correlation was found for susceptibility for epilepsy in 783 epilepsy patient and 359 healthy controls [11]. Moreover, a separate study failed to show any association between 13 common RLIP76 polymorphisms and drug-response in epilepsy in a prospective cohort of 503 epilepsy patients [12]. In this study, to further investigate the association between RLIP76 and drug response in epilepsy, we performed genotype analysis for the *RLIP76* gene and statistical analyses for any association in refractory and drug-responsive groups.

MATERIALS AND METHOD

Patients. A total of 146 (81 male and 65 female) refractory and 155 (79 male and 76 female) non refractory patients were recruited for the study. The mean age for refractory and non refractory groups were 34.4 ± 17.8 (ranging from 3 to 83 years) and 25.4 ± 17.5 (ranging from 2 to 78 years), respectively. One-hundred and forty-three the patients were above the age of 18, while 158 patients were below the age of 18.

Patients who were seizure free for at least 1 year were regarded as non refractory epilepsy patients. Patients, who had seizures, in spite of taking at least three different AEDs with maximum tolerable doses, were considered to be refractory epilepsy patients.

This study was approved by the Akdeniz University Ethics Committee (Antalya, Turkey). Information about the study was given to all patients. A signed, informed consent was obtained from all patients or from adults responsible for them. Patients were recruited from Akdeniz University Hospital, Departments of Pediatrics and Neurology (Antalya, Turkey). All patients were Turkish.

Genotyping. DNA isolation from peripheral leukocytes was performed by a standard salting-out method [13]. All coding exons (exons 2-10) and exon-intron boundaries of the *RLIP76* (RALBP1, NM_006788.3) gene were screened for sequence changes by denaturing high performance liquid chromatography (HPLC). The polymerase chain reaction (PCR) primer sequences are given in Table 1. The PCR reactions were set up in 20 ML volume with 10-20 ng DNA, 0.5 U Optimase Taq DNA polymerase, using a PE9700 thermal cycler [Applied Biosystems Inc. (ABI), Foster City, CA, USA]. Amplifications were done by touch-down PCR with annealing temperature with decreasing increments of 0.5°C in each cycle starting from 61°C to 54°C. Denaturing HPLC analyses were performed on a Transgenomic (Omaha, NE, USA) Wave HS nucleic acid fragment analysis system with DNASep cartridges, WAVE Optimized buffers and standards. To increase the sensitivity of these analyses, they were performed on at least two different oven temperatures for each PCR fragment. All regions that have denaturing HPLC patterns suggesting a sequence change were sequenced successfully to define the sequence change. The PCR products were purified using a Roche High Pure PCR product purification kit (catalog no. 11 732 676 001; Roche Applied Science, Indianapolis, IN, USA). A direct sequencing protocol was applied and BigDye Terminator v.1.1 cycle sequencing system was used (Applied Biosystems). Sequencing samples were separated using an ABI 3130™ system (Applied Biosystems).

Statistical Analysis. When minimum expected values were less than 5, the Fisher's Exact test was used. When the minimum expected value was between 5 and 25, the Yate's Continuity Correction test was used. In all other circumstances, the Pearson $\chi 2$ (Chi-square) test was used. Statistical analysis was performed with SPSS version 15 (SPSS Inc., Chicago,

IL, USA); p <0.05 was considered to be statistically significant.

RESULTS

We have not detected any sequence change in the coding regions of the gene. However, two substitutions within the 5'UTR (5' untranslated region) (c.160-4G>A, c.187 C>G); eight substitutions within the intronic regions (c.1562-38G>A, c.1670+107G>A, c.1670+93G>A, c.1670+96G>A, c.1670+100C>T, c.1670+130C>T, c.1670+131G>C, c.1670+140G>C) were detected. The c.187G>C change was detected in two refractory and one non refractory patients, the c.1670+93G>A change was detected in four refractory and six non refractory patients, the c.1670+96G>A and c.1670+100C>T changes were detected in one refractory patient and the c.1670+131G>C and c.1670+140G>C changes in one non refractory patient each. Allele frequencies and statistical analyses results for all sequence changes are presented in Table 2. Allele distributions that were analyzed with regard to age (p 0.774, Pearson $\chi 2$ analysis), gender (p 0.433, Pearson $\chi 2$ Chi-Square analysis), drug response status and allele frequencies revealed no statistically significant correlation.

DISCUSSION

Approximately one-third of epilepsy patients remain untreated even though currently available drugs are used. Understanding the mechanism underlying drug response is crucial for better management strategies for drug resistant patients and may even lead to new drug discoveries. RLIP76 is one of these proteins that are proposed to have a role in AED transport mechanisms [4]. Our study is the first to investigate polymorphisms of the *RLIP76* gene in Turkish patients. The Turkish population has a heterogeneous genetic background and high frequency of consanguinity which is around 33.9% in the Antalya region [14]. The polymorphisms we examined were mostly different from those cited by Soranzo *et al.* [11] and Leschziner *et al.* [12]. We found no correlation between the *RLIP76* gene polymorphisms and drug response which agreed with both these studies. However, Awasthi *et al.* [4], showed that RLIP76 was expressed preferentially in the luminal surface of endothelial cell membranes of brain tissue and RLIP76 knockout mice had deficient phenytoin extrusion mechanisms in the blood-brain barrier. It was concluded that AED transport is done mainly by RLIP76 in the blood brain-barrier, therefore, it might be involved in drug resistance in epilepsy. Moreover, there is controversy regarding the location of RLIP76 in the cell and thus its function [15,16]. Our finding no linkage between drug response and the *RLIP76* gene polymorphisms does not necessarily exclude RLIP76 having a possible role in drug response, given that the effects of nucleotide substitutions are not known.

Several other factors may be involved in the drug resistance phenomena in epileptic patients [2,3]. However, the reported results are conflicting. A striking example is the synonymous SNP, C3435T [17]. However, a recent meta-analysis, in which genotype association results for 3,231 drug-resistant patients and 3,524 drug-responsive or healthy controls were analyzed, failed to show any association between the

Table 1. Primer sequences designed for *RLIP76* gene exons 2-10

Exon	Forward Primer (5'>3')	Reverse Primer (5'>3')
2	GAT TTG TAA ATG CTG TGG TG	AAT ATC TAG GCA TAG GAA AA
3	AGC ATA TGG ACA GTT TGA CA	AAA ACC ATG AAG TGA AG
4	CTT TTC TCA GTA TCT TCT CT	CAG TTC CCC AAT CTC AGT AC
5	GTA ATA GAC ACA AAT AAG TG	CAC CAC TGG ACT CCA CAA CA
6	TTG CAC GTG TTG TTA GTT TA	AAA TGA CCA TTA AGA GCC TTG C
7	CGT GTA GAT TGG TGG TTG GTG	AAA TGA CCA TTA AGA GCC AAG C
8	GAG ACC CTA AAC AAG TGA CA	TGT AGG AAA GTC TGA GAG CT
9	GTC ACA GAC AAG CAC ATC CA	ATA TGT GGT CTC TAC AAC TT
10	GTC CTC TGA CTT CCT TAA GT	ACA GTA AGA TGC ACG GGT C

Table 2. Allele frequencies in the *RLIP76* gene

Sequence Change	Allele	Number of hromosomes in Refractory Patients (%)	Number of Chromosomes in Non Refractory Patients (%)	Number in Both Groups (%)	*p* Value	Test Applied
c.160-4G>A	G A	130 (44.5) 162 (55.5)	126 (40.6) 184 (59.4)	256 (42.5) 346 (57.5)	0.3795	Pearson $\chi 2$
c.187C>G	C G	2 (0.7) 290 (99.3)	1 (0.3) 309 (99.7)	3 (0.5) 599 (99.5)	0.6135	Fisher's exact
c.1562-38G>A	G A	260 (89.0) 32 (11.0)	280 (90.3) 30 (9.7)	540 (89.7) 62 (10.3)	0.7018	Pearson $\chi 2$
c.1670+107G>A	G A	274 (93.8) 18 (6.2)	296 (95.5) 14 (4.5)	570 (94.7) 32 (5.3)	0.4720	Pearson $\chi 2$
c.1670+93G>A	G A	288 (98.6) 4 (1.4)	304 (98.1) 6 (1.9)	592 (98.4) 10 (1.6)	0.7530	Fisher's exact
c.1670+96G>A	G A	291 (99.7) 1 (0.3)	310 (100.0) 0 (0.0)	601 (99.8) 1 (0.2)	0.9761	Fisher's exact
c.1670+100C>T	C T	291 (99.7) 1 (0.3)	310 (100.0) 0 (0.0)	601 (99.8) 1 (0.2)	0.9761	Fisher's exact
c.1670+130C>T	C T	262 (89.7) 30 (10.3)	283 (91.3) 27 (16.1)	545 (90.5) 57 (9.5)	0.6059	Pearson $\chi 2$
c.1670+131G>C	G C	292 (100.0) 0 (0.0)	309 (99.7) 1 (0.3)	601 (99.8) 1 (0.2)	1.00	Fisher's exact
c.1670+140G>C	G C	292 (100.0) 0 (0.0)	309 (99.7) 1 (0.3)	601 (99.8) 1 (0.2)	1.00	Fisher's exact

ABCB1 gene C3435T genotype and drug response in epilepsy [18]. Thus, this polymorphism seems unlikely to have an effect in predisposition to drug resistance. Zang *et al.* [19] have shown that human P-glycoprotein can transport phenytoin and phenobarbital in a concentration dependent fashion. However, the antiepileptic drugs carbamazepine, vaproic acid, phenytoin, lamotrigine and primidone were shown not to interact with ABCB1, ABCC1 and ABCC2 transporters in drug-resistant cancer cell lines [20]. Likewise, carbamazepine, valproate, levetiracetam, phenytoin, lamotrigine and phenobarbital have been shown not to be substrates of human MRP1, 2 and 5 in kidney cell lines [21].

These controversial data may result from factors such as the drugs used, patient demographic characteristics and epilepsy subtypes. Moreover, other genetic variants have been shown to be associated with drug response in epileptic patients. A recent study of the association between polymorphisms of the CYP2C9, CYP2C19, UGT1A1, UGT2B7, ABCB1, SCN1A genes and drug resistance found no significant association, but stratification by patient age and etiology yielded significant correlation between certain ABCB1 genotypes, patients with symptomatic epilepsy and risk of drug resistance [22].

Although there are many promising results available in the current literature, the exact factors involved in drug resistance in epilepsy have yet to be identified. Important obstacles to progress in this field may be the patient selection criteria and the definition of drug responsiveness and of refractory patients [23]. These might differ between publications and might create bias. Indeed, 20.42% of 191 drug refractory epilepsy patients were found to be pseudo refractory. That is, factors causing seizures are extraneous and re-

evaluation of treatment would result in reduction in the number of refractory epilepsy cases [24]. In addition, optimal doses of AEDs show great variation between individuals. Therefore, it might be useful to evaluate genetic variations from two perspectives: ***1)*** that might affect clinical efficacy of AEDs such as drug absorption, distribution and targets, and ***2)*** that might affect tolerability and safety that are adverse reactions of AEDs, as suggested by Löscher *et al.* [17]. For instance, an allele of the MRP2 gene c.1247G>A polymorphism was found to be strongly associated with the adverse drug reactions of carbamazepine in 146 patients with epilepsy and in 279 patients in a replication study [25]. Indeed, in a study, which included 809 patients, to evaluate adverse effects of AEDs and their relationship with a number of co-prescribed AEDs and AED loads, it was suggested that adverse effects are more prominent with individual susceptibility, AED type, and skills of the physicians than the number of co-prescribed AEDs and their load [26]. Multiple mechanisms probably underlie the phenotype. Therefore, combined effects of multiple genes and other factors, should be investigated with large families and larger sample sizes, and therefore, multi-centric collaborations are needed, especially for polymorphisms with minor allele frequencies.

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