



MDR-1 and MRP2 gene polymorphisms in Mexican epileptic pediatric patients with complex partial seizures

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Although the Pgp efflux transport protein is overexpressed in resected tissue of patients with epilepsy, the presence of polymorphisms in MDR1/*ABCB1* and MRP2/*ABCC2* in patients with antiepileptic-drugs resistant epilepsy (ADR) is controversial. The aim of this study was to perform an exploratory study to identify nucleotide changes and search new and reported mutations in patients with ADR and patients with good response (CTR) to antiepileptic drugs (AEDs) in a rigorously selected population. We analyzed 22 samples in Material and Methods, from drug-resistant patients with epilepsy and 7 samples from patients with good response to AEDs. Genomic DNA was obtained from leukocytes. Eleven exons in both genes were genotyped. The concentration of drugs in saliva and plasma was determined. The concentration of valproic acid in saliva was lower in ADR than in CRT. In *ABCB1*, five reported SNPs and five unreported nucleotide changes were identified; rs2229109 (GA) and rs2032582 (AT and AG) were found only in the ADR. Of six SNPs associated with the *ABCC2* that were found in the study population, rs3740066 (TT) and 66744T > A (TG) were found only in the ADR. The strongest risk factor in the *ABCB1* gene was identified as the TA genotype of rs2032582, whereas for the *ABCC2* gene the strongest risk factor was the T allele of rs3740066. The screening of SNPs in *ACBC1* and *ABCC2* indicates that the Mexican patients with epilepsy in this study display frequently reported *ABCC1* polymorphisms; however, in the study subjects with a higher risk factor for drug resistance, new nucleotide changes were found in the *ABCC2* gene. Thus, the population of Mexican patients with AED-resistant epilepsy (ADR) used in this study exhibits genetic variability with respect to those reported in other study populations; however, it is necessary to explore this polymorphism in a larger population of patients with ADR.

Keywords: drug-resistant, epilepsy, *ABCB1*, *ABCC2*, Mexican patients, single-nucleotide polymorphisms, anti-epileptic drugs

INTRODUCTION

Epilepsy is a chronic non-communicable disorder of the brain that affects approximately 70 million people worldwide (1, 2). The administration of anti-epileptic drugs (AEDs) is the treatment of choice. In 30% of patients with epilepsy, seizures persist despite polytherapy with more than one AED, adequate monitoring of serum drug levels and the watchful care of a neurologist (3). Hence, the timely identification of patients who do not respond favorably to AEDs is crucial because these patients are affected both by the treatment and by continuing seizures. Although the pathophysiological basis of drug resistance in epilepsy is not clear, some mechanisms for this resistance have been proposed. Following seizures, the overexpression of drug-transporter proteins in the blood brain barrier (BBB) could be mediated by the nuclear receptor PXR (4); alternatively, polymorphisms in these proteins could

abrogate their function. The main function of carrier proteins is to allow the passage of compounds through biological barriers such as the BBB. Using ATP, the ABC transporter family of proteins actively transports a wide variety of compounds, including toxins and xenobiotics, across cell membranes. ABC transporter proteins have two functional domains. The first domain is anchored to the cell membrane by its alpha-helical structure; the second, which is a nucleotide-binding domain (NBD), binds ATP, which provides the energy necessary for the conformational change that result in transport of the compound across the membrane. All ABC transporters have at least two transmembrane domains and two NBDs that are highly conserved between species (5).

MDR-1 (P-glycoprotein or P-gp) is an ATP-dependent efflux pump protein that controls the flow of toxins and drugs such as felbamate (FBM), gabapentin (GBP), lamotrigine (LTG),

phenytoin (PTH), and topiramate (TPM) into the brain (6–9). The gene encoding MDR-1 is *ABCB1*, which is located on chromosome 7q21.12 and consists of 209,461 base pairs (bp) and 29 exons (NCBI reference sequence NG_011513.1). The most extensively studied single-nucleotide polymorphisms (SNPs) in the *ABCB1* gene are rs1128503 (C1236T), rs2032582 (G2677T/A), and rs1045642 (C3435T); the latter has received increased attention as a critical variable in resistance to AEDs (10–16). MRP2 is another efflux transporter protein present in the BBB. Its gene, *ABCC2*, is located on chromosome 10q24 and contains 69,000 bp representing 33 exons (NG_011798.1). The MRP2 transporter protein recognizes CBZ, LTG, and FBM; its most relevant polymorphisms are rs2273697 (G1249A) and rs3740066 (C3972T). The latter polymorphism reduces the transport of carbamazepine (17–20). The aim of this study was to perform a non-inferential exploratory study to identify reported nucleotide changes and explore new ones in a rigorously selected Mexican population of patients with AED-resistant epilepsy (ADR) and patients with good response to AEDs (CTR).

MATERIALS AND METHODS

PATIENTS AND SAMPLE COLLECTION

This project was approved by the National Research Committee of the Instituto Mexicano del Seguro Social (Mexican Social Security Institute, IMSS). An observational study with 22 patients with ADR (cases) and 7 patients with good response to AEDs (controls) was performed, while avoiding inbreeding between the patient's biological parents.

The inclusion criteria for ADR were patients with demonstrable epileptic focus through EEG and radiological focal structural lesion to discard and classified as resistant to pharmacological treatment, treated with two or more drugs (Table 1) at appropriate doses, serum levels within therapeutic range for at least 6 months of continuous treatment, and under the supervision of a neurologist pediatrician, with a frequency of three seizures per month, who attend Neurology Service, Hospital of Pediatrics, National Medical Center, Century XXI, who is of Mexican origin like their parents and grandparents, from 1 to 16 years of age, both genders, whereas for asymptomatic control patients was seizure-free for at least 6 months before the study.

Both groups had complex partial seizures, showed similar clinical variables, were previously selected under medical supervision, and gave written informed consent. Exclusion criteria were all types of seizures that are not complex partial, little adherence to treatment.

A total of 15 ml of blood was collected from each patient via antecubital vein using an EDTA tube, and two fractions of 0.5–2.0 ml of saliva were collected upon stimulation with citric acid. Samples that were used to monitor drug concentrations were taken before the first dose in the morning.

AMPLIFICATION AND SEQUENCING

Genomic DNA was extracted from leukocytes in patient blood samples using a commercial kit (Genomic DNA Purification kit, Thermo Scientific®, USA), according to the supplier's recommendations. The polymorphisms (SNPs) from exons 1 (rs9282564), 10

Table 1 | Demographics and clinical data of patients with AEDs-resistant epilepsy and patients with good response to AEDs.

Clinical data	ADR	CTR
Age (years ± SD), range	10.45 ± 3.762 (1–15)	11.50 ± 3.017 (1–15)
Gender (F/M)	12/8	3/3
Onset of seizures	4.715 ± 3.835 (1–14)	4.833 ± 3.656 (2–10)
Number of seizures/month	63.45 ± 27.76 (3–450)	–
Years of seizures	5.85 ± 0.8438 (1–12)	–
Controlled time (years ± SD) range	–	1.9 ± 0.28 (0.6–2)
Valproic Ac. dose (mg/day)	598.0 ± 129.7	814.3 ± 212.1
Valproic Ac. concentration/saliva (mg/l)	0.57 ± 0.12	1.68 ± 0.59
Valproic Ac. concentration/plasma (mg/l)	31.62 ± 8.03	35.17 ± 18.04
Carbamazepine dose (mg/day)	900.0 ± 100.0	600.0 ± 200.0
Carbamazepine concentration/saliva (mg/l)	0.7244 ± 0.3232	1.017 ± 0.034
Carbamazepine concentration/plasma (mg/l)	5.900 ± 0.156	6.76 ± 0.29
Phenytoin dose (mg/day)	145.0 ± 55.00	
Phenytoin concentration/saliva (mg/l)	0.9337 ± 0.06814	
Phenytoin concentration/plasma (mg/l)	3.150 ± 1.750	
Levetiracetam dose (mg/day)	1250 ± 322.7	1000 ± 0.0
Levetiracetam concentration/saliva (mg/l)	1.661 ± 0.8825	
Levetiracetam concentration/plasma (mg/l)	4.657 ± 0.5897	

Mean ± SD.

F, female; M, male.

(rs2229109), 11 (rs1128503), 13 (rs28381902), 20 (rs2032582), and 25 (rs1045642) were located in GenBank accession CCDS5608.1, corresponding to the *ABCB1* gene, and exons 10 (rs2273697), 25 (rs8187692, rs17222723), 28 (rs3740066), 29 (rs7899457), and 32 (rs8187710) were located in GenBank accession CCDS7484.1, corresponding to the *ABCC2* gene. These polymorphisms were selected based on their frequency in the Hispanic or Mexican population, as noted in NCBI GenBank. Each amplification reaction was performed with 5 µl (5 ng/µl) of genomic DNA in a 50 µl total reaction volume containing 5 µl of 10× reaction buffer, MgCl₂ (concentration depending on the primer set), 1 µl 10 mM dNTPs

(Thermo Scientific®, USA), 1 µl of each 10 mM flanking primer (Invitrogen®, USA), and 1 µl of Taq polymerase (Thermo Scientific®, USA). PCR was performed in a DNA engine system®thermocycler (Bio-Rad®, USA) with a cycle program at 94°C for 3 min, 38 cycles of 94°C for 30 s, T_h° for 35 s, 72°C for 35 s, and one extension cycle of 10 min at 72°C. The primers, MgCl₂ concentration, and T_h° for each amplification reaction are listed in **Table 2**.

The amplification products were purified with the GeneJET Gel Extraction kit (Thermo Scientific®, USA) and sequenced in a 5 µl total reaction volume using 100 ng of amplicon, Fw or Rv primer, and Big Dye 3.1 sequencing Ready Reaction Kit (Applied Biosystems, USA) according to the manufacturer's recommendations. Sequencing was performed in a DNA engine system®thermocycler (Bio-Rad®, USA) with a cycle program at 94°C for 1 min, 30 cycles of 94°C for 10 s, 50°C for 5 s, 60°C for 4 min, and finally the fragments were analyzed in Automated Sequencing ABI Prism 310 Gene Analyzer (Applied Biosystems, USA), previous purification with DyeEx 2.0 Spin Kit (Qiagen, Germany).

DRUG MONITORING

Plasma samples were used for VPA and CBZ detection by immunofluorescence with a polarized light system (FPIA, Dade Behring, USA), according to the supplier's recommendations.

Saliva samples were used to detection concentration of CBZ and PHT (determined by AxSYM®, Abbot Laboratories®, USA) according to the supplier's recommendations, VPA, and LEV [determined by high performance liquid chromatography (HPLC)]. For VPA, 30 µl of an internal standard solution (98% octanoic acid diluted 1/500 with methanol), 200 µl of phosphoric acid, and 5 ml of cyclohexane were added to 1 ml of saliva and vortexed for 2 min. After centrifugation, a back-extraction was performed by adding 100 µl of 1% TEA to the organic phase supernatant. The mixture was vortexed and centrifuged. Finally, 20 µl of the aqueous phase was directly injected into a Dionex Ultimate 3000 Series. A Phenomenex Luna 5 µm CN column (15 cm × 4.6 mm) was used as the stationary phase. The mobile phase was a mixture of 40 mM phosphate potassium, monobasic, pH 3.4: Acetonitrile (90:10). The column compartment was kept at 40°C, and the wavelength of detection was 210 nm. For LEV, 50 µl of internal standard solution (diethylbarbituric acid, 12 mg/l) was added to 500 µl of saliva. For the extraction, 1.5 ml of ethyl acetate was added, and the mixture was vortexed for 1 min. After centrifugation, the supernatant was separated and dried under nitrogen stream at 37–40°C. Dry residue was dissolved with 100 µl of mobile phase, and 20 µl was injected into a Shimadzu LC-6A.A Phenomenex® Luna C18 column (5 µm, 100 Å, 150 mm × 4.6 mm), which was used as a reversed stationary phase. The mobile phase was a mixture of 50 mM phosphate buffer and acetonitrile (72:8).

STATISTICAL ANALYSIS

Clinical data from patients were analyzed using non-parametric and unpaired tests ($p < 0.05$); allelic and genotype relative frequencies were analyzed using the Fisher exact test in Graph Pad Prism software version 4.00 (GraphpadPrisma Software Inc.® San Diego, CA, USA). Hardy–Weinberg equilibrium (HWE) values were derived from chi-square tests, and the association between

odds ratios (ORs) and confidence intervals was determined using software available at <http://ihg.gsf.de/cgi-bin/hw/hwa1.pl>.

RESULTS

CLINICAL DATA

Because previous studies suggest that external factors such as gender, age of onset, number of seizures, and other factors influence the response to AED treatment, the selection of a homogeneous population for the study was an important aspect of this work (21, 22). There was no significant difference in male: female frequency between the two groups used in the study. Neither the average age of the patients (10.57 ± 1.56 for patients with good response to AEDs and 10.23 ± 0.78 for patients with ADR; $p = 0.42$) or the average age of onset of seizures (4.57 ± 1.43 years in patients with good response to AEDs and 4.38 ± 0.81 patients with ADR; $p = 0.34$) differed significantly. Furthermore, the average seizure-free time in patients with good response to AEDs was 1.9 years, while the average time with seizures in patients with ADR was 5.8 years with a frequency of 63.4 seizures per month. The AEDs VPA, CBZ, LTG, PHT, and LEV were used with relative frequencies of 0.71, 0.14, 0.29, 0.0, and 0.14, respectively, in patients with good response to AEDs and with relative frequencies of 0.59, 0.09, 0.0, 0.18, and 0.23, respectively, in patients with ADR. The most commonly used AED was VPA, but VPA and CBZ were prescribed to both groups (**Table 1**). The daily dose of VPA (814.3 ± 212.1 mg/day in patients with good response to AEDs and 598.0 ± 129.7 mg/day in patients with ADR; $p = 0.206$) was similar in the two groups. The two groups showed no significant difference in mean plasma VPA concentrations (35.17 ± 18.04 mg/l patients with good response to AEDs and 31.62 ± 8.03 mg/l in patients with ADR; $p = 0.42$); however, the average saliva drug concentration was lower in patients with ADR (1.68 ± 0.59 mg/l patients with good response to AEDs and 0.57 ± 0.12 mg/l in patients with ADR; $p = 0.01$).

POLYMORPHISMS IN THE *ABCB1* AND *ABCC2* GENES IN HISPANIC AND MEXICAN POPULATIONS

In this study, chromatograms from amplicons were analyzed to identify expected polymorphisms and to determine the relative frequencies of alleles and genotypes, as described in **Table 3**. For *ABCB1*, only the polymorphisms rs2229109, rs1128503, rs28381902, rs2032582, and rs1045642, but not rs9282564, were detected, whereas rs3740066 but not rs2273697, rs8187692, rs17222723, rs7899457, or rs8187710 were found in *ABCC2* in our patients, unlike the Hispanic/Mexican populations initially selected by the NCBI GenBank. In addition to these known polymorphisms, we identified rs2214102, rs2235047, and rs2235048 of *ABCB1* by sequencing the entire fragment including the exon and parts of the introns.

Another comparison was made between the relative frequencies of the minor allele (MAF) in our population and in the population represented by the NCBI GenBank data. The most significant similarities were found in rs1128503 and rs3740066 in ADR and in rs1045642 and rs2235048 in CTR (**Table 3**).

An important aspect of genetic alteration is whether a nucleotide change results in a change in an amino acid residue and whether this, in turn, structurally modifies the protein. In

Table 2 | Primers used on ABCB1 and ABCC2 fragments amplification.

GEN	EXON	Primer		MgCl ₂ (mM)	T _h ^o	Size (bp)
		Fw	RV			
ABCB1	2	5'-AGGTTAGTCTCACCTCCAGCG-3'	5'-GGCTAGCTTGCCTTTCTTAAA-3'	1.0	57	270
	11	5'-ATTCGAAGAGTGGGCACAAA-3'	5'-TCATCTCACCATCCCCTCTGT-3'	1.0	52	398
	12	5'-ATTCGAAGAGTGGGCACAAA-3'	5'-TCATCTCACCATCCCCTCTGT-3'	1.0	52	398
	14	5'-TTGGGCTGTGTATAGGATTCC-3'	5'-AAGCCTCACTGACCTTATCCA-3'	1.5	55	274
	21	5'-CAGCATTCTGAAGTCATGGAA-3'	5'-TCCAAGAAGTGGCTTTGCTA-3'	1.75	56	576
	26	5'-TGTGCTGGTCTGAAGTTGAT-3'	5'-TGGTCGAACACTTTCATCCCT-3'	1.50	62	472
ABCC2	10	5'-TTAGGCATTGACCCTATCCA-3'	5'-GCCCAAACCTCCATTAAGAA-3'	1.25	56	366
	25	5'-CGGGACTGGCTGATTCTTTA-3'	5'-ATGGGTAAATACCAGGGGAA-3'	1.0	54	273
	28	5'-TTCTATGACACGAGTCCTGGG-3'	5'-CATCCAGGCCTTCTTCACT-3'	1.25	56	258
	29	5'-CCCCAAGAATTATTTGTGGAA-3'	5'-GCATGTGCCGAGTAAGTT-3'	1.5	57	238
	32	5'-GCCTAGACTTGAGATGCTGC-3'	5'-TGGGGCCTTCTGCTAGAATT-3'	1.5	55	198

The primer design was performed using the web page <http://www.yeastgenome.org/cgi-bin/web-primer>, T_h^o were selected by a temperature gradient, and MgCl₂ concentration was selected initially with 2.0 mM and performed gradually until obtained a single sharp band.

this respect, the SNPs rs2229109 and rs2032582 are of interest due to the possible modifications that they may produce in protein MDR1 (**Table 3**).

The relative frequencies of the studied alleles were also compared with their frequencies in the Hispanic and Mexican reference populations reported in the NCBI GenBank (**Table 4**). In our population, SNP rs2229109 in exon 10 of *ABCB1* occurred with a relative frequency of 0.09 and was only present in heterozygous form in the ADR group (**Table 3**). Although a reference for the Mexican population was not available, we observed that our results showed a twofold higher minor allele frequency with respect to sample ss48292345 from the Hispanic population (0.043) in a similar number of individuals (**Table 4**). The SNPs rs1128503 and rs2032582 in exons 11 and 20, respectively, of the *ABCB1* gene showed similar relative frequencies in the CTR (0.71) and ADR (0.82) patient groups; however, there was a difference in the frequencies of homozygotes and heterozygotes in the CTR group (**Table 3**). Furthermore, there were no Mexican individuals with AG heterozygous mutations in SNP rs2032582, and the AT heterozygous mutation was absent from both populations (**Table 4**).

In studies of patients with ADR, SNP rs1045642 in exon 25 has been shown to be relevant in association studies with drug resistant; in our population, the relative frequencies of this SNP were similar in the CTR and ARE groups at both the allelic and the genotypic levels. Furthermore, the SNPs rs2235047 and rs2235048 were identified in an intron between exons 25 and 26. The heterozygous form of rs2235047 showed a higher frequency ADR than in CTR (0.400 vs. 0.286), as seen in **Table 3**; however, the most outstanding difference in this SNP was in comparison to reference populations, relative to which it was more than three times as frequent (**Table 4**); this is more significant in our population than rs1045642. The relative frequency of SNP rs2235048 was the same as that of rs1045642; when SNP rs1045642 showed the TT, TC, or CC genotype, we found the genotype CC, CT, or TT, respectively, in the position of SNP rs2235048. This association may be

relevant to our population, which so far has not shown a clear similarity to reference populations.

SNP rs3740066 in exon 28 of the *ABCC2* gene occurred with a relative frequency of 0.13 as a heterozygous polymorphism in the controlled group and with a relative frequency of 0.41 as homozygous and heterozygous polymorphisms in the drug-resistant group; the observed heterozygous ratio (ADR: CTR) was 2:1. In addition, the unexpected SNP rs2214102 located in the 5'UTR region of the *ABCB1* gene was present in the homozygous form in both groups; only 5% of the drug-resistant patients had the heterozygous form. However, this finding is not relevant because it is similar to a previous report in a Mexican population.

Some other differences observed in this study are worth noting. At SNP rs2032582, the allelic and genotypic distribution in the controlled group was more similar to reported populations in NCBI GenBank than was that of the ADR; in particular, the heterozygous A > T mutation was not found in other populations similar to ours. Furthermore, although allele A of SNP rs2229109 was present at a lower frequency, our population showed an allele frequency more than 2.5-fold greater than the reported MAF (0.045 vs. 0.017).

NEW POLYMORPHISMS OF THE *ABCC2* GENE IN MEXICAN EPILEPTIC PEDIATRIC PATIENTS

By sequencing amplicons from exons 28 and 29 of the *ABCB1* and *ABCC2* genes, we found other single-nucleotide changes (SNCs) that have not been reported in the NCBI GenBank database (**Table 2**, RefSeqGene NG_011798.1). The SNC located in position 66744T > G adjacent to rs3740066 in exon 28 is present as a homozygous wild-type or heterozygous mutation, allowing the formation of four different triplets: ATC, ATT, AGT, and AGC. The ATC and ATT triplets, which show relative frequencies of 0.929 and 0.682 for triplet ATC and 0.071 and 0.205 for triplet ATT in CTR and ADR, respectively, are not associated with an amino acid change (Ile1324Ile). The AGT and AGC triplets produce an amino acid change (Ile1324Ser, **Figure 1A**); these occur

Table 3 | Genotype and allelic relative frequencies of SNPs, MAF value, amino acid changes, and OR found in *ABCB1* and *ABCC2* genes.

Gene	SNP	Sample	Allele frequency	Genotype frequency	MAF	Protein association		Odds ratio		
						aa, change	Localization	Allele	Genotype	
									Heterozogous	Homozygous
<i>ABCB1</i>	rs2214102	CTR	G = 1.000	G/G = 1.000	A = 0.040/88		5'UTR region	[G] ↔ [A], 2.446	[GG] ↔ [GA], 2.692	[GG] ↔ [AA], 0.385
		ADR	G = 0.971	G/G = 0.955						
	rs2229109	CTR	G = 1.000	G/G = 1.000	A = 0.017/36	400; G > A; Ser > Asn	Cytoplasmatic; binding site; Second domain	[G] ↔ [A], 1.706	[GG] ↔ [GA], 1.829	[GG] ↔ [AA], 0.366
		ADR	G = 0.955	G/G = 0.909						
	rs1128503	CTR	T = 0.571	C/T = 0.571	T = 0.422/919	412; T > C; Gly > Gly	Cytoplasmatic; beta chain; second domain	[T] ↔ [C], 1.543	[TT] ↔ [TC], 1.200	[TT] ↔ [CC], 3.000
		ADR	T = 0.455	C/T = 0.545						
	rs2032582	CTR	G = 0.500	G/T = 0.429	A = 0.340/741	893; T > A; Ser > Thr 893; T > G; Ser > Ala	Cytoplasmatic; Alfa-helix chain; third domain	[T] ↔ [A], 2.143	[TT] ↔ [TA], 2.778	[TT] ↔ [AA], 0.526
		ADR	G = 0.523	G/T = 0.364						
	rs1045642	CTR	T = 0.429	TT = 0.143	T = 0.397/864	1145; T > C Ile > Ile	Cytoplasmatic, fourth domain	[T] ↔ [C], 0.825	[TT] ↔ [TC], 0.500	[TT] ↔ [CC], 0.600
		ADR	T = 0.475	TT = 0.255						
	rs2235047	CTR	T = 0.857	TT = 0.714	G = 0.179/389		Intron between 25 and 26 exon	[T] ↔ [G], 1.020	[TT] ↔ [TG], 1.026	[TT] ↔ [GG], 0.407
		ADR	G = 0.143	TG = 0.286						

(Continued)

Table 3 | Continued

Gene	SNP	Sample	Allele frequency	Genotype frequency	MAF	Protein association		Odds ratio		
						aa, change	Localization	Allele	Genotype	
									Heterozogous	Homozygous
	rs2235048	CTR	C = 0.429 T = 0.571	CC = 0.143 CT = 0.571 TT = 0.286	C = 0.398/867	Intron between 25 and 26 exon	[C] ↔ [T], 0.682	[CC] ↔ [CT], 0.417	[CC] ↔ [TT], 0.417	
		ADR	C = 0.525 T = 0.472	CC = 0.300 CT = 0.450 TT = 0.250						
ABCC2	66744 T > G	CTR	T = 1.000	T/T = 1.000	No reported	1323; T > G; Ile > Ser	Cytoplasmatic region, fourth domain	[T] ↔ [G], 4.038	[TT] ↔ [TG], 4.1714	[TT] ↔ [GG], 0.429
		ADR	T = 0.890 G = 0.110	T/T = 0.770 T/A = 0.230						
	rs3740066	CTR	C = 0.928 T = 0.072	C/C = 0.857 C/T = 0.143	T = 0.304/663	1324; C > T; Ile > Ile	Cytoplasmatic region, fourth domain	[C] ↔ [T], 4.875	[CC] ↔ [CT], 2.769	[CC] ↔ [TT], 3.370
		ADR	C = 0.727 T = 0.273	C/C = 0.591 C/T = 0.273 T/T = 0.136						
	68049 T > A	CTR	T = 0.790 A = 0.210	TT = 0.570 TA = 0.430	No reported	1373; T > A; Leu > His	Cytoplasmatic region, fourth domain	[T] ↔ [A], 0.733	[TT] ↔ [TA], 0.667	[TT] ↔ [AA], 0.310
		ADR	T = 0.840 A = 0.160	TT = 0.680 TA = 0.320						
	67967C > A	CTR	C = 0.500 A = 0.500	CA = 1.000	No reported	1342; C > A; Ser > Ser	Cytoplasmatic region, fourth domain	[C] ↔ [A], 0.760	[CC] ↔ [CA], 0.371	[CC] ↔ [AA], 0.143
		ADR	C = 0.570 A = 0.430	CC = 0.140 CA = 0.860						
	68072C > A	CTR	C = 0.500 A = 0.500	CA = 1.000	No reported	1381; C > A; Pro > Thr	Cytoplasmatic region, fourth domain	[C] ↔ [A], 0.750	[CC] ↔ [CA], 0.352	[CC] ↔ [AA], 0.143
		ADR	C = 0.590 A = 0.410	CC = 0.180 CA = 0.820						
	68088 G > C	CTR	G = 0.710 C = 0.290	GG = 0.710 CC = 0.290	No reported	Intron between 29 and 30		[G] ↔ [C], 0.147	[GG] ↔ [GC], 0.314	[GG] ↔ [CC], 0.147
		ADR	G = 0.890 C = 0.110	GG = 0.840 CC = 0.160						

Relative frequencies are presented for the five SNPs reported on NCBI Gene Bank database of ABCB1 and ABCC2 genes among 14 controlled and 44 drug-resistant patients (2n), obtained by direct sequencing. The position indicates the nucleotide change at ABCC2 gene from RefSeqGene NG_011798.1. Amino acid change was obtained from SNP page and its localization from <http://www.uniprot.org/> website. Odds ratio (OR) represent the allele risk or mutated using the software on line through the web page <http://ihg.gsf.de/cgi-bin/hw/hwa1.pl>. Bold font: allele OR > 1.0. DR, drug-resistant; CTR, controlled; MAF, Minor Allele Frequency.

Table 4 | Genotype and allelic population frequencies of SNPs found in ABCB1 and ABCC2 genes.

Gene	SNP	NCBI assay ID	Population	Sample (2n)	Allele frequency	Genotype frequency
ABCB1	rs2235048	ss35072278	Hispanic	44	T = 0.455 C = 0.545	TT = 0.182 TC = 0.545 CC = 0.273
		ss48292340	Hispanic	38	T = 0.553 C = 0.447	TT = 0.369 TC = 0.368 CC = 0.263
		ss44833502	Mexican	100	T = 0.540 C = 0.460	TT = 0.260 TC = 0.560 CC = 0.180
	rs2235047	ss35072277	Hispanic	44	T = 0.932 G = 0.068	TT = 0.864 TG = 0.136
			Mexican	100	T = 0.900 G = 0.100	TT = 0.840 TG = 0.126 GG = 0.011
		ss48292339	Hispanic	42	T = 0.952 G = 0.048	TT = 0.905 TG = 0.095 GG = 0.00
	rs1045642	ss35072275	Hispanic	42	T = 0.452 C = 0.548	TT = 0.238 TC = 0.429 CC = 0.333
			Mexican	100	C = 0.540 T = 0.460	TT = 0.180 TC = 0.560 CC = 0.260
	rs2214102	ss12675208	Hispanic	46	G = 0.957 A = 0.043	G/G = 0.913 A/G = 0.087
		ss35071953	Hispanic	44	G = 0.905 A = 0.095	G/G = 0.810 A/G = 0.190
		ss52073858	Mexican	77	G = 0.980 A = 0.020	G/G = 0.960 A/G = 0.040
	rs2229109	ss35072104	Hispanic	44	G = 1.000 A = 0.000	G/G = 1.000 A/G = 0.000
			ss48292345	Hispanic	46	G = 0.978 A = 0.022
	rs1128503	ss35072107	Hispanic	44	T = 0.545 C = 0.455	C/T = 0.545 T/T = 0.273 C/C = 0.182
			ss48292343	Hispanic	46	C = 0.609 T = 0.391
		ss52068966	Mexican	77	C = 0.540 T = 0.460	C/T = 0.560 C/C = 0.260 T/T = 0.180
	rs2032582	ss12675210	Hispanic	46	G = 0.565 T = 0.392 A = 0.043	G/T = 0.522 G/G = 0.261 T/T = 0.130 A/G = 0.08
			ss35072218	Hispanic	44	G = 0.545 T = 0.455
		ss12675210	Mexican	77	G = 0.570 T = 0.430	G/T = 0.540 G/G = 0.300 T/T = 0.160

(Continued)

Table 4 | Continued

Gene	SNP	NCBI assay ID	Population	Sample (2n)	Allele frequency	Genotype frequency
ABCC2	rs3740066	ss48292395	M	44	C = 0.614 T = 0.386	C/T = 0.500 C/C = 0.364 T/T = 0.136

Hispanic populations from ss12675208, ss48292345, ss48292343, ss12675210, and ss48292395 samples belong to HISP-MULTI-NATIONAL Population ID Class, obtained of 23 anonymized individuals of self-described HISPANIC heritage. Hispanic populations from ss35071953, ss35072104, ss35072107 and ss35072218 samples belong to EGP_HISP-PANEL Population ID Class, obtained of 22 individuals with Coriell Cell Repository (CCR) ID. Mexican population belongs to HAPMAP-MEX Population ID Class, obtained of 100 individuals with Mexican ancestry in Los Angeles, California.

at relative frequencies of 0.068 and 0.045, respectively, but only in drug-resistant patients.

For the SNC 67967C > A in exon 29, no amino acid change (Ser1342Ser) was observed due to nucleotide substitution (**Figure 1B**); however, SNC 68072C > A resulted in the amino acid change Pro1381Thr (**Figure 1D**). This allele was present as a heterozygous mutation in both groups (1.0 in the CTR group, 0.86 in the ADR group). The SNC 68049T > A in exon 29 resulted in a Leu1373His change (**Figure 1C**); this allele was heterozygous (0.43 in the CTR group, 0.32 in the ADR group), but the wild-type allele predominated in both groups (>50%). These nucleotide changes are located in the region of the gene coding for the fourth domain of the protein, corresponding to its cytoplasmic region. In contrast, the SNC 68088G > C (**Figure 1E**), which is located between exons 29 and 30, occurred in our study population as a homozygous mutation with a relative frequency of 0.29 in the CTR group and a relative frequency of 0.05 in ADR group.

DISCUSSION

The pediatric epilepsy patients admitted to the Neurology Service, Hospital of Pediatrics, National Medical Center, Century XXI, were referred from family clinics and general hospitals for inadequate control of epileptic seizures. In most cases, the patients received dosage adjustments or changes in drug regimen, drug monitoring, and electroencephalographic studies and were followed closely for adequate control of their seizures. However, some patients continued to interictal epileptiform discharges on EEG and/or the occurrence of seizures, and some gradually deteriorated. The results obtained with a small group of patients who did not respond to at least three different drug regimens but were followed for 4.7 ± 3.8 (CI = 1–14) years are presented in this work.

Fagiolino et al. (23) suggested that the quantitation of drugs in saliva can be used to identify drug-resistant patients. The hypothesis presented by these authors is based on the drug concentration circulating in free plasma, which irrigates the glandular capillary tissue, resulting in equilibrium with the aqueous spaces. Considering our results with respect to this hypothesis, we found a lower concentration of free VPA in drug-resistant patients, suggesting a possible deficiency in drug transport in these patients.

The overexpression of P-gp and MRP2 in resected tissue from patients who have undergone surgery for epilepsy suggests that this phenomenon may be conditional upon the presence of polymorphisms in the genes *ABCB1* and *ABCC2* and not simply to

exposure to xenobiotics that recognize these transporter proteins. However, the association between polymorphisms of the *ABCB1* and *ABCC2* genes and anti-epileptic drug-resistant epilepsy is controversial; SNPs rs1128503 (C1236T), rs2032582 (G2677T/A), and especially rs1045642 (C3435T) in the *ABCB1* gene have not shown a clear relationship to anti-epileptic drug-resistant epilepsy from different populations or ethnic groups (13, 24–27). This may be because, as has been reported, SNP rs1045642 is more relevant as a haplotype with rs1128503 and rs2032582. Zimprich et al. (28), studying European patients with temporal lobe epilepsy, demonstrated a relationship between the CGC haplotype at SNPs rs1128503, rs2032582, and rs1045642, respectively, and resistance to several AEDs. In another report, Seo et al. (10) demonstrated that Japanese with anti-epileptic drug-resistant epilepsy showed a tendency to possess the haplotype TTT at SNPs rs1128503, rs2032582, and rs1045642 when CBZ was employed. To enable comparison with those reports, haplotype frequency was calculated in our population. Both haplotypes (CGC and TTT) were found in our population. Within each group, the two haplotypes occurred with similar frequencies (CGC = 0.619, TTT = 0.523 in ADR and CGC = 0.714, TTT = 0.714 in CTR), but both were present at higher frequencies in the CTR group.

For patients who exhibit a mutated allele, OR values can be used to represent relative risk factors among the ADR and CTR patients. The polymorphisms or nucleotide changes, in descending order of risk factor magnitude, are rs2214102 > rs2032582 [T ↔ A] > rs2229109 > rs1128503 > rs2032582 [T ↔ G] > rs2235047 in *ABCB1*, and rs3740066 > 66744 T > G in *ABCC2*; relative to heterozygote genotype rs2032582 [T ↔ A] > rs2214102 > rs2229109 > rs2032582 [T ↔ G] > rs1128503 > rs2235047 in *ABCB1*, and 66744 T > G > rs3740066 in *ABCC2*; finally, with regard to the homozygote genotype, rs1128503 > rs2032582 [T ↔ G] in *ABCB1* and rs3740066 in *ABCC2* (**Table 3**).

Although the number of patients is lower in our work, the results are similar to studies with larger numbers of patients (25, 29, 30), where the lack of association of *ABCB1* polymorphisms with anti-epileptic drug resistance (indicated by us as a risk factor) it has been pointed out, including two Indian studies (31, 32) and a meta-analysis (26). In contrast, Taur et al. (33) and Siddiqui et al. (34) reported association with anti-epileptic drug resistance. On the other hand, we find that the *ABCC2* is a greater risk factor for increased anti-epileptic drug resistance than *ABCB1*, although

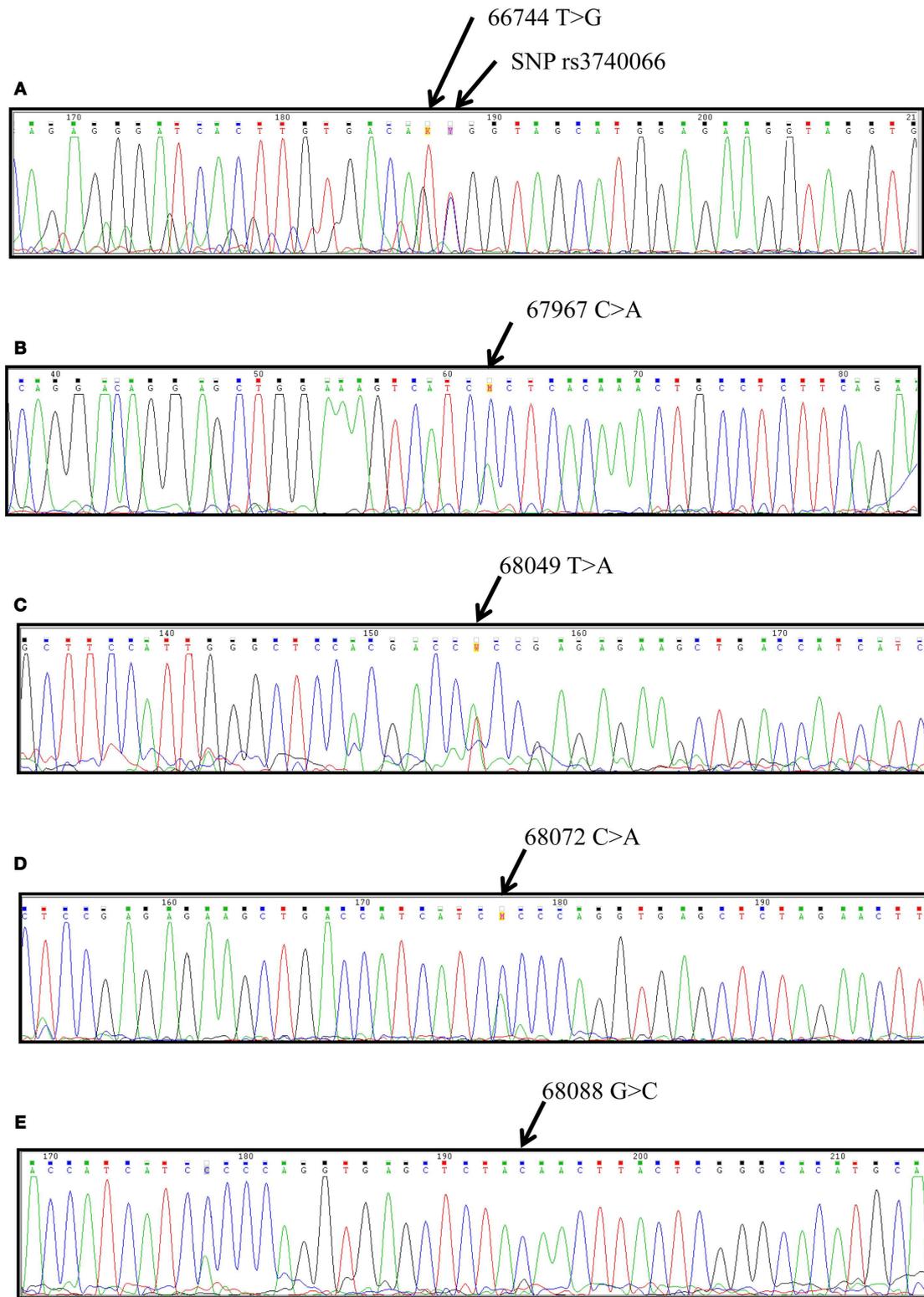


FIGURE 1 | Chromatograms of single-nucleotide changes found in the *ABCC2* gene from Mexican pediatric patients with epilepsy. (A) The 66744 T > G change in located in exon 28. (B) The 67967 C > A change in located in exon 29. (C) The 68049 T > A

change in located in exon 29. (D) The 68072 C > A change in located in exon 29. (E) The 68088 G > C change in located in intron 29. The position indicates the nucleotide change in the *ABCC2* gene (RefSeqGene NG_011798.1).

in previous studies, we only share the SNP rs3740066, not representing a major risk factor as the high activity promoter variant c-24C > T; these data suggest that *ABCC2* polymorphism may affect the response to AEDs.

This study employed a different strategy, rigorously selecting pediatric patients with ADR and patients with good seizure control to decrease the potential influence of confounding variables associated with the disease, to develop a study utilizing a non-inferential exploratory search based on the polymorphisms already reported and on unreported new characteristics of the Mexican population. Because our population has been little studied, this will allow us to conduct association studies with new SNPs and AEDs used to treat different forms of epilepsy. To avoid sequencing entire genes, we selected exons containing polymorphisms that have been previously reported in Mexican and Hispanic populations, including the P-gp and MRP2 proteins, which are associated with drug resistance. The most important outcome of this study was the identification of five new changes in the *ABCC2*; these are 66744T > G, 67967C > A, 68072C > A, 68049C > A, and G68088C. The nucleotide change 66744T > G was found only in ADR, and the risk factor associated with it was one of the highest found in the study. Moreover, the genetic variability in SNP rs2032582 in ADR was different and unexpected based on previous reports in the Mexican population in that the A allele was found. This result requires replication in a larger pool of Mexican patients.

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