

**Title: Association of genetic variants in six candidate genes with valproic acid  
therapy optimization**

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## **Abstract**

**Objective** Valproic acid (VPA) is one of the most widely used antiepileptic drugs.

The aim of the present study is to investigate whether polymorphisms in genes related to pharmacokinetic and pharmacodynamic pathways of VPA were associated with the large interindividual variability in dosages and concentrations.

**Methods and results** Genetic polymorphisms in six candidate genes were detected in 162 epileptic patients under maintenance VPA mono-therapy and stable seizure control by realtime-PCR and PCR-RFLP. Results of statistical analysis demonstrated that the *UGT1A6* 19T>G, 541A>G, 552A>C and *GRIN2B* -200T>G were significantly associated with dosage and concentration-dose-ratio of VPA (all  $p<0.0001$ ). In addition, the regression model of concentration-dose-ratio of VPA also revealed that genetic variants in *UGT1A6*, *GRIN2B* and *UGT2B7* genes interactively affect concentration-dose-ratio of VPA and could explain 47% variation.

**Conclusion** Although with limited sample size, the present study identified genetic factors associated with VPA therapy optimization which has not been revealed and provided useful information for individualized VPA therapy in epileptic patients.

**Keywords** Valproic acid, *UGT1A6*, *GRIN2B*, *UGT2B7*, polymorphism, pharmacogenomics

## Introduction

Valproic acid (VPA; 2-propylpentanoic acid) is one of the most widely used antiepileptic drugs (AEDs) for treatment of both partial and generalized seizures in adults and children. It was also found to be useful in bipolar disorder, migraine, neuropathic pain, and cancer therapy [1-2]. However, with broad recommended dose range and a wide effective therapeutic plasma level (50–100 mg/L), large interindividual variability in doses and concentrations were observed in the epilepsy treatment with VPA.

There are many proposed mechanisms contributed to the numerous effects of VPA in treating epilepsy. Its anticonvulsant activity was thought to be related to the increased levels of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) in the brain. The GABA-elevating effect of VPA was primarily attributed to inhibition of nerve-terminal GABA transaminase, encoded by the *ABAT* gene. In addition, reduction of *GRIN* gene encoded *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptors mediated neuronal excitation may be another important mechanism for its antiepileptic effect [3].

In terms of pharmacokinetics, VPA is an 8-carbon 2-chain fatty acid that may absorb via intestinal fatty acid-binding proteins (I-FABP). The *FABP2* gene encoded I-FABP is only expressed in the intestine absorptive simple columnar epithelial cells

[4]. The most extensively studied polymorphism, 163G>A, is the alanine to threonine substitution at codon 54 in exon 2 of the human *FABP2* gene that may affect primary protein structures in the small helical regions [5]. Previous report indicated that this polymorphism was associated with VPA effective doses in Russians [6]; however, the effect of 163G>A on VPA steady-state concentrations and concentration-dose ratios (CDRs) was not evaluated.

VPA is almost entirely metabolized by the liver and about 30 to 70% of the administered dose excreted in the urine is directly glucuronidated to form its ester glucuronide conjugate [7]. This conjugative reaction was carried out by human UDP-glucuronosyl-transferases (*UGTs*) system and UGT1A3, UGT1A4, UGT1A6, UGT1A8, UGT1A9, UGT1A10 and UGT2B7 have been reported to be related to VPA glucuronidation [8-10]. Among these isozymes, UGT2B7, UGT1A6 and UGT1A9 count for the major VPA intrinsic clearance [11]. The *UGT2B7* gene contains 6 exons with a total length of about 16 kb [12]. UGT2B7 glucuronidates many endogenous compounds, such as steroid hormones and bile acid, as well as numerous clinically important drugs, including naproxen, ketoprofen, ibuprofen, morphine, propranolol, zidovudine, and VPA [13-16]. The *UGT2B7* gene has been reported to be polymorphic in the proximal promoter region, such as -327A>G, -161T>C, -138G>A, and -125T>C, in addition, in the coding region, 211G>T and

802C>T were identified as non-synonymous polymorphisms [17-18]. Although these genetic variants have been suggested to affect gene expression, the clinical effects on VPA maintenance doses, concentrations and CDRs were not yet been evaluated.

The UGT1A9 and UGT1A6 are major UGT isoforms in human liver that glucuronidate a large spectrum of drugs, including VPA, steroids, anticancer agents, fibrates and antiarrhythmics [19-20]. Previous studies indicated that there was interindividual variability in the liver glucuronidation of UGT1A6 and UGT1A9 substrates which may result in important pharmacological and physiological consequences [21]. Recent studies have identified several polymorphisms in the *UGT1A6* gene, such as 19T>G, 552A>C and 541A>G, as well as I399T>C and -1887T>G in *UGT1A9* gene, were associated with the variability in glucuronidation of their substrates in the in vitro models [19-20, 22]. Furthermore, in a study of Chinese epilepsy patients, the mean value of sodium valproate serum concentration was higher in the patients with A allele in 552A>C of *UGT1A6* gene than that of the patients with C allele [23]. Results of these studies indicated that genetic polymorphisms in *UGT1A6* and *UGT1A9* may play an important role in VPA therapy. Nonetheless, the combined effect of polymorphisms in *UGT1A6*, *UGT1A9* and *UGT2B7* genes on the dosages, concentrations and CDRs of VPA has not been investigated.

To our knowledge, there was no previous study had evaluated the effect of multiple genes on VPA therapy. Besides, the joint effect of pharmacokinetic and pharmacodynamic genes on VPA concentration-dose ratio was not considered before. By comprehensive analyses of genetic polymorphisms related to the pharmacokinetic and pharmacodynamic pathways of VPA, the present study aimed to identify the multiple genetic effect and the interactions between genes regarding influences on VPA concentration-dose ratio in epilepsy patients.

## **Methods**

### **Subjects**

The study was approved by the Ethics Committee of the Department of Neurology, National Taiwan University Hospital. Blood samples for genotyping were collected after informed consents were obtained from all subjects. All patients had electroencephalogram and magnetic resonance imaging brain scans. The classifications of epilepsies and epileptic syndromes were conducted according to the guidelines of ILAE. The temporal lobe epilepsy (TLE) referred to all temporal lobe epilepsies. For each patient the following clinical information was recorded: gender, weight (kg), age, epilepsy classification, etiology, valproic acid maintenance dose (mg/kg/day), valproic acid serum concentration at maintenance dose (mg/L). The maintenance dose of valproic acid was defined as the dosage which has not been changed for at least one year under good compliance and well seizure control (seizure attack less than three times in one year). Concentration-dose ratios (CDRs) were calculated by dividing the steady state VPA serum concentration by the VPA daily dose.

A total of 162 patients with epilepsy under valproic acid mono-therapy treatment (90 men, 72 women, age  $36.23 \pm 1.02$  (mean  $\pm$  SD)) were included and each of them reached a maintenance dose for at least six months (VPA dose:  $19.95 \pm 0.72$

mg/kg/day; concentration:  $68.81 \pm 1.94$  mg/L). Of these patients, 146 subjects (90.1 %) were localization-related epilepsies and TLE was the major type (126 subjects, 86.3%) (Table 1). As normal controls, 190 healthy volunteers were genotyped for comparison (98 men, 92 women, age  $39.1 \pm 1.52$ ). All patients and controls were recruited from the same center, of the same ethnic background and unrelated.

### **Genotyping**

Genomic DNA was isolated from peripheral blood sample by use of a QIAamp DNA Mini Kit. *UGT1A9* -1887T>G, I399T>C; *UGT2B7* -842A>G, -161C>T, 735A>G; *FABP2* 163G>A and *ABAT* 167A>G were genotyped by ABI Prism 7900 HT Sequence Detection System (Applied Biosystems). PCRs were performed in 20  $\mu$ l volume, containing allele-specific probes, assay-specific primers, TaqMan Universal PCR Master Mix, and genomic DNA (50 ng). Genotypes analyses were estimated by SDS 2.2 software (Applied Biosystems).

For *UGT1A6* 19T>G, 552A>C, 541A>G; *UGT2B7* 802T>C, and *GRIN2B* -200T>G, -421C>A, PCRs were conducted in 25  $\mu$ l volumes containing 50 ng genomic DNA, 2.5  $\mu$ M/ $\mu$ L dNTPs, 10  $\mu$ M/ $\mu$ L each primers, 1 $\times$ reaction Buffer, and 5 unit Taq DNA polymerase (Fermentas, Inc.). PCR conditions were denaturation at 94  $^{\circ}$ C for 5 min followed by 35 cycles at 94  $^{\circ}$ C for 20 s, annealing at 60 $^{\circ}$ C for 20 s, 72  $^{\circ}$ C for 20 s with a final elongation step at 72  $^{\circ}$ C for 5 min. The mutant alleles were

examined by restriction fragment length polymorphism (RFLP).

### **Statistical Analysis**

Prior to statistical analysis, the normality of data set, such as VPA dosage, concentration and CDR, were tested. The CDR of VPA was not demonstrated normal distribution before natural log transformation. Therefore, lnCDR of VPA was applied in the statistical analysis. For the association between dosages, concentrations and lnCDRs with each genetic polymorphism were analyzed by Pearson's chi-square test or one way ANOVA followed by Bonferroni post hoc test. Identification of haplotypes was performed using EM algorithm [24]. The standardized linkage disequilibrium values were calculated for measurement of the linkage disequilibrium among these loci [25-26]. A p-value less than 0.05 was considered to indicate statistical significance. Multiple comparisons were corrected using Bonferroni's method.

Further multiple regression models were conducted to detect the joint effect of *UGT1A6*, *UGT1A9*, *UGT2B7*, *FABP2*, *GRIN2B*, and *ABAT* polymorphisms on logCDR of VPA with the adjustment for the epilepsy syndrome, age and weight. The model selection procedures were undergone based on the Akaike Information Criterion (AIC) [27]. All data analyses were performed using SAS version 9.1.3 (SAS Inc, Cary, NC, USA).

## Results

The allele and genotype frequencies of the *UGT1A6*, *UGT1A9*, *UGT2B7*, *FABP2*, *GRIN2B*, and *ABAT* polymorphic loci for patients under VPA maintenance mono-therapy and normal controls were listed in Table 2 and Table 3. The genotypic distributions were all consistent with Hardy-Weinberg equilibrium proportions and genotype frequencies were not significantly different between patients and healthy controls. Significant linkage disequilibrium was detected among *UGT2B7* -161C>T, -842A>G, and 802T>C, among *UGT1A9* 1399T>C, -1887T>G, *UGT1A6* 19T>G, 541A>G, and 552A>C and between *GRIN2B* -200T>G and -421C>A as indicated by high values of  $D'$  ( $>50$ ;  $r^2>0.45$ ; all  $p$ -values  $< 0.0001$ ).

### Association of genetic variants with VPA Dosage

The statistical analysis revealed significant association between the VPA dosage (mg/kg/day) with the *UGT1A6* 19T>G, 541A>G, 552A>C or *GRIN2B* -200T>G (all  $p<0.0001$ ) (Table 2 and Table 3). Carriers of the variant *UGT1A6* 19T>G allele tended to require higher VPA dosage than noncarriers ( $p<0.0001$ ) and the homozygous carriers also seemed to require higher VPA dosage ( $p<0.0001$ ). Carriers of *UGT1A6* 541A>G and 552A>C revealed similar results. Patients with the variant *UGT1A6* 541A>G allele were more likely to require higher dosage of VPA than patients with wild types ( $p<0.0001$ ) and the homozygous variants carriers seemed to require higher dosage as well ( $p=0.0001$ ). For the patients with the variant *UGT1A6* 552A>C allele,

higher dosages of VPA were needed than patients with wild types ( $p < 0.0001$ ) and the homozygous variants carriers also seemed to need higher dosages ( $p < 0.0001$ ). On the other hand, carriers of the variant *GRIN2B* -200T>G allele were more likely to require lower VPA dosage than noncarriers ( $p < 0.0001$ ) and the homozygous carriers also tended to require lower dosage ( $p < 0.0001$ ).

### **Association of genetic variants with VPA Concentration-Dose Ratio**

Among the six candidate genes, genetic polymorphisms in *UGT1A6* and *GRIN2B* genes were significantly associated with lnCDRs of VPA (Table 2 and Table 3). Carriers of the variant *UGT1A6* 19T>G allele tended to have lower lnCDRs than noncarriers ( $p < 0.0001$ ) and the homozygous carriers also seemed to have lower lnCDRs ( $p < 0.0001$ ). Similar results were observed in carriers of *UGT1A6* 541A>G and 552A>C. Carriers of the variant *UGT1A6* 541A>G allele were more likely to have lower lnCDRs than noncarriers ( $p < 0.0001$ ) and the homozygous carriers seemed to have lower lnCDRs as well ( $p = 0.0001$ ). Carriers of the variant *UGT1A6* 552A>C allele tended to have lower lnCDRs than noncarriers ( $p < 0.0001$ ) and the homozygous carriers also seemed to have lower lnCDRs ( $p < 0.0001$ ). On the other hand, carriers of the variant *GRIN2B* -200T>G allele were more likely to have higher lnCDRs than noncarriers ( $p < 0.0001$ ) and the homozygous carriers also tended to have higher lnCDRs ( $p < 0.0001$ ).

Haplotype analysis demonstrated that comparison of haplotype pattern distributions revealed that patients with TAATT, TAACT and TAACG haplotypes composed of *UGT1A6* 19T>G, 541A>G, 552A>C, *UGT1A9* 1399T>C and -1887T>G were more likely to have higher lnCDRs (all  $p<0.0009$ ; Table 4). For haplotypes in the *GRIN2B* gene, patients with GC haplotype composed of *GRIN2B* -200T>G and -421C>A were more likely to have higher lnCDRs ( $p<0.003$ ; Table 5).

### **Regression model analysis**

Multiple regression analysis was applied to evaluate the combined effect of pharmacokinetic and pharmacodynamic related genes on lnCDRs of VPA under adjustment of cofactors, such as epilepsy syndromes, weight, and age. Among the included cofactors, only temporal lobe epilepsy combined with genetic variants in *UGT1A6*, *GRIN2B* and *UGT2B7* genes demonstrated significant effect on lnCDR of VPA. The most fitted model indicated that temporal lobe epilepsy, genetic variants in *UGT1A6* and *GRIN2B* genes and interaction terms of *UGT2B7* -161C>T, -842A>G and 802T>C work synergistically on the effect of lnCDRs of VPA (adjusted  $r^2=47\%$ ; Table 6).

## Discussion

The dosage optimization plays an important role in epilepsy therapy and identifying genetic factors which may have impact on the pharmacokinetic and pharmacodynamic of VPA could help better individualized therapy. The present study suggests that multiple genes affect lnCDR of VPA not only separately but also synergistically in epileptic patients. In summary, the present study demonstrated that the *UGT1A6* 19T>G, 541A>G, 552A>C and *GRIN2B* -200T>G were significantly associated with lnCDR of VPA (all  $p < 0.0001$ ). In addition, the regression model of lnCDR of VPA also revealed that genetic variants in *UGT1A6*, *GRIN2B* and *UGT2B7* genes interactively affect lnCDR of VPA and could explain 47% variation. These results indicated that multiple genetic effects were participated in the VPA therapy.

Our results should not be over-interpreted because of the limited sample size. However, the clinical impact of multiple genetic polymorphisms on VPA pharmacokinetics and pharmacodynamics was evaluated for the first time. Moreover, the genetic polymorphisms in *UGT1A6* and *GRIN2B* genes have not been demonstrated to be associated with CDR of VPA before. The functional significance of the association between lnCDR of VPA and *UGT1A6* 19T>G, 541A>G, 552A>C and *GRIN2B* -200T>G may be interpreted from our results. Patients with variant genotypes in *UGT1A6* 19T>G, 541A>G and 552A>C showed lower lnCDRs of VPA than those with wild-types, suggesting higher activities of the UGT1A6 enzymes in

patients with the variant genotypes. Consequently, patients with the variant genotypes composed of *UGT1A6* 19T>G, 541A>G and 552A>C may need higher VPA maintenance dosages than those with wild-types and this phenomenon was observed in our patients. Our results were supported by previous in vitro study, in which HEK293 cells expressed variant genotypes of *UGT1A6* 19T>G, 541A>G and 552A>C displayed lower  $K_M$  values toward VPA, indicating a higher affinity of the UGT1A6 enzyme and higher metabolic activities of the cells with variant genotypes [20]. Therefore, *UGT1A6* 19T>G, 541A>G and 552A>C were suggested to be important genetic predictors of VPA therapy and may need to be confirmed in a larger population.

As for *GRIN2B* -200T>G, patients with the variant genotypes showed higher  $\ln CDRs$  of VPA than those with wild-types, indicating higher transformation rate of dose to concentration in patients with variant genotypes. Thus, patients with variant genotypes of *GRIN2B* -200T>G may need lower doses of VPA to achieve target therapeutic levels than patients with wild-types and this was also observed in our data. The functional effect of *GRIN2B* -200T>G was demonstrated in an in vitro study, the transcriptional activity of the human NMDA receptor 2B subunit gene in PC12 cells expressed the variant -200G allele were down-regulated compared to that of the G allele [28]. The -200G allele was significantly associated with the susceptibility of

schizophrenics in a case control study [28]. These findings suggested that the -200T>G genetic polymorphism may cause NMDA receptor dysfunction, therefore, the glutamate mediated neuronal excitation and the effective dose of VPA to control epilepsies may both be reduced.

The effect of genetic polymorphisms in *UGT2B7*, such as -161C>T, 802T>C and 842A>G, on the CDR of VPA was not identified in the single gene analysis in the present study. However, in the multiple regression model, under the adjustment of epilepsy syndromes, weight, and age, the combined effect of *UGT2B7* -161C>T, 802T>C and 842A>G was demonstrated to be correlated with the lnCDR of VPA. These findings suggested that multivariate analysis may be more informative than single variant in clinical studies. In a study of 14 healthy subjects with the *UGT2B15*\*2/\*2 genotype, AUC of VPA after a dose of 600 mg once daily for 4 days tended to increase in subjects with variants of *UGT2B7* -161C>T and 802T>C [29]. The functional significance of *UGT2B7* genetic polymorphisms was controversial. Several studies have demonstrated that the activities of *UGT2B7* enzymes may be higher, similar or lower in cells or subjects with variant *UGT2B7* -161C>T and 802T>C [30-33]. Both methodological and substrate factors may contribute to the differences. Our results were consistent with the previous study of VPA [29] and both studies indicated that subjects with variants of *UGT2B7* -161C>T and 802T>C may

have lower UGT2B7 enzyme activities.

Population trough CDR of antiepileptic drugs has been suggested to be a clinical reference to estimate the dose required to achieve a target concentration [34]. It has been also proposed for the pharmacokinetic monitoring of cyclosporine trough concentration [35]. Therefore, factors that contributed to interindividual variability of CDR may be important in clinical application.

In conclusion, the present study identified genetic polymorphisms in *UGT1A6*, *GRIN2B* and *UGT2B7* genes may explain part of the interindividual variability in VPA treatment. Further larger population studies may need to confirm our findings.

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