Pharmacogenetic evaluation of *ABCB1, Cyp2C9, Cyp2C19* and methylene tetrahydrofolate reductase polymorphisms in teratogenicity of anti-epileptic drugs in women with epilepsy

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Abstract

Aim: Pregnancy in women with epilepsy (WWE) who are on anti-epileptic drugs (AEDs) has two- to three-fold increased risk of fetal malformations. AEDs are mostly metabolized by *Cyp2C9*, *Cyp2C19* and *Cyp3A4* and transported by *ABCB1*. Patients on AED therapy can have folate deficiency. We hypothesize that the polymorphisms in *ABCB1*, *Cyp2C9*, *Cyp2C19* and methylene tetrahydrofolate reductase (*MTHFR*) might result in differential expression resulting in differential drug transport, drug metabolism and folate metabolism, which in turn may contribute to the teratogenic impact of AEDs. **Materials and Methods:** The *ABCB1, Cyp2C9, Cyp2C19* and *MTHFR* polymorphisms were genotyped for their role in teratogenic potential and the nature of teratogenecity in response to AED treatment in WWE. The allelic, genotypic associations were tested in 266 WWE comprising of 143 WWE who had given birth to babies with WWE-malformation (WWE-M) and 123 WWE who had normal offsprings (WWE-N). **Results:** In WWE-M, CC genotype of Ex07 + 139C/T was overrepresented ($P = 0.0032$) whereas the poor metabolizer allele *2 and *2 *2 genotype of CYP2C219 was significantly higher in comparison to WWE-N group ($P = 0.007$ and $P = 0.005$, respectively). All these observations were independent of the nature of malformation (cardiac vs. non cardiac malformations). **Conclusion:** Our study indicates the possibility that *ABCB1* and *Cyp2C19* may play a pivotal role in the AED induced teratogenesis, which is independent of nature of malformation. This is one of the first reports indicating the pharmacogenetic role of *Cyp2C19* and *ABCB1* in teratogenesis of AED in pregnant WWE.

Key Words

ABCB1, anti-epileptic drugs, *Cyp2C19*, *Cyp2C9*, epilepsy, methylene tetrahydrofolate reductase, pharmacogenomics, South India, teratogenic

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Introduction

In India, there are over 2.5 million women with Epilepsies (WWE).[1] Pregnancy in WWE who are taking anti-epileptic drugs (AEDs) have two to three fold increased risk of fetal malformations.[2,3] The excess risk of congenital malformations in offspring's of WWE can be potentially attributed to teratogenic potential of AED.[4,5] Several AEDs such as phenobarbitone (PB), phenytoin (PHT), carbamazepine (CBZ) and valproate (VPA) have been shown to have teratogenic potential in studies in humans.[6] Due to the great deal of overlap in the malformations reported with diverse AEDs, a term "Fetal Anti-epileptic Drug Syndrome" has been proposed[7] to encompass the common mechanism of AED induced teratogenicity.[8-10] These AEDs are mostly metabolized by *Cyp2C9*, *Cyp2C19* and *Cyp3A4* and transported by *ABCB1*. Patients on AED therapy are also known to develop folate deficiency,[11] which in turn may predispose to fetal malformation.[9,12] The most important risk factor for malformations in infants of WWE is antenatal exposure to AEDs. These backgrounds had led us to investigate the role of pharmacogenetic variability of genes involved in drug transport, drug metabolism and folate metabolism on risk of fetal malformation. Insight into these factors involving AED

metabolism and transport might provide a rational basis for prevention by adjustment of medication and in the future, for individual predictive testing for safest therapy.

P-glycoprotein (Pgp), encoded by the human *ABCB1* gene, is a membrane protein that is involved in active transport of various AEDs across tissue barriers such as blood brain barrier and placenta. Pgp expression has been found to be influenced by *ABCB1* polymorphisms. The polymorphisms of *ABCB1* Pro T-129C, C1236T, G2677T/A and C3435T were also associated with variations in the activities of Pgp and/or in the expression of *ABCB1* mRNA.[13,14] Similarly, *Cyp2C9* and *Cyp2C19* are involved in metabolism of various AEDs. Polymorphisms in these genes can impact the pharmacokinetics, metabolism, safety and efficacy of drugs.[15] Defects in methylene tetrahydrofolate reductase gene (*MTHFR*), major enzyme in folate metabolism is known to impair the ability to process folate, which has been linked to decreased conversion of homocysteine to methionine resulting in various adverse pregnancy outcomes.[16]

Until date, no studies have been carried out to understand the role of teratogenicity of AEDs in WWE based on pharmacogenetic evaluation. We hypothesize that the polymorphisms in *ABCB1*, *Cyp2C9*, *Cyp2C19 and MTHFR* might result in differential expression resulting in differential drug transport, drug metabolism and folate metabolism, which in turn may result in teratogenic impact of AEDs in WWE.

Materials and Methods

WWE were enrolled in the pre pregnant stage or first trimester of pregnancy in Kerala Registry of Epilepsy and Pregnancy (KREP) and were followed-up through pregnancy and delivery until their offsprings were 6 years of age. Epilepsy was classified according to the 1981 International League against Epilepsy classification. Details of AED's, folic acid usage, other substance exposure and seizure frequency during pregnancy were recorded prospectively on a monthly basis. All newborns were examined for malformations by clinical examination at birth and by echocardiography and abdomen ultrasonography at three months of age.[17,18] For the purpose of this study, we had defined a malformation as any defect or variation from normal in one or more systems of the infant. For example, small defect in interatrial septum of 2-4 mm size, which amounts to patent foramen ovale was also classified as a malformation. From 1200 pregnancies in KREP registry, we identified 143 WWE who had given birth to infants with WWE-malformations (WWE-M). Out of the 1057 WWE who have had healthy babies (WWE-N), we selected 123 women as controls.[19] The study was set on conventional guidelines of treatment of epilepsy in pregnant women. All WWE were using folic acid (5 mg) supplementation prior to conception and during pregnancy. The details of AED and other medication, seizure frequency, pregnancy outcome and fetal outcome were abstracted from the clinical records of the registry. The study had the approval of the Institutional Ethics Committee with appropriate consent in all the cases and controls.

Genotyping method

Deoxyribonucleic acid (DNA) was extracted by the standard organic extraction protocol from 10 ml of peripheral blood samples collected in ethylenediaminetetraacetic acid vials. We screened for *CYP2C9* *1, *2 and *3 and *CYP2C19* *1, *2 and *3 polymorphisms in the drug metabolism genes and seven *ABCB1* polymorphisms, which included Pro129T/C, Ex03-1G/A, Ex07 + 139C/T, Ex13 1236C/T, Ex18-76T/A, Ex22 2677G/T and Ex27 3435C/T in the drug transporter gene and *MTHFR* C677T and A1298C in folate metabolism gene. *ABCB1* Ex22 2677A was absent in our population.[20] The details regarding the rsIDs, location, primers and restriction enzymes, are given in Table 1. All the polymerase chain reaction (PCR)'s were carried out with 50 ng of template DNA, 10 pmols of each primer, 0.2 mM dNTP, PCR buffer containing 2 mM $MgCl_2$ and 0.5 U Taq DNA polymerase. The PCR amplifications consisted of an initial denaturation for 5 min at 95°C, followed by 35 cycles of denaturation at 94°C for 30s and extension at 72°C for 30s. The annealing temperature for each single nucleotide polymorphism (SNP) is mentioned in Table 1. The terminal extension was done at 72°C for 10 min. The restriction digestion products were resolved in 3% agarose gel and visualized by ethidium bromide staining, in a ultraviolet transilluminator. Representative samples were also validated for their restriction fragment length polymorphism patterns by sequencing. Sequencing was carried out as per the manufacturer's protocol using Big Dye Terminator kit version 3.1 from Applied Biosystems, Foster City, CA, USA.

Statistical analysis

The deviations from Hardy-Weinberg equilibrium for genotype distributions were examined using the Pearson's Chi-square test and exact test implemented in the Finetti program.[101] Allele, genotype and haplotype frequency analysis for association was performed using the COCAPHASE program in the UNPHASED v3.011 package, which uses standard unconditional logistic regression analysis.[21,102] Correction for multiple testing was performed using permutation correction by the COCAPHASE program. This approach corrects for multiple testing but takes into account the correlation between markers. It is thus less conservative than a Bonferroni correction, which is appropriate for independent tests such as unlinked markers. For the single-marker analyses, 10,000 permutations were carried out to estimate the significance of the best results, correcting for the number of loci tested for each gene. To estimate the linkage disequilibrium (LD) between pairs of loci in the WWE-N and WWE-M populations, the standardized disequilibrium coefficient (*D'*) and the squared correlation coefficient (*r²*) were calculated using the Haploview 4.1.[22,103] LD blocks were defined in accordance with Gabriel's criteria.[20] We further tested the power of the allelic and genotypic association using the software power for association with error (PAWE) version 1.2. PAWE is a free software. (http://linkage.rockefeller.edu/pawe/. Gordon D, Finch SJ, Nothnagel M, Ott J. Power and sample size calculations for case-control genetic association tests when errors present: application to single nucleotide polymorphisms. *Human Heredity* 54 (1): 22-33(2002).[23,104] PAWE considers several parameters such as relative risk, prevalence of the disease, frequency of SNP or marker, whether that is in LD or not. We used a model-free approach for calculations of power for a fixed sample size, where the allele and genotype frequencies of SNP markers were known in both the WWE-N

Table 1: Primer sequences used to amplify PCR fragments, including the single nucleotide polymorphisms and RE

SNP location	rsID	Primer sequence (5' → 3')	Temperature (°C)	RE
MDR1	rs3213619	TCAGCATTCAGTCAATCCGG	62	MspA1I
Pro T129C		TTTGCGTGCCCCTACCTC		
MDR1	rs2214102	TCTTACTGCTCTCTGGCTTCG	60	FokI
Ex02-1G/A		CATTTATTTCAGAGCTGGAGGC		
MDR1	rs1202168	5'-AGGTTTCATTTTGGTGCCTG	61	SspI
Ex07+139C/T		GAACAAAAGGATGCACACGACA		
MDR1	rs1128503	TACCTGTGTCTGTGAATTGCC	59	HaeIII
Ex 13C 1236T		CCTGACTCACCACACCAATG		
MDR1	rs1922242	TTTGTCAACATTTTTTTGAAGC	69	ApoI
Ex 18-76T/A		TATTATTGCAAATGCTGGTTGC		
MDR1	rs2032582	TGCAGGCTATAGGTTCCAGG	68	BanI
Ex22G2677T/A		TTTAGTTTGACTCACCTTCCC		
MDR1	rs1045642	TGTTTCAGCTGCTTGATGG	59	Sau3AI
Ex27 C3435T		AAGGCATGTATGTTGGCCTC		
MTHFR C677T	rs1801133	TGAAGGAGAAGGTGTCTGCGGGA	64	HinfI
		AGGACGGTGCGGTGAGAGTGG		
MTHFRA 1298C	rs1801131	CTGGGCATGTGGTGGCACTGC	66	MboII
		CGCAGCCTGGCCTGCAGCTGG		
CYP2C9*2	rs1799853	CACTGGCTGAAAGAGCTAACAGAG	48	Sau96I
		GTGATATGGAGTAGGGTCACCCAC		
CYP2C9*3	rs1057910	AGGAAGAGATTGAACGTGTGA	59	StyI
		TGCATGGGGCAGGCTGGTGGGGAGAAGGGCAA		
CYP2C 19*2	rs4244285	AATTACAACCAGAGCTTGGC	55	SmaI
		TATCACTTTCCATAAAAGCAAG		
		ACTTCAGGGCTTGGTCAATA		
CYP2C 19*3	rs4986893	TATTATTATCTGTTAACTAATATGA	46	BamHI

PCR = Polymerase chain reaction, SNP = Single nucleotide polymorphism, RE = Restriction enzymes

and WWE-M groups. The power calculations were carried out assuming the Douglas Skol Boehnke error model with parameter settings of γ (0.01) and η (0.01) at 5% level of significance.[24] The criterion for significance was set at $P < 0.05$. The allele and genotype associations were further evaluated according to the nature of teratogenic malformation within the WWE-M group. Nature of teratogenic malformations was further classified into cardiovascular malformations and other types.

Results

The *ABCB1, Cyp2C9, Cyp2C19* and *MTHFR* polymorphisms were genotyped in a total of 266 WWE. Out of these 2656 WWE, 143 WWE whose offspring had malformations and 123 WWE with normal offspring were selected from a total of 1200 WWE who were observed in the pregnancy registry (mean age, 25 years) belonging to WWE-M (*N* = 143) and WWE-N (123). The birth defects in the infants pertained to the cardiovascular system (85), central nervous system (7), gastrointestinal system (6), genito urinary system (7), skeletal system (3), cleft lip or palate (2), combination of cardiac and gastro intestinal defects (3), combination of cardiac and genito urinary system (3), combination of gastrointestinal and genito urinary system (2), or Central nervous system, skeletal and other defects (3).

The clinical characteristics of the WWE-N and WWE-M groups are given in Table 2. The AEDs used included CBZ, VPA, PHT,

Table 2: Clinical characteristics in epileptic control (WWE-N) and malformation (WWE-M) groups of WWE

	WWE-N group	WWE-M group
N	123	143
Age mean±SD	25±3.5	25±3.9
AED usage		
Mono therapy	80	93
Poly therapy	43	50
Malformation type		
Cardiovascular	Nil	97
Other	Nil	46
Seizure frequency mean	10.6	13.1
No seizure	55	62
≤3	31	40
>3	37	41

WWE = Women with epilepsy, AED = Anti-epileptic drug, SD = Standard deviation

clonazepam and PB. Among the WWE-M, 93 individuals were on monotherapy and 50 on polytherapy.

The allele and genotype frequencies of the *ABCB1, Cyp2C9, Cyp2C19* and *MTHFR* variants were compared between WWE-M and WWE-N groups [Table 3]. We observed significant allelic and genotypic association with *Cyp2C19* and significant genotypic association with *ABCB1* Ex07 + 139C/T. With reference to *Cyp2C19* gene, the patients in the WWE-M

Table 3: Allele and genotype frequencies of *MDR1, MTHFR, Cyp2C9* and *Cyp2C19* polymorphisms in WWE with (WWE-M) and without (WWE-N) malformed offsprings with OR and 95% CI

MDR1	Genotype			P	Allele		OR (95% CI)	P
	CC	CT	TT		C	T		
Pro T129C								
WWE-N	1 (0.01)	6 (0.05)	116 (0.94)	0.957	8 (0.04)	246 (0.96)	1.101 (0.3930-3.085)	1
WWE-M	1 (0.01)	5 (0.04)	116 (0.95)		7 (0.03)	237 (0.97)		
	GG	GA	AA		G	A		
Ex 03-1G/A								
WWE-N	115 (0.93)	8 (0.07)	0	0.46	238 (0.97)	8 (0.03)	0.712 (0.2813-1.802)	0.493
WWE-M	111 (0.91)	11 (0.09)	0		233 (0.95)	11 (0.05)		
	CC	CT	TT		C	T		
In 07+139C/T								
WWE-N	19 (0.15)	64 (0.52)	40 (0.33)	0.0032	102 (0.41)	144 (0.59)	0.7497 (0.5310-1.058)	0.115
WWE-M	44 (0.31)	49 (0.35)	48 (0.34)		137 (0.49)	145 (0.51)		
	CC	CT	TT		C	T		
Ex 13C 1236T								
WWE-N	16 (0.13)	43 (0.35)	64 (0.52)	0.405	75 (0.30)	171 (0.70)	1.328 (0.9056-1.948)	0.171
WWE-M	13 (0.09)	44 (0.31)	84 (0.60)		70 (0.25)	212 (0.75)		
	TT	TA	AA		T	A		
In 17-76 T/A								
WWE-N	75 (0.61)	40 (0.32)	8 (0.07)	0.637	190 (0.77)	56 (0.23)	1.223 (0.8026-1.863)	0.39
WWE-M	91 (0.66)	42 (0.30)	6 (0.04)		224 (0.79)	54 (0.21)		
	GG	GT	TT		G	T		
Ex 22G2677T								
WWE-N	16 (0.13)	52 (0.42)	55 (0.45)	0.734	84 (0.34)	162 (0.66)	1.162 (0.8066-1.674)	0.456
WWE-M	15 (0.11)	57 (0.40)	69 (0.49)		87 (0.31)	195 (0.69)		
	CC	CT	TT		C	T		
Ex 27C3435T								
WWE-N	4 (0.03)	63 (0.51)	56 (0.46)	0.965	71 (0.29)	175 (0.71)	1.043 (0.7136-1.523)	0.847
WWE-M	4 (0.03)	71 (0.50)	66 (0.47)		79 (0.28)	203 (0.72)		
	CC	CT	TT		C	T		
MTHFRC677T								
WWE-N	103 (0.84)	19 (0.15)	1 (0.01)	0.839	225 (0.91)	21 (0.09)	0.8467 (0.4676-1.533)	0.653
WWE-M	115 (0.82)	24 (0.17)	2 (0.01)		254 (0.90)	28 (0.10)		
	AA	AC	CC		A	C		
MTHFRA 1298C								
WWE-N	29 (0.24)	59 (0.48)	35 (0.28)	0.851	117 (0.48)	129 (0.52)	0.907 (0.6441-1.277)	0.601
WWE-M	36 (0.25)	69 (0.49)	36 (0.26)		141 (0.50)	141 (0.50)		
	*1*1	*1*2	*2*2		*1	*2		
CYP2C9								
WWE-N	118 (0.96)	4 (0.03)	1 (0.01)	0.083	240 (0.98)	6 (0.02)	0.5173 (0.1935-1.383)	0.242
WWE-M	128 (0.91)	13 (0.09)	0		269 (0.95)	13 (0.05)		
	*1*1	*1*2	*2*2		*1	*2		
CYP2C19								
WWE-N	51 (0.42)	62 (0.50)	10 (0.08)	0.005	164 (0.66)	82 (0.34)	1.639 (1.150-2.335)	0.007
WWE-M	46 (0.32)	63 (0.45)	32 (0.23)		155 (0.55)	127 (0.45)		

WWE = Women with epilepsy, OD = Odds ratio, CI = Confidence interval

group had significantly increased poor metabolizer allele *2 (*P* = 0.007) and *2*2 genotypes (*P* = 0.005) when compared with the patients in the WWE-N group. Similarly, with reference to Ex07 + 139C/T the patients in the WWE-M group were more likely to have the CC genotype in comparison to the patients with normal offspring (*P* = 0.0032). The power of Ex07 + 139C/T genotypic test to predict association with AED teratogenicity in pregnant mother with epilepsy using PAWE was observed to be

87%. The C allele of Ex07 + 139C/T was also found to be strongly associated with malformation in haplotypic combination with its neighboring SNPs [Table 4]. This haplotypic association was observed to extend to four loci haplotypic combination more specifically with Ex 03-1G/A, Ex 07 + 139C/T and Ex13C1236T. The LD plot between the WWE-M and WWE-N groups displayed shifts in the extent of LD between Ex 07 + 139C/T, Ex13 1236C/T and Ex 17-76T/A SNPs [Figure 1].

Table 4: *MDR1* **haplotypes in malformation (WWE-M) and no malformation (WWE-N) groups of WWE**

Marker	Haplotype	WWE-M	WWE-N	P
+139C/T-C1236T	C-T	0.2937	0.133	3.34×10^{-5}
Ex 02-1G/A-+139C/T-C1236T	G-C-T	0.2867	0.1177	2.18×10^{-5}
+139C/T-C1236T-In 17-76 T/A	C-T-T	0.2041	0.09547	9.94×10^{-4}
T 129C-Ex 02-1G/A-+139C/T-C1236T	T-G-C-T	0.2777	0.1175	4.91×10^{-5}
Ex 02-1G/A-+139C/T-C1236T-In 17-76 T/A	G-C-T-T	0.2049	0.0836	1.97×10^{-4}

WWE = Women with epilepsy
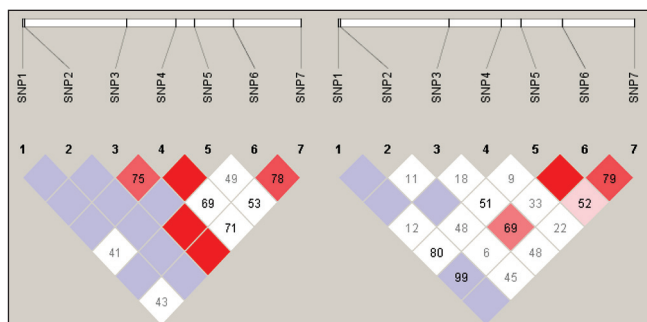
Figure 1: Linkage disequilibrium status between pair of SNP's in *ABCB1* between WWE-N and WWE-M groups

We further investigated the role of *ABCB1*, *Cyp2C9*, *Cyp2C19* and *MTHFR* SNP's in influencing the nature of teratogenic malformation (cardiac vs. non cardiac) within the WWE-M group. When the allele and genotype comparisons were made within the WWE-M group for cardiovascular malformations and other malformations no significant association of *ABCB1*, *Cyp2C9*, *Cyp2C19* and *MTHFR* SNPs were observed with any of the malformations [Table 5]. However, variations in the pattern of LD of the *ABCB1* gene could be observed between malformation types, treatment regimen, and seizure frequency.

Discussion

The setting of the pregnancy registry gave us the unique opportunity to compare the DNA polymorphism in a cohort of WWE-M with that of WWE-N with reference to the AED usage. The commonly used AEDs are mostly metabolized by *Cyp2C9*, *Cyp2C19* and *Cyp3A4* and transported by drug transporter *ABCB1*. *Cyp3A4* was found to be highly monomorphic in our study sample. Among the SNPs screened across *ABCB1*, *Cyp2C9*, *Cyp2C19* and *MTHFR* genes, we found *Cyp2C19* and *ABCB1* Ex07 + 139C/T to be of good clinical significance. We observed that WWE who gave birth to infants having malformations were more likely to have the C allele and CC genotype of Ex07 + 139C/T when compared to WWE who gave birth to normal offspring. C allele of Ex07 + 139C/T was in strong haplotypic combination with a synonymous polymorphism at 1236C/T and regulatory region of Ex 03-1G/A. The functional relevance of these polymorphisms is presently unclear. It would be worthwhile to investigate the role of these polymorphisms with respect to MDR expression. None of the earlier studies have investigated this polymorphism for its clinical or functional significance. This observation is interesting as most of the earlier studies within the *ABCB1* gene have concentrated on the synonymous polymorphism 3435C>T and 1236C>T and a non-synonymous polymorphism in exon 22 2677G>TA. The synonymous 3435C>T polymorphism has been reported to

be in LD with a synonymous SNP in exon 13 (1236C>T) and a non-synonymous SNP in exon 22 (2677G>TA), suggesting that the observed functional differences in Pgp, initially attributed to the exon 27 synonymous SNP, may be the result of the associated non-synonymous polymorphism in exon 22, which results in amino acid change (Ala893Ser or Ala893Thr).[13] Most convincing evidence for an association between *ABCB1* genotype and Pgp expression, function and therapeutic drug response came from a prospective study on 3435C/T polymorphism and its role on brain uptake of PB in patients with generalized epilepsy[25] CC genotype of C3435T polymorphism was noted to have two-fold higher expression when compared with that in individuals with the TT genotype.[26] Since C3435T is a silent mutation, the effect of C3435T polymorphism on Pgp-mediated transport may result from linkage to other polymorphic sites in *ABCB1* gene affecting amino acid residue or altered conformation. However, many studies have also contradicted these observations.[13,27]

Most of the AEDs that are currently in clinical use, are known to induce major congenital malformations such as cardiac malformations, coarctation of aorta, spina bifida and other neural tube defects (NTDs), hypospadias, cleft lip or palate and limb reduction defects.[4] In the present study, because of the limited number of subjects under each category of malformation, we broadly divided the nature of malformations into cardiac malformations and non-cardiac malformations. In this series, we could not find any relation between *ABCB1* polymorphisms and these two broad categories of malformations. *ABCB1* polymorphisms are also known to influence the plasma concentrations of these AEDs and therapeutic response in epileptic patients.[25,28-30] It has been reported that PHT and CBZ dose requirements were influenced by the genotype in position 3435C/T and 2677G/T of the *ABCB1* gene.[30] Clinical data indicates that frequency of major malformations is more with polytherapy than with monotherapy.[4] Patients often end-up on polytherapy when the seizures are difficult to control, although this may not be true in every situation.

We further investigated whether the malformation was due to *ABCB1* polymorphisms resulting in differential drug transport or could also be due to altered drug metabolism as a result of differential expression of *Cyp2C19* and *Cyp2C19* polymorphic variants. *Cyp2C19* *2 (Arg144/Cys) and *Cyp2C19* *3 (Ile359/Leu) lead to impaired metabolic activity associated with poor metabolizer phenotype. The homozygous mutant genotype *2 *2 was observed to be in increased frequency in women with malformation. *Cyp2C19* allelic or genotypic variants (*Cyp2C19* *2/*2, 3/3, or 2/3) lead to a truncated and completely inactive enzyme and impairs the metabolism of AEDs. Variations in

Table 5: Allele and genotype frequencies of *MDR1*, *MTHFR*, *Cyp2C9* and *Cyp2C19* polymorphisms within the malformed offsprings (WWE-M) group based on type of malformations with odds ratios

MDR1	Genotype			P	Allele		OR (95% CI)	P
	CC	CT	TT		C	T		
Pro T129C								
CVDs	1 (0.01)	3 (0.03)	87 (0.96)	0.661	5 (0.03)	177 (0.97)	1.299 (0.2453-6.881)	1
Others	0	2 (0.06)	31 (0.94)		2 (0.03)	66 (0.97)		
	GG	GA	AA		G	A		
Ex 03-1G/A								
CVDs	83 (0.91)	8 (0.09)	0	0.96	174 (0.96)	8 (0.04)	0.9655 (0.2483-3.755)	1
Others	30 (0.91)	3 (0.09)	0		63 (0.96)	3 (0.04)		
	CC	CT	TT		C	T		
In 07+139 C/T								
CVDs	34 (0.35)	29 (0.30)	34 (0.35)	0.265	97 (0.50)	97 (0.50)	1.091 (0.6639-1.793)	0.8
Others	12 (0.25)	20 (0.44)	14 (0.31)		44 (0.48)	48 (0.52)		
	CC	CT	TT		C	T		
Ex 13C 1236T								
CVDs	10 (0.10)	27 (0.28)	60 (0.62)	0.543	47 (0.24)	147 (0.76)	0.8569 (0.4871-1.507)	0.662
Others	4 (0.09)	17 (0.37)	25 (0.54)		25 (0.27)	67 (0.73)		
	TT	TA	AA		T	A		
In 17-76 T/A								
CVDs	65 (0.68)	25 (0.26)	6 (0.06)	0.054	155 (0.80)	37 (0.20)	0.892 (0.4796-1.659)	0.75
Others	26 (0.58)	19 (0.42)	0		71 (0.79)	19 (0.21)		
	GG	GT	TT		G	T		
Ex 22 G2677T								
CVDs	11 (0.11)	36 (0.37)	50 (0.52)	0.457	58 (0.30)	136 (0.70)	0.7996 (0.4717-1.356)	0.416
Others	5 (0.11)	22 (0.47)	19 (0.42)		32 (0.35)	60 (0.65)		
	CC	CT	TT		C	T		
Ex 27 C3435T								
CVDs	4 (0.04)	46 (0.48)	47 (0.48)	0.221	54 (0.28)	140 (0.72)	0.9286 (0.5369-1.606)	0.781
Others	0	27 (0.59)	19 (0.41)		27 (0.30)	65 (0.70)		
	CC	CT	TT		C	T		
MTHFR C677T								
CVDs	78 (0.80)	18 (0.19)	1 (0.01)	0.628	174 (0.90)	20 (0.10)	1.207 (0.5105-2.853)	0.832
Others	39 (0.85)	6 (0.13)	1 (0.02)		84 (0.91)	8 (0.09)		
	AA	AC	CC		A	C		
MTHFR A1298C								
CVDs	23 (0.24)	48 (0.50)	26 (0.36)	0.75	94 (0.48)	100 (0.52)	0.8249 (0.5018-1.356)	0.527
Others	13 (0.28)	23 (0.50)	10 (0.22)		49 (0.53)	43 (0.47)		
	*1*1	*1*2	*2*2		*1	*2		
CYP2C9								
CVDs	86 (0.89)	11 (0.11)	0	0.174	183 (0.94)	11 (0.06)	2.705 (0.5869-12.47)	0.185
Others	44 (0.96)	2 (0.04)	0		90 (0.98)	2 (0.02)		
	*1*1	*1*2	*2*2		*1	*2		
CYP2C19								
CVDs	32 (0.33)	50 (0.52)	15 (0.16)	0.535	114 (0.59)	80 (0.41)	1.197 (0.7182-1.995)	0.49
Others	16 (0.35)	26 (0.57)	4 (0.09)		58 (0.63)	34 (0.37)		

WWE = Women with epilepsy, OD = Odds ratio, CI = Confidence interval, CVD = Cardiovascular disease

PHT metabolism were reported for allelic variants of *CYP2C19* in a Japanese population. PB total plasma clearance was reported to be 19% less in patients with *CYP2C19* *2/*2 and *2/*3 than in those with *1/*1. In our study, we could not compare the plasma clearance of any of these AEDs, but the *Cyp2C19* variants were found to be associated with teratogenicity, but not with the nature of malformation. Since, the study was set up in a naturalistic background; the prescribed dosage might have controlled the seizure, but have resulted in teratogenic

malformation. None of the SNPs in *ABCB1*, *Cyp2C9*, *Cyp2C19* and *MTHFR* genes were observed to be associated with nature of malformations. *Cyp2C9* *2 (Arg144/Cys) and *Cyp2C9* *3 (Ile359/Leu) are the two most common variant alleles, which leads to impaired metabolic activity. In our population we had very small frequency of *Cyp2C9* *3 but for the analysis we have pooled the poor metabolizer phenotype represented by *Cyp2C9* *2 and *Cyp2C9* *3. *Cyp2C9* gene mutations in the coding region have been reported with PHT toxicity. In a small number of

patients with epilepsy the heterozygous *Cyp2C9 *3* allele had significantly lower PHT elimination rate and reduced required dosage than its wild type counterpart. However, *Cyp2C9* variants were not found to be associated with teratogenic effects in our study. It is important to note that *Cyp2C19* is the major drug metabolizing gene that is involved in metabolizing majority of AEDs used in conventional practice.

AEDs like VPA are involved in the inhibition of folate metabolism by having adverse effects on the folate pathway genes like that of the MTHFR enzyme. Defective *MTHFR* due to C677T and A1298C mutations may results in decreased activity of the enzyme conversion of homocysteine to methionine. Increased prevalence of C677T mutation in *MTHFR* gene correlates with the incidence of NTDs. However, in our study we did not find any association of any of these polymorphisms with malformation, nature of malformation, nature of drug therapy or seizure frequency. We had only three cases of NTDs of which two were homozygous wild type (CC genotype) and one was heterozygous mutant (CT). Folate supplementation was a part of the therapy possibly this might have prevented some of the patients who were at genetic risk for developing malformations.

Novel allelic, genotypic, and haplotypic association of Ex7 + 139C/T which is upstream to most of the earlier reported *ABCB1* associations, provides a new direction which needs to be investigated further in identifying the role of *ABCB1* in AED response. This study is possibly one of the first observations that indicate a role of *ABCB1* gene with malformation due to AED usage during pregnancy. The study supports the hypothesis that certain *ABCB1* polymorphisms play a potential role in the development of congenital malformations, which is independent of the nature of malformations or seizure frequency. In addition, the role of *Cyp2C19* in malformation opens up a crucial task of integrating the pharmacogenetic testing in AED treatment. The study hints at a potential interaction among the drug transport, drug metabolizing and folate pathway genes. Registries for WWE are relatively rare therefore a combined strategy can be evolved to validate these observations in other population based registries. It is important to bear that we cannot make strong clinical recommendations as this is a purely observational study that is inherently weak in its design.

Conclusion

The present study had shown genotypic effects of *ABCB1, Cyp2C9, Cyp2C19* and *MTHFR* polymorphisms on AEDs on teratogenic effects in pregnant mothers with epilepsy in a conventional clinical set up. Pharmacogenetic testing is likely to become an important instrument that helps the clinician to make appropriate prescriptions for epilepsy in pregnancy. However, these findings from observational study needs further confirmation in a larger sample.

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Ethical conduct of research

The authors state that they obtained appropriate institutional review board approval as per the Indian Council of Medical Research guidelines and principles outlined in the declaration of Helsinki for all human or animal experimental investigations. In addition for investigations involving human subjects, informed consent has been obtained from participants involved.