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UGT Gene polymorphism and Linkage Disequilibrium in Padiatric Epilepsy

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Abstract

Objective of the observational cohort study was to evaluate UGT1A6 gene polymorphisms and their association with sodium valproate monotherapy. The extent of linkage disequilibrium in pairwise combinations of single nucleotide polymorphisms (SNPs) were also calculated. Selection of the SNPs was carried out using bioinformatics tools. Children aged 2-18 years diagnosed with epilepsy and treated with sodium valproate monotherapy were recruited. Five ml of venous blood sample was collected. EDTA samples were used to evaluate the gene polymorphism using PCR-RFLP method and serum sample used to estimate the serum drug concentration by HPLC. Linkage Disequilibrium was carried out using the SHEsisplus program platform. Out of 231 missense mutations, three SNPs, rs1105879 (A552C) rs6759892 (T19G) and rs2070959 (A541G) were selected for the wet lab analysis. rs1105879 (A552C) was found to be deleterious with a SIFT score of 0.026. Apart from UGT1A6 (T19G) pattern, none of the genotype frequency distributions for UGT1A6 variants deviated significantly from HWE ($P<0.05$), suggesting that alleles were in equilibrium. There was no statistically significant association between patients of UGT1A6 (A552C, T19G, A541G) gene polymorphism and the therapeutic range of sodium valproate at different intervals (basal, six months, and one year). The SNPs of UGT1A6 showed a strong LD between T19G and A541G, A541G and A552C of UGT1A6. None of the pairwise haplotype associations were statistically significant except for one haplotype, TAACGT, with a chi-square value of 7.77, $p=0.005$, and an OR of 0.121 [95% CI = 0.021-0.689]. This indicates a favorable response to treatment among these haplotypes. There was a higher pattern of mutant carrier alleles for the genes UGT1A6 (T19G, A541G, A552C). There was no association between the gene UGT1A6 and sodium valproate concentration although the mean concentration of sodium valproate was high in wild type of UGT1A6 (T19G, A541G, A552C).

Keywords: Bioinformatics, gene, linkage disequilibrium, epilepsy

Full length article **Corresponding Author*, e-mail: *ushachidu@yahoo.com*

1. Introduction

Seizures are the temporary disruptions of brain function resulting from abnormal, excessive neuronal activity; Epilepsy is a chronic condition of repeated seizures [1,2]. The treatment of epilepsy in children is highly individualized at each major step in the management. Despite prompt treatment, approximately 20% of patients will experience recurrent seizure episodes. Sodium valproate is a broadly used broad-spectrum anti-epileptic drug. Glucuronide conjugation and β-oxidation are the major metabolic pathways, while CYP-mediated oxidation is a minor pathway.

Uridine-5-bisphosphate glucuronosyltransferase (UGT) is the enzyme that initiates the glucuronidation reaction. Steady-state serum drug concentrations may vary as a result of polymorphisms affecting genes encoding UGT enzymes that alter sodium valproate metabolism. In order to maintain therapeutic levels, this may necessitate adjusting the dosage of sodium valproate in epileptic patients. Before taking up wet lab-based approaches, if disease-associated SNPs can be identified from neutral SNPs, it would be of great use. When subsequent independent studies fail to identify disease associations, in silico analysis is useful [3,4]. As a result, predictive tools-derived independent evidence of SNP

functionality may also be a useful resource for distinguishing between genuine associations and false positives. In evolutionary biology and medical genetics, the analysis of linkage disequilibrium (LD) patterns has emerged as a significant topic [5]. Within a given population, LD occurs when two alleles at distinct loci are genetically related and exhibit non-random association on the same chromosome. Selection, recombination rate, mutation rate, genetic drift, mating system, population structure, and genetic linkage all have an impact on LD. Due to the fact that the range of possible values is dependent on the frequencies of the involved alleles, the coefficient of LD(D) is not always a suitable method for determining linkage disequilibrium.

UGT1A6 and CYP polymorphisms in Chinese, Japanese, Caucasian, and Iranian populations have been the focus of previous research. Only one North Indian study of the UGT1A6 polymorphism in epileptic children has been conducted for sodium valproate monotherapy. However, studies conducted in a number of Asian nations have produced contradictory reports. These studies' findings cannot be applied to the general population. Objectives of the study are to select the SNPs of UGT1A6 for polymorphism evaluation by bioinformatics tools

- To evaluate the pattern of UGT1A6 polymorphism in paediatric epileptics on sodium valproate monotherapy.
- To determine the association between genetic polymorphism and serum concentration of sodium valproate.
- To calculate the extent of linkage disequilibrium in pairwise combinations of SNPs

2. Materials and methods

Part A. Evaluation of the functional impact of missense SNPs using SIFT.

The query sequence for the UGT1A6 FASTA amino acid sequence is protein accession ID NP_001063.2. The web-based tool Sort the Intolerant from Tolerant (Seven) was used to examine the rs number as well as the position of the SNP on the chromosome. SIFT, Polyphen and I-mutant bioinformatics tools were used to filter the suitable SNPs for wet analysis.

Part B: Genotyping and assay of sodium valproate

Study design: Observational study

Study setting:

Clinical Work: Paediatric OPD/IPD of Justice K S Hegde Charitable Hospital, Deralakatte, Mangalore.

Laboratory work: Central Research Laboratory, KSHEMA, Deralakatte and Biochemical laboratory of Justice K S Hegde Charitable Hospital, Deralakatte, Mangalore.

Study subjects: Children aged 2-18 years diagnosed with epilepsy and treated with sodium valproate monotherapy from the past 30 days irrespective of their gender.

Ethical considerations:

The Central Ethics committee of NITTE (Deemed to be University) approved the study (Approval number: NU/CEC/2018/0174)

Inclusion criteria:

- Epileptic Patients of 2-18 years of age are diagnosed clinically by EEG
- Patients on a stable dose of 20mg/kg/day sodium valproate on a frequency of twice / thrice a day for the past one month

Exclusion criteria:

- History or evidence of hepatitis or impaired renal functions
- Patients who are already on treatment with any other antiepileptic drugs or drugs which induce or inhibit enzymatic action of sodium valproate metabolism

Specimen collection: After 30 days of taking the prescribed sodium valproate medication, an aseptic sample of five milliliters of whole blood was taken. There were three parts to this sample: 2 milliliters of EDTA whole blood were stored at -70 degrees Celsius for genetic polymorphism analysis, and 2 milliliters of plain whole blood were centrifuged to extract serum, which was then stored at -20 degrees Celsius to estimate serum drug concentration. Miller et al.'s modified method was used to extract DNA from human blood. Using an Eppendorf Nano-drop spectrophotometer at 260 nm, isolated DNA was measured. The ratio of 260 to 280 nm was used to determine the DNA sample's purity. The sealed DNA was kept at -20°C until further examination.

The UGT1A6 (T19G, A541G, A552C) polymorphisms were investigated by the Polymerase Chain Response Limitation Piece Length Polymorphism (PCR-RFLP) technique (Table 1). Each restriction enzyme, Hha I, NsiI, and Fnu4HI, was used to digest the amplified PCR product for the UGT1A6 (T19G, A541G, and A552C) genes. The MJ-Mini Thermal cycler, made by Bio-Rad and located in Tokyo, Japan, was used for the amplification process. Using ethidium bromide, the restricted fragment was separated on a 3% agarose gel. Based on the findings of the digestion patterns analysis, the genotype was assigned.

Serum sodium valproate was assayed by HPLC.

Part C: Linkage disequilibrium

D’ and R^2 were analyzed to evaluate the linkage disequilibrium between haplotypes of UGT1A6 (T19G, A541G, A552C) (sites 1,2,3) using the SHEsis-plus program

platform. The analysis did not include haplotypes with frequencies that were lower than 0.03.

Statistical analysis:

For qualitative data, frequency, percentage, and mean SD were used to summarize the information that was gathered. The Kolmogorov-Smirnov test was used to determine the data's normal distribution. Using an online "SNP stat" calculator, the Hardy-Weinberg equilibrium (HWE) of the genotype frequencies at each locus was evaluated. We used the SHEsis analysis platform to determine haplotype frequencies and calculate the linkage disequilibrium index based on the observed frequencies of UGT1A6 and UGT2B7. Polymorphism phenotyping (PolyPhen) and Sorting Intolerant From Tolerant (SIFT) were utilized to locate potentially harmful SNPs. Gene polymorphism and Sodium valproate levels were compared using One Way ANOVA, Kruskal Wallis test and the student "t' test. The Chi-Square test assessed the association between genetic polymorphism and sodium valproate. To calculate the extent of LD in pairwise combinations of SNPs, we have calculated D' and R^2. LD between haplotypes of UGT1A6 (T19G, A541G, A552C) (sites 1,2,3) and UGT2B7 (C161T, A268G, G211T) (sites 4,5,6) were determined using the SHEsis-plus program platform. Haplotypes with frequencies of less than 0.03 were omitted from the analysis.

3. Results and Discussions

Part A: Bioinformatics analysis

Upon carrying out the SIFT analysis of SNPs of UGT1A6 gene, it was observed that coding variants were 100%, but predicted ones were 98% (227 of 231), tolerated were 46% (106/227), damaging were 54% (121/227). Ninety-nine percent (229 of 231) were non-synonymous and only 1% (2 of 231) were synonymous. Eighty-three percent (192 of 231) of them were novel. Out of 231 missense mutations, three SNPs were selected, rs1105879 (A552C) rs6759892 (T19G) and rs2070959 (A541G). rs1105879 (A552C) was found to be deleterious with a SIFT score of 0.026. These SNPs were analyzed by the PolyPhen tool as well. I mutant suite 3.0 was used to predict the effects of single point protein mutation of above three SNPs of UGT1A6. The functional impacts of SNPs of the UGT gene (UGT1A6 and UGT2B7) were investigated using computational prediction tools and results were as described before (Table 2). Three SNPs each rs6759892, rs2070959, rs1105879 (UGT1A6) were selected for the wet lab analysis. Although the selected variants were found to be tolerant, studies have shown the significant role of those SNPs in different populations.

Bioinformatics: SNPs with a SIFT score of less than or equal to 0.05 are thought to be harmful, while those with a SIFT score greater than or equal to 0.05 are thought to be tolerant. The information's median value is between 2.75 and 3.5, ideally. The diversity of the prediction sequences can be measured with this. A warning indicates that the prediction was based on closely related sequences, as indicated by a value greater than 3.25. The number of sequences that contain an amino acid at the position of prediction is known as sequences at position. SIFT automatically selects sequences, but this column indicates that if the substitution is at the beginning or end of the protein, only a small number of sequences may be represented there. Polyphen scores of 0.0-0.15 suggest benign mutation,0.15-1.0 possibly damaging and scores 0.85-1.0 are damaging, predicted confidently. On I mutant analysis, DDG values of binary classification of all the three SNPs of UGT1A6 showed values <0 implying a decreased stability. Two missense mutations in UGT1A6's exon 1 cause the substitutions Thr181Ala and Arg184Ser [6]. Although the Arg184Ser allele, the single mutation, has been identified, the mutations are typically linked to a single allele (UGT1A6*2). UGT1A6 can glucuronidate many xenobiotic phenols. The rate of metabolism of phenols by recombinant UGT1A6*2 was lower than that for the wild-type enzyme [7]. The three common cSNPs of UGT1A6 define the alleles encoded Ser7Ala (T19G), Thr181Ala (A541G), and Arg184Ser (A552C) amino acid changes.

Part B: Wet lab analysis results

Patient characteristics:

One hundred twenty-two patients were screened, out of which a hundred patients fulfilled inclusion criteria and enrolled in the study after signing informed consent. Hundred patient samples were collected during the time of enrolment, 75 patients during six months follow-up investigations were performed, and one-year follow-up was carried out for 72 patients. Eleven patients shifted from sodium valproate treatment to other anti-seizure drug therapy, and in eight patients, another anti-seizure drug was added along with sodium valproate because of recurrent seizure attacks. One patient died because of complications of congenital heart disease. We could not collect samples of eight patients at six months and one year; however, they were seizure-free during this period. The mean age of study participants was 8.5 ± 4.3 years (2.2 years – 17.3 years), and the average BMI was 16.5 ± 4.3 (7.81 – 32.84) during enrolment. Fifty-seven patients were males, and forty-three patients were females. Patients were categorized into 3 groups based on age (i.e 2-6 yrs, 6-12yrs and 12-18 yrs). There was a statistically significant difference observed in BMI between different age groups. However, the BMI was in the normal range in different age group patients. (Table 7). The frequency distribution of various alleles in UGT1A6 (T19G, A541G, A552C are shown in Figures 2.

It was observed in the UGT1A6 gene that a higher frequency of mutant carrier allele type (Heterozygous and recessive pattern) was observed at T19G, A541G, and A552C loci than the wild type. Apart from UGT1A6 (T19G) pattern, none of the genotype frequency distributions for UGT1A6 variants deviated significantly from HWE ($P<0.05$), suggesting that alleles were in equilibrium. (Table 4).

Ethnic differences account for the disparity in prevalence between Asian, Caucasian, and Chinese populations in UGT1A6 (T19G, A541G, and A552C type). However, due to the high prevalence of these alleles, a variation in the genetic pattern may have a significant clinical impact. Because of this, genotyping will soon be required for more individualized treatment. When determining the drug's dosage, clinicians can thus collaborate with patients from a

variety of ethnic backgrounds. The role of gene polymorphisms in sodium valproate's pharmacokinetics and pharmacodynamics has been the subject of numerous studies. In terms of UGT1A6 (T19G, A541G, and A552C type), the Indian population's pattern of polymorphism was slightly different from the Chinese populations [6-12]. The wild type of genetic polymorphism was the most common in the Chinese pediatric epileptic population, whereas the mutant carrier allele type was the most common in the current study population. UGT1A6 (A541G & A552C) gene polymorphism had a predominant pattern of mutant carrier allele type in a Caucasian population study that was comparable to ours. However, there was no difference in the genetic pattern between our South Indian and North Indian pediatric epileptic patients [9]. One-way ANOVA followed by Bonferroni's post hoc test. Values in (Mean ± SD) Kruskal Wallis test, Values are expressed in Median (interquartile range). It was observed that there was no statistically significant difference in the SV concentration, SV dose, and SVCs at the time of enrolment of patients among different genotypes of UGT1A6 (T19G). (Table 5). Mean sodium valproate concentration at different intervals did not change significantly among various UGT1A6 (T19G) gene polymorphisms. (Table 6)

There was no statistically significant association between patients of UGT1A6 (T19G) gene polymorphism and the therapeutic range of sodium valproate at different intervals (basal, six months, and one year) (Table 7). It was observed that there was no statistically significant difference in the SV concentration, SV dose, and SVCs at the time of enrolment of patients among different genotypes of UGT1A6 (A541G) (Table 8). Mean sodium valproate concentration at different intervals did not significantly change among various UGT1A6 (A541G) gene polymorphisms (Table 9). There was no statistically significant association between patients of UGT1A6 (A541G) gene polymorphism and the therapeutic range of sodium valproate at different intervals (basal, six months, and one year) (Table 10). It was observed that there was no statistically significant difference in the SV concentration, SV dose, and SVCs at the time of enrolment of patients among different genotypes of UGT1A6 (A552C) (Table 11). Mean sodium valproate concentration at different intervals did not change significantly among various UGT1A6 (A552C) gene polymorphisms (Table 12). There was no statistically significant association between patients of UGT1A6 (A552C) gene polymorphism and the therapeutic range of sodium valproate at different intervals (basal, six months, and one year) (Table 13). One of the most important UGT isoforms involved in the metabolism of sodium valproate is UGT1A6. Therefore, the purpose of this study was to determine the prevalence of various UGT1A6 polymorphisms and their impact on sodium valproate concentrations in pediatric epileptic patients receiving sodium valproate monotherapy.

Our study population's valproate dose requirements, serum valproate concentrations, and standardized serum valproate concentrations were unaffected by the UGT1A6 polymorphisms. The effect of UGT gene polymorphism on sodium valproate concentration, for instance, has been the subject of numerous studies, each of which has produced distinct findings. Jain and co., Chu and others, as well as Wang et al detailed that there was no huge impact of polymorphisms on serum valproate fixations [9,10,14]. Hung and co. announced in Chinese epileptic patients on sodium valproate monotherapy that transporters of the variation UGT1A6 (T19G, A541G, and A552C) allele would in general require higher sodium valproate measurements and lower logarithmic fixation to-portion proportions (lnCDRs) than non-transporters and the homozygous transporters likewise appeared to require higher sodium valproate doses and lower lnCDRs. However, in our study, we could not observe any significant difference between sodium valproate dosages and lnCDR. Guo et al. reported in their study among Chinese children with epilepsy on sodium valproate monotherapy that UGT1A6 double heterozygous at nucleotide positions T19G, A541G, and A552C are associated with increased sodium valproate metabolism. It suggests that UGT1A6 mutations may be mainly responsible for the differences in plasma sodium valproate concentrations among different genotypic groups [7]. Our study also supported the above study as mutant variants had a lower mean sodium valproate concentration than wild type.

Jain and co. in the North Indian population of epileptic children, there was no significant correlation found between sodium valproate doses, serum valproate concentration, or UGT1A6 genotypes [9]. The common heterozygous of each of the three UGT1A6 polymorphisms were found in nearly half of the children with epilepsy. Xing and co. carried out a systematic screening of gene polymorphisms as part of an SNP analysis on three ethnic Chinese subjects. Six SNPs and ten mutations, nine of which were previously unknown, were found. T19G, A315G, A541G, and A552C showed significant linkage disequilibrium, suggesting that genetic polymorphisms in UGT1A6 may contribute to differences between individuals and within ethnic groups [12]. Saeki et al. studied on Japanese population and reported on several novel SNPs of UGT1A6. Among the studied thirteen SNPs, C308A confers translation termination at codon 103, resulting in an immature protein that probably lacks enzymatic activity. High linkage disequilibrium was observed among T19G, A541G, and A552C as reported in Caucasian and African- American populations [13].

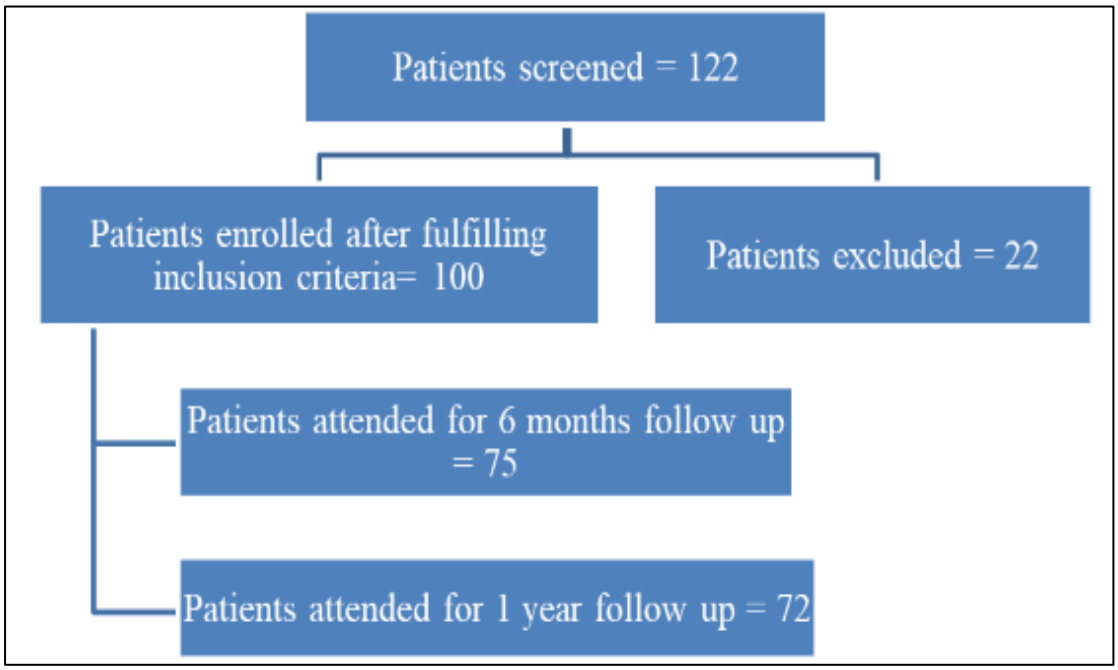
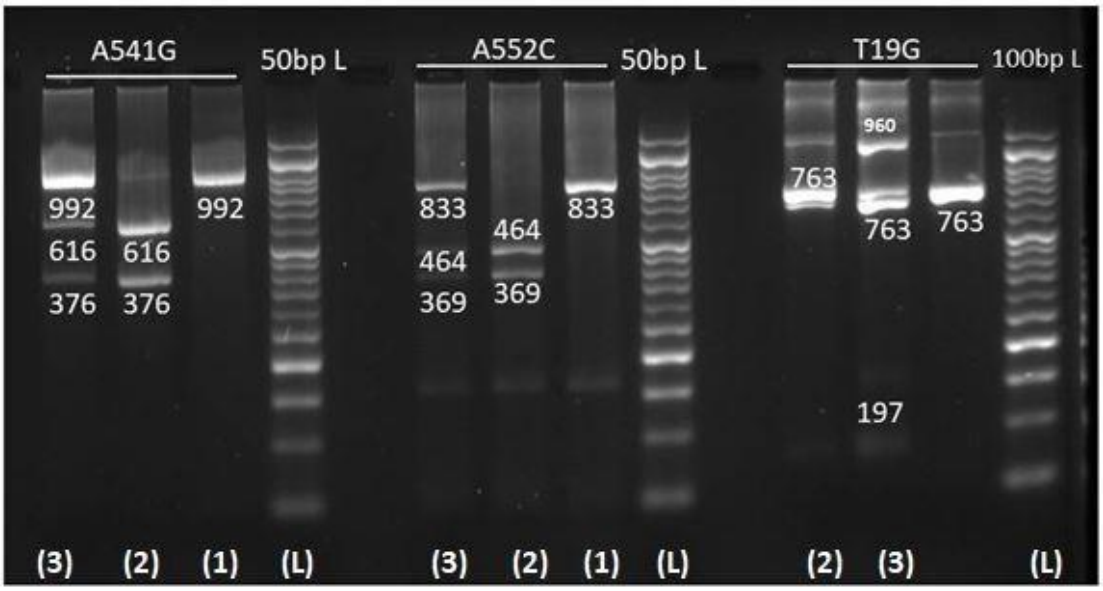


Figure 1: Recruitment of patients



Electrophoresis gel image patterns of UGT1A6 (A541G, A552C & T19G) gene after digestion with *NsiI, Fnu4HI, HhaI* enzymes respectively. L: Ladder, Lane (1): homozygosity for variant allele, Lane (2): Homozygosity for common allele, Lane (3): Heterozygosity.

Figure 2: Electrophoresis image patterns of UGT1A6 (A541G, A552C &T19G)

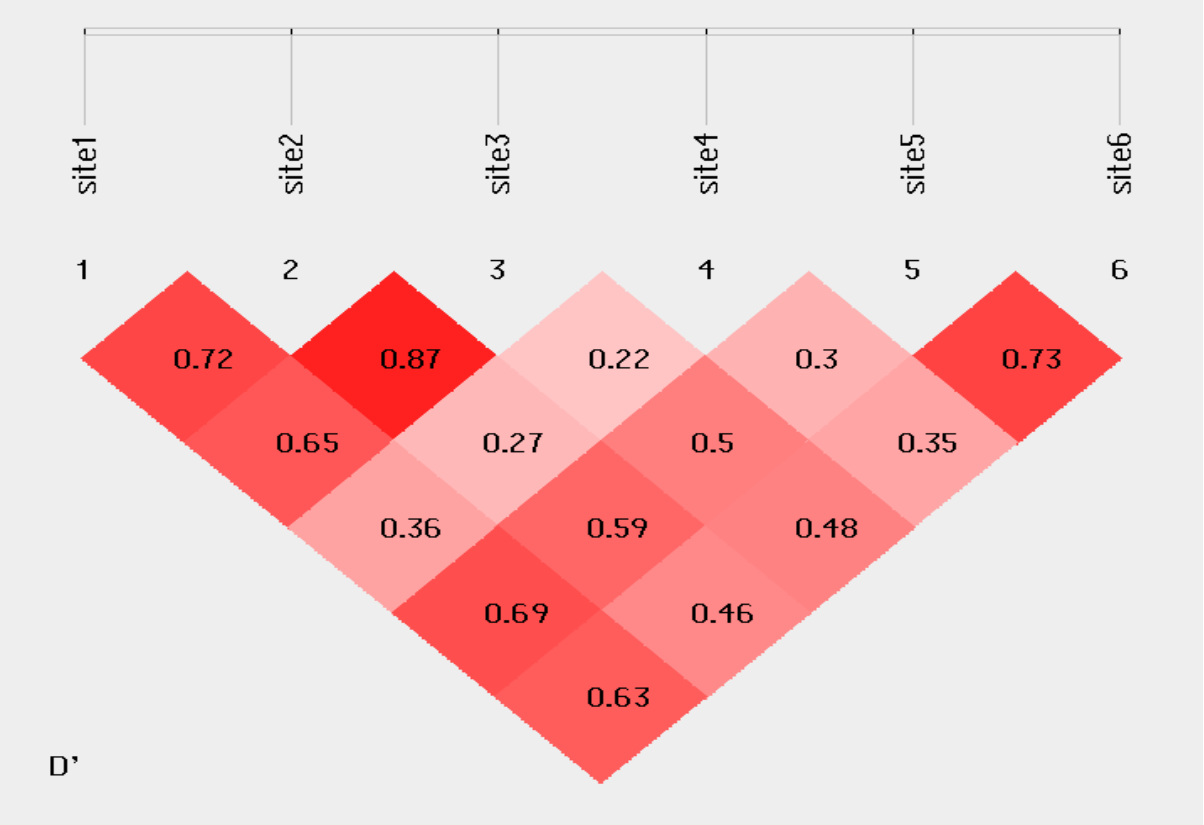


Figure 3: Linkage disequilibrium between SNPs of UGT1A6

Table 1: Primers and Restriction enzymes used for a particular gene

Gene	Polymorphism	Primer Sequence	Length (bp)	Annealing Temp (◦C)	Restriction Enzyme
UGT1A6	19 T>G rs-6759892	F: 5´-GATTTGGAGAGTGAAAACTCTTT-3´ R: 5´-CAGGCACCACCACTACAATCTC-3´	237	55	*Hha*I
	541 A>G rs-2070959	F: 5´-GGAAATACCTAGGAGCCCTGTGA-3´ R: 5´-AGGAGCCAAATGAGTGAGGGAG-3´	992	64	*Nsi*I
	552 A>C rs-1105879	F: 5´-GGAAATACCTAGGAGCCCTGTGA-3´ R: 5´-AGGAGCCAAATGAGTGAGGGAG-3´	992	64	*Fnu*4HI

Table 2: Bioinformatics of SNPs OF UGT1A6

SNP	AMINO ACID CHANGE	SIFT SCORE	SIFT MEDIAN	SIFT Prediction	Polyphen score	sensitivity	specificity	I-mutant-DDG (Kcal/mol/stability
rs6759892	S7A	1	3.02	TOLERATED	0.038	0.94	0.82	-1.19/decr stability
rs2070959	T181A	0.773	2.51	TOLERATED	0.0	1	0	-0.68/decr stability
rs1105879	R184S	0.026	2.51	DELETERIOUS	0.0	1	0	-1.17/decr stability

Table 3: Demographic data of patients in different age groups at baseline.

Characteristics	2-6 years (N=25)	6-12 years (N=60)	12-18 years (N=15)	P-value
Gender-Male:	17	32	08	
Female:	08	28	07	
Seizure type-GTCS:	17	55	11	
CPS:	08	05	04	
BMI (Mean ± SD)	14.08 ± 3.5	16.08 ± 3.8	21.04 ± 4.4	0.000*
Sodium Valproate Concentration (µg/ml) (Mean ± SD)	95.7 ± 43.6	104.2 ± 28.5	112.3 ± 23.3	0.315

One-way ANOVA followed by Bonferroni's post hoc test. *P-value <0.05 considered statistically significant

Table 4: Hardy-Weinberg Equilibrium (HWE) for UGT1A6 gene

Gene variant		Frequency pattern of UGT1A6 gene (%)		
		T19G	A541G	A552C
Wild type	Observed	32	43	38
	Expected	39.06	46.24	38.44
Heterozygous	Observed	61	50	48
	Expected	46.87	43.52	47.12
Homozygous recessive	Observed	07	07	14
	Expected	14.06	10.24	14.44
Chi-square value		9.08*	2.217	0.035

*P≤0.05, df=4, wpcalc online calculator used.

Table 5: Comparison of sodium valproate (SV) concentration, SV dose, SV concentration (SVCs) in UGT1A6 (T19G) at baseline.

UGT1A6 - T19G (N=100)				
	TT (31)	TG (61)	GG (08)	P- Value
SV Concentration(µg/mL)	108.0 ± 33.8	100.9 ± 35.8	103.6 ± 18.5	0.643
SV-dose (mg/kg/day)	20.6 ± 7.6	22.6 ± 8.5	24.3 ± 8.3	0.477
SVCs [(µg/mL)/(mg/kg)]	5.5 (4.2 - 6.7)	5.1 (4.4 -5.7)	5.3 (3.7 – 6.9)	0.286

Table 6: Comparison of SV concentrations in patients with different genotypes of UGT1A6 (T19G) at different intervals.

Sodium valproate concentration (µg/mL) (Mean ± SD)				
UGT1A6 (T19G)				
	TT	TG	GG	P-Value
Basal (N=100)	108.1 ± 33.9 (31)	100.9 ± 35.8 (61)	103.6 ± 18.5 (08)	0.643
6 Months (N=75)	97.4 ± 43.5 (23)	89.6 ± 41.7 (46)	92.2 ± 42.6 (06)	0.770
1 Year (N=72)	82.1 ± 30.5 (22)	76.6 ± 37.5 (44)	73.8 ± 37.4 (06)	0.802

One-way ANOVA followed by Bonferroni's post hoc test, df=2.

Table 7: Association between UGT1A6 (T19G) gene polymorphism and different ranges of SV concentration.

UGT1A6 (T19G) No. of patients									
Range (µg/mL)	Basal			Six months			One year		
	TT	TG	GG	TT	TG	GG	TT	TG	GG
<50	03	10	00	04	09	00	04	09	07
50-100	08	10	03	09	21	17	11	20	15
>100	20	41	05	00	04	02	01	04	01
χ^2 value	3.87			7.92			1.31		
P-value	0.423			0.095			0.858		

Statistical test used is Fisher's exact test.

Table 8: Comparison of SV concentration, SV dose, and SVCs at baseline in UGT1A6 (A541G) gene.

UGT1A6 (A541G)				
	AA (43)	AG (50)	GG (07)	P-Value
SV Concentration(µg/mL)	106.3 ± 34.2	101.1 ± 33.6	100.7 ± 40.7	0.748
SV-dose (mg/kg/day)	21.8 ± 8.1	22.3 ± 8.7	17.9 ± 4.7	0.410
SVCs [(µg/mL)/(mg/kg)]	5.3 (4.3 – 6.2)	6.1 (5.1 - 7.0)	3.7 (2.1 – 6.2)	0.203

One-way ANOVA followed by Bonferroni's post hoc test. Values in (Mean ± SD) Kruskal wallis test, Values are expressed in Median (interquartile range)

Table 9: Comparison of SV concentrations in patients with different genotypes of UGT1A6 (A541G) at different time intervals

Sodium valproate concentration (µg/mL) (Mean±SD)				
UGT1A6 (A541G)				
	AA	AG	GG	P-Value
Basal (N=100)	106.3 ± 34.2 (43)	101.1 ± 33.6 (50)	100.7 ± 40.7 (07)	0.748
6 Months (N=75)	97.5 ± 41.7 (34)	87.1 ± 41.2 (36)	93.2 ± 43.4 (05)	0.586
1 Year (N=72)	77.9 ± 30.8 (33)	79.9 ± 40.1 (34)	71.9 ± 29.7 (05)	0.919

One-way ANOVA followed by Bonferroni's post hoc test, df=2.

Table 10: Association between UGT1A6 (A541G) gene polymorphism and different ranges of SV concentration

UGT1A6 (A541G) No. of patients									
Range (µg/mL)	Basal			Six months			One year		
	AA	AG	GG	AA	AG	GG	AA	AG	GG
<50	04	07	02	06	06	01	05	10	01
50-100	10	11	00	09	18	13	17	14	02
>100	29	32	05	01	02	02	09	12	02
χ^2 value	3.47			3.64			2.19		
P-value	0.483			0.457			0.699		

Statistical test used is Fisher's exact test.

Table 11: Comparison of SV concentration, SV dose, and SVCs at baseline in UGT1A6 (A552C).

UGT1A6 (A552C)				
	AA (38)	AC (48)	CC (14)	P-Value
SV Concentration(µg/mL)	108.5 ± 32.3	97.7 ± 36.2	108.6 ± 30.7	0.289
SV-dose (mg/kg/day)	21.5 ± 7.7	21.9 ± 7.2	22.1 ± 12.4	0.963
SVCs [(µg/mL)/(mg/kg)]	5.98 ± 2.76	4.91 ± 2.36	6.46 ± 2.37	0.192

One-way ANOVA followed by Bonferroni's post hoc test. Values in (Mean ± SD)

Table 12: Comparison of SV concentrations in patients with different genotypes of UGT1A6 (A552C) at different time intervals.

Sodium valproate concentration (µg/mL) (Mean±SD)				
UGT1A6 (A552C)				
	AA	AC	CC	P-Value
Basal (N=100)	108.5 ± 32.3 (38)	97.7 ± 36.2 (48)	108.6 ± 30.7 (14)	0.289
6 Months (N=75)	102.3 ± 37.5 (30)	82.5 ± 40.8 (36)	97.6 ± 43.5 (09)	0.145
1 Year (N=72)	77.3 ± 28.3 (29)	80.8 ± 40.8 (34)	69.0 ± 26.6 (09)	0.666

One-way ANOVA followed by Bonferroni's post hoc test, df=2.

Table 13: Association between UGT1A6 (A552C) gene polymorphism and different ranges of sodium valproate concentration.

UGT1A6 (A552C) No. of patients									
Range (µg/mL)	Basal			Six months			One year		
	AA	AC	CC	AA	AC	CC	AA	AC	CC
<50	03	08	02	03	09	01	04	09	03
50-100	09	11	01	08	17	04	16	14	03
>100	26	29	11	18	11	04	07	13	03
χ^2 value	3.36			7.43			3.54		
P-value	0.499			0.115			0.471		

Statistical test used is Fisher‟s exact test.

Table 14: Haplotype association of UGT1A6 T19G and A541G with response (n=100, adjusted by Age + Sex)

	T19G	A541G	Freq	OR (95% CI)	P-value
1	T	A	0.5595	1.00	---
2	G	G	0.2545	0.60 (0.22 - 1.67)	0.33
3	G	A	0.1205	0.93 (0.27 - 3.21)	0.91
4	T	G	0.0655	0.75 (0.15 - 3.70)	0.73
Global haplotype association p-value: 0.77					

Table 15: Haplotype association of UGT1A6 A541G and A552C with response (n=100, adjusted by Age+Sex)

	A541G	A552C	Freq	OR (95% CI)	P-value
1	A	A	0.5923	1.00	---
2	G	C	0.2923	0.63 (0.27 - 1.49)	0.3
3	A	C	0.0877	0.73 (0.18 - 2.90)	0.65
4	G	A	0.0277	0.74 (0.10 - 5.49)	0.77
Global haplotype association p-value: 0.73					

Table 16: Haplotype association of UGT1A6 T19G and A552C with response (n=100, adjusted by Age+Sex)

	T19G	A552C	Freq	OR (95% CI)	P-value
1	T	A	0.5285	1.00	---
2	G	C	0.2835	0.76 (0.27 - 2.12)	0.6
3	T	C	0.0965	0.48 (0.11 - 2.15)	0.34
4	G	A	0.0915	0.70 (0.18 - 2.69)	0.6
Global haplotype association p-value: 0.65					

Munisamy and co reported that the UGT1A6 A552C polymorphism plays a significant role in the toxicity of sodium valproate in the North Indian epileptic population, with poor metabolizers having a longer elimination half-life and a lower clearance rate at steady-state concentrations of sodium valproate [15]. Variability in sodium valproate

pharmacokinetics is caused by genetic polymorphisms in the drug-metabolizing enzymes UGT1A6, which may result in an increased risk of valproate toxicity. However, the heterogeneity of valproate pharmacokinetics may also be caused by beta-oxidation and CYP-mediated oxidation.

The analysis of the combined genotype data for both the cases and the controls was used to investigate LD between pairs of SNPs. Controls and cases of pediatric epileptics who did not respond to VPA monotherapy were considered. Two LD measures were developed: the global statistic R^2 and the statistic D' that takes into account the limitations placed on R^2 by the marker pair's various allele frequencies. The SNPs of UGT1A6 showed a strong LD between T19G and A541G, A541G and A552C of UGT1A6. as suggested by high D' values (fig 3). However, the co-inheritance of the aforementioned alleles is not supported by the low R^2 values (0.58,0.41,0.03). The low allele frequencies could be the cause of the observed low R^2 values. A stronger disequilibrium between the alleles is suggested when the D' and R^2 values are closer to 1, indicating a high likelihood of co-inheritance. It suggests that the alleles are independent if the values are closer to zero or equal to zero. Even though a higher D' values were noted for different pairs of SNPs, high LD could not be established due to low R^2 values. Binary analysis revealed that none of the gene interactions were statistically significant. Using haplotype frequencies predicted by Shesisplus for association with therapy response, all genes were subjected to a haplotype analysis. Except for the haplotype TAACGT, which had an OR of 0.121 [95% CI =0.0210.689], none of the pairwise haplotype associations were statistically significant (table 2). This proposes a decent reaction to treatment among these haplotypes. Plot of the linkage disequilibrium between the analyzed SNPs and the UGT gene, with each square representing a pairwise linkage disequilibrium relationship between the two SNPs (with R^2 values written within the box representing R^2 values 100 as the linkage disequilibrium measure range). $R^2 = 1$ indicates high linkage disequilibrium in the darkest colored squares. R^2 values between 0 and 1 are indicated by the mid-colored squares. Low linkage disequilibrium (R^2 0) is indicated by the lightest colored squares. However, both D' and R^2 are taken into account when predicting the co-inheritance of the alleles.

After LD analysis, it was possible to conclude that the alleles were not in equilibrium. Due to low R^2 values, their coinheritance was not, however, established. The response to therapy was not correlated with the UGT1A6 haplotypes in a way that was statistically significant.

The haplotype association with the responsiveness to therapy was evaluated using SNP Statonline tool in pairwise manner between the SNPs. The associations were insignificant with $p>0.05$ (tables 3-7). However statistically significant ($p=0.014$) association was observed between the haplotypes of UGT2B7, C161T and G211T and responsiveness to Valproate monotherapy (table 14).

4. Conclusions

The mutant carrier alleles for the genes UGT1A6 (T19G, A541G, and A552C) were more prevalent. Although the wild type of UGT1A6 (T19G, A541G, and A552C) had a high mean sodium valproate concentration, there was no co-inheritance of UGT1A6 SNPs. However, there was no association between sodium valproate concentration and the genotypes of the gene UGT1A6 that were examined.

5. Limitations of the study

- A few enrolled patients' samples for estimation of sodium valproate could not be collected that has compromised the final sample size.
- Functional and protein expression studies were not carried out to ascertain the role of studied SNPs in patients.
- Dietary patterns, drug formulation and brands of sodium valproate in our study participants were not streamlined which could have influenced the sodium valproate concentration.

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