Effects of UGT1A6, UGT2B7, and CYP2C9 genotypes on plasma concentrations of valproic acid in Chinese children with epilepsy

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a) Running title: VPA concentrations and genetic polymorphisms

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c) This paper includes 20 pages, three tables and four figures.
Summary:

Valproic acid (VPA) is one of the most commonly prescribed drugs for the treatment of epilepsy. Interindividual variability in VPA dose and plasma concentration may reflect functional consequences of genetic polymorphisms in genes encoding drug-metabolizing enzymes. The aim of this study was to determine the relationship between plasma concentrations of VPA and single nucleotide polymorphisms (SNPs) involving uridine diphosphate glucuronosyltransferase (UGT) 1A6 (UGT1A6), UGT2B7, and cytochrome P450 2C9 (CYP2C9) genes in Chinese children with epilepsy. UGT1A6, UGT2B7, and CYP2C9 polymorphisms were identified by polymerase chain reaction-restriction fragment length polymorphism approach or direct automated DNA sequencing in 98 epileptic patients treated with VPA monotherapy. Patients with double heterozygosities at nucleotide positions T19G, A541G and A552C in the UGT1A6 gene, were associated with higher VPA doses compared to those with wild type or single heterozygosity (\(P=0.010\)). Lower adjusted plasma VPA concentrations were also observed in patients with UGT1A6 double heterozygoses than those with single heterozygosity (\(p=0.027\)). There were no differences in VPA dose or adjusted plasma VPA concentrations among the UGT2B7*2 or CYP2C9*3 genotypic groups. These results suggest that UGT1A6 mutations affect VPA metabolism in epileptic children. It needs to be further investigated in a larger cohort of patients.

Key Words: epilepsy; valproic acid; genetic polymorphisms; UGT1A6; UGT2B7; CYP2C9
**Introduction**

Valproic acid (VPA) is widely used for treating children with epilepsy. Although VPA is affordable and effective in the control of seizures, there is large interindividual variability in pharmacokinetics and pharmacodynamics. Therefore, its plasma concentration needs to be monitored as a guide of dose adjustment during the course of therapy.\(^1\) These differences in VPA dose and plasma concentration may reflect functional consequences of genetic polymorphisms in genes encoding drug-metabolizing enzymes.

The metabolism of VPA involves three major metabolic pathways, glucuronidation conjugation, mitochondrial \(\beta\) -oxidation, and CYP-catalyzed terminal desaturation and hydroxylation.\(^2\) The most extensive biotransformation pathway for VPA is conjugation with glucuronic acid, mainly mediated by UGT1A6, UGT1A8, and UGT2B7.\(^3\) Approximately 20% to 70% of a dose of VPA is excreted in the urine as glucuronide conjugates. CYP-catalyzed metabolism of VPA accounts for about 10% of the administrated dose, mainly mediated by CYP2C9.\(^4\) The genes coding VPA-metabolizing enzymes are highly polymorphic. It has been reported that the three most common single nucleotide polymorphisms in the coding region of the \(UGT1A6\) gene are T19G, A541G and A552C with allele frequencies of 0.238, 0.220, and 0.247, respectively, in Chinese population.\(^5\) The combinations of different variants (UGT1A6*2 to *4) result in increased glucuronidation activities.\(^6-7\) UGT2B7*2 with C802T is the most extensively studied \(UGT2B7\) variant with allele frequency of 32.8% in Chinese population.\(^8\) In terms of the contribution of UGT2B7*2 to different drug metabolisms, conflicting results have been reported.\(^9-11\) CYP2C9*3 with A1075C, the major \(CYP2C9\) variant with allele frequency of about 3.3% in Chinese population,\(^12\) had been demonstrated to significantly impair the catalytic activities of the wild-type CYP2C9 toward various substrates both in vitro and in vivo.\(^13\)

Previous studies reported potential contributions of the SNPs in genes encoding the major VPA-metabolizing enzymes to VPA disposition.\(^14-20\) However, the results were not entirely consistent among the studies. For example, two Chinese reports demonstrated an association between CYP2C9*3 genotype and VPA metabolism,\(^16, 18\) but the association was not observed in an Iranian study.\(^14\) These conflicting results reinforce the need to further...
determine the functional significance of the SNPs of UGT1A6, UGT2B7 and CYP2C9 on VPA disposition in patients with epilepsy. Moreover, VPA is metabolized by multiple UGTs and CYPs. Thus, previous studies were limited in accessing the influence of these gene polymorphisms on VPA disposition since most of studies only assayed the influence of the polymorphisms of one metabolizing enzyme in adult patients with epilepsy. The present study was designed to comprehensively evaluate the effects of UGT1A6 variants including the combinations of T19G, A541G and A552C, UGT2B7*2, and CYP2C9*3 on plasma VPA concentrations in a cohort of Chinese children with epilepsy.
Methods

Patients

A total 98 epileptic patients (56 males and 42 females, mean age 7.8±7.5 years, mean body weight 27.3±15.5 Kg) were recruited at the Shengjing Hospital, China Medical University in Shenyang, China. Patients diagnosed as partial seizures or generalized seizures were treated with VPA as monotherapy. After minimum one month of continuous VPA treatments, 2-3mL blood samples were drawn from each patient to measure plasma VPA concentration. The mean VPA daily dose was 17.2±15.5 mg/kg per day, and the mean VPA plasma concentration was 65.2 ±26.8 µg/ml. Due to large interindividual variations in VPA metabolism, steady-state plasma concentrations of VPA were adjusted by dose and body weight of each patient. Patients previously treated with any drugs known to modulate the activities of UGT1A6, UGT2B7 and CYP2C9 or with liver or renal disorders, were excluded in this study. Informed consent was provided to all the patients participated in the study. Sample handling and data analysis protocols were approved by the Medical Ethical Committee of China Medical University.

VPA Quantitation

Blood samples were drawn from each patient after minimum one month of continuous VPA treatments to ensure that plasma VPA concentrations were at steady state. Peripheral blood samples were collected from patients before VPA administration and plasma VPA concentrations were quantified using the fluorescence polarization immunoassay of the Abbott TDx system, according to the instructions of the manufacturer. The assay had a coefficient of variation (C.V.) lower than 4%, and a sensitivity of 0.7µg/ml. Results are presented as means of duplicates for each sample.

Genotyping

Genomic DNA was extracted from clotted whole blood. Prior to extraction, clotted samples were thawed, and then dispersed into small pieces through nylon mesh (pore size 77 µm). Genomic DNA was then extracted by phenol–chloroform extraction and ethanol precipitation, as previously described. The UGT1A6 T19G, A541G, A552C and CYP2C9 A1075C polymorphisms were analyzed by polymerase chain reaction-restriction fragment
length polymorphism (PCR-RFLP) method, as described previously.\textsuperscript{7,22-23} \textit{UGT2B7} C802T polymorphism was determined by direct automated DNA sequencing after PCR amplification using forward (5'-CTGCATAATTCTAGGACAAC-3') and reverse (5'-CTACCATAACAATCAGTTG-3') primers.

\textit{Statistical analysis}

Statistical analysis was performed using the SPSS software (version 16.0; SPSS Inc., IL, USA). The patients’ demographic characteristics including age and body weight, VPA doses, plasma VPA concentrations, and adjusted plasma VPA concentrations were compared between two genotypic groups using the Student’s \textit{t}-test or one way ANOVA. Deviation of Hardy-Weiberg equilibrium (HWE) was examined by Chi-square test. Linkage disequilibrium between SNP pairs was carried out by using the online software SHEsis (http://analysis.bio-x.cn/myAnalysis.pnp). A two-tailed \textit{p}-value of less than 0.05 was considered to be statistically significant.
Results

To explore the effects of genetic factors on interindividual variabilities in VPA doses and plasma concentrations in our cohort of Chinese children with epilepsy, the *UGT1A6* T19G, A541G, A552C, *UGT2B7* C802T, and *CYP2C9* A1075C polymorphisms were analyzed by PCR-RFLP or automated DNA sequencing (Figure 1). The frequencies of each genotype are shown in Table 1. The frequency of each genotype was consistent with the Hardy-Weinberg equilibrium. Significant linkage disequilibrium was detected among T19G, A541G, and A552C of *UGT1A6* gene. The D’coefficients between T19G and A541G, T19G and A552C, and A541G and A552C were 0.938, 0.941, and 1.000, respectively. There were no significant differences in the frequencies of the *UGT1A6*, *UGT2B7* and *CYP2C9* genotypes between our study and other studies in Chinese populations.5, 8, 12)

Furthermore, associations between *UGT1A6*, *UGT2B7* and *CYP2C9* SNPs and VPA doses or plasma VPA concentrations were observed in all studied epileptic children (Table 2). None of the demographic characteristics was significantly different in each genotype, indicating that these characteristics did not influence the variations of VPA dose or plasma concentrations between the genotypes (Table 2). For *UGT1A6*, patients with double heterozygosities at nucleotide positions T19G, A541G and A552C, had significantly higher VPA doses than those with wild type or single heterozygosity (Table 2 and Figure 2). Although lower adjusted VPA concentrations were also observed in patients with *UGT1A6* double heterozygosities than those with wild type or single heterozygosity (Table 2), only the differences between double and single heterozygosities were statistically significant (Figure 1). However, these results could be biased by the 3 years mean age of the patients with *UGT1A6* double heterozygosities compared to the 8 years mean age of the patients with either wild type or *UGT1A6* single heterozygosity (Table 2). To test this possibility, additional statistical analyses were performed only in 0-4 year old children. Interestingly, younger patients with *UGT1A6* double heterozygosities still maintained higher VPA doses and lower adjusted plasma VPA concentrations than those with either wild type or single heterozygosities (Table 3).

In contrast, no significant associations were observed between VPA doses, VPA
concentrations or adjusted VPA plasma concentrations and *UGT2B7*2, or *CYP2C9*3 genotypes in this patient cohort (Table 2, Figures 3 and 4).
Discussion

Genetic polymorphism may be an important source of interindividual variability in pharmacokinetics and pharmacodynamics of VPA. This study demonstrated that *UGT1A6* double heterozygosities at nucleotide positions T19G, A541G and A552C were associated with higher VPA doses and lower adjusted plasma VPA concentrations compared to those with wild type or single heterozygosity in epileptic children. These results suggest that the increased VPA doses and the decreased adjusted plasma VPA concentrations may be attributed to increased glucuronidation rate of the drug. Previous *in vitro* studies also demonstrated that cells expressing recombinant *UGT1A6* variants including eight possible combinations of T19G, A541G and A552C had higher VPA glucuronidation rates than those expressing wild type. Especially UGT1A6*2 (19G/541G/552C) showed the highest enzyme activity and intrinsic clearance values of VPA. This was further supported by two *in vivo* studies about A552C genotype and VPA concentration, but not supported by the other study about A541G genotype and VPA concentration in China. Interestingly, our results also showed that *UGT1A6* single heterozygosity did not decrease, rather slightly increased plasma VPA concentrations, compared with the wild type genotypic group. This tendency was also previously observed that human liver tissue samples with heterozygous expression of UGT1A6 *1/*2 exhibited lower activity than those homozygous for *1 or *2. It is explained that single heterozygous *UGT1A6* variants may perturb UGT1A6 localization to the ER membrane, dimerization or stability, thus, resulting in low activity. However, due to a limited sample size, the results need to be further investigated in a larger cohort of patients.

Otherwise, it is the first study for identifying genetic factors of the interindividual variability of VPA metabolism in epileptic children. Some studies reported the effect of age on the metabolism of VPA. One study showed that there was no statistically significant difference in the rate of VPA glucuronidation in the human liver microsome bank between the younger patients (2-56 years) and elderly patients (65 years onward). Another study showed that UGT1A6 and UGT2B7 had no age dependent changes at the transcript levels and the protein levels between the paediatric liver samples (aged 7-24 months) and adult liver samples (aged 25-75 years). UGT1A6 had comparatively higher activity at birth and
increased to adult levels at 14 months. These results suggest that the protein levels and activities of UGT1A6 have reached the adult levels in young children after 14 months. Our results showed that UGT1A6 double heterozygosity were associated with higher VPA doses and lower VPA plasma concentrations in either the entire patient cohort or only in those aged 0-4 years. These data strongly suggest that the significant differences of VPA doses and adjusted plasma VPA concentrations between the UGT1A6 genotypic groups are age-independent. Our findings are supported by a recent study in adult epileptic patients which also showed that homozygous carriers of the variant UGT1A6 T19G, A541G and A552C allele tend to require higher VPA doses and lower concentration-to-dose ratios(CDRs) than noncarriers.

Besides UGT1A6, UGT2B7 also play an important role in the intrinsic clearance of VPA. To date, reports on the influence of UGT2B7 polymorphisms on substrate drug metabolism still remains inconsistent. In our study, UGT2B7*2 genotype had no effects on plasma VPA concentrations. This is consistent with previous two studies, which also showed an insignificant difference between UGT2B7*2 genotype and AUCs of VPA. Thus, UGT2B7*2 may have no or minor effects on plasma VPA concentrations.

CYP2C9 is responsible for the majority (75-80%) of VPA terminal desaturation and hydroxylation. Two previous studies demonstrated an association between CYP2C9*3 genotype and VPA metabolism. One indicated that the genetic polymorphisms of CYP2C19 and CYP2C9 were highly significant factors for VPA pharmacokinetic. The other indicated that patients with CYP2C9*3 heterozygous genotype had higher plasma VPA concentrations than those with the wild-type genotype. However, no association was observed between CYP2C9*3 genotype and the plasma VPA concentrations in our study and an Iranian study. The discrepancy may be attributed to the sample size, age and race. Otherwise, the other CYP genes that could theoretically affect VPA plasma levels in epilepsy, including CYP2A6, CYP2B6, may mitigate the magnitude of the CYP2C9-based VPA interactions.

In conclusion, our study demonstrates that UGT1A6 double heterozygosities at nucleotide positions T19G, A541G and A552C are associated with increased VPA metabolism, whereas UGT2B7*2 and CYP2C9*3 had no obvious impacts in Chinese children with epilepsy. It suggests that UGT1A6 mutations may be mainly responsible for the differences in
plasma VPA concentrations among different genotypic groups. It might provide usefully genetic information for personalized VPA therapy in epileptic children.

**Acknowledgments**

We are grateful to Dr. Yubin Ge for helpful comments on the manuscript.

**References**


Table 1 Genotype frequencies of the *UGT1A6*, *UGT2B7* and *CYP2C9* polymorphisms

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<th>Genotype</th>
<th>Number of patients</th>
<th>Frequency (%)</th>
<th>95% Confidence Interval (%)</th>
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Table 2 Effects of the UGT1A6, UGT2B7 and CYP2C9 genotypes on VPA doses, VPA concentrations and adjusted VPA concentrations in all studied epileptic children

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<th>Genotype</th>
<th>NO.</th>
<th>Age (Years)</th>
<th>Weight (Kg)</th>
<th>VPA dose (mg/Kg per day)</th>
<th>VPA Concentration (µg/ml)</th>
<th>Adjusted VPA Concentration (µg/ml per mg/Kg)</th>
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<td>0.17</td>
<td>0.56</td>
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\(^a\)Wild type in UGT1A6 gene is defined as *1/*1; \(^b\)Single heterozygosity include *1/*2 (n=33), *1/*3 (n=3), *1/*4 (n=1), and *1/*8 (n=2); \(^c\)Double heterozygosities include *2/*2 (n=4), *2/*4 (n=1), and *2/*10 (n=1); \(^d\)*2 is defined as 19G/541G/552C; \(^e\)*3 is defined as 19G/541A/552A; \(^f\)*4 is defined as 19G/541A/552C; \(^g\)*8 is defined as 19T/541G/552C; \(^h\)*10 is defined as 19G/541G/552A.
<table>
<thead>
<tr>
<th>Genotype</th>
<th>NO.</th>
<th>Age (Years)</th>
<th>Weight (Kg)</th>
<th>VPA dose (mg/Kg per day)</th>
<th>VPA Concentration (µg/ml)</th>
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<td>29.35±13.64</td>
<td>52.14±29.90</td>
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**P value**

- ANOVA: 0.431 0.469 0.012 0.825 0.129
- WT vs SingH: 0.199 0.253 0.704 0.647 0.412
- WT vs DoubH: 0.733 0.551 0.004 0.591 0.045
- SingH vs DoubH: 0.556 0.811 0.010 0.842 0.159

a Wild type in UGT1A6 gene is defined as *1/*1; b Single heterozygosity include *1/*2 (n=11), *1/*3 (n=1), and *1/*4 (n=1); c Double heterozygosities include *2/*2 (n=3), *2/*4 (n=1), and *2/*10 (n=1), d *2 is defined as 19G/541G/552C; *3 is defined as 19G/541A/552A; *4 is defined as 19G/541A/552C; *10 is defined as 19G/541G/552A; e WT refers to Wild type; f SingH refers to single heterozygosity; g DoubH refers to double heterozygosities.
Legends for figures

Figure 1 Genotyping of the UGT1A6 T19G, A541G, A552C, UGT2B7 C802T, and CYP2C9 A1075C by PCR-RFLP and DNA sequencing. Segments of the UGT1A6, UGT2B7 and CYP2C9 genes were PCR amplified from genomic DNA. Panel A: PCR amplicons were digested by HhaI, NsiI, Fnu4HI, and KpnI to genotype the T19G, A541G, A552C of UGT1A6 and A1075C of CYP2C9*3, respectively. Panel B: DNA sequencing results for UGT2B7*2. (1), (ii) and (iii) represent the DNA sequences of wild type, heterozygotes, and homozygotes. Mutated base pairs are underlined.

Figure 2 Differences in VPA dose, VPA concentration and adjusted plasma concentration in various UGT1A6 genotypic groups in Chinese epileptic children. A: VPA dose, B: VPA concentration, C: adjusted VPA concentration. Horizontal lines indicate mean value in each group. WT: Wild type; SingH: single heterozygosity; DoubH: double heterozygosities.

Figure 3 Differences in VPA dose, VPA concentration, and adjusted plasma concentration in various UGT2B7 genotypic groups in Chinese epileptic children. A: VPA dose, B: VPA concentration, C: adjusted VPA concentration. Horizontal lines indicate mean values of each group.

Figure 4 Differences in VPA dose, VPA concentration, and adjusted plasma concentration in various CYP2C9 genotypic groups in Chinese epileptic children. A: VPA dose, B: VPA concentration, C: adjusted VPA concentration. Horizontal lines indicate mean values of each group.
Figure 1

A

\[
\begin{array}{c|c|c|c}
\text{HinfI} & \text{NciI} & \text{PvuII} & \text{KpnI} \\
\hline
\text{TT} & \text{AA} & \text{AA} & \text{AA} \\
\text{TG} & \text{GG} & \text{AC} & \text{RC} \\
\text{GG} & \text{AG} & \text{GC} & \text{IC} \\
\end{array}
\]

B

\[
\begin{array}{c|c|c|c|c}
\text{UGT1A6} & \text{UGT2B7} & \text{CYP2C9} & \text{C802T} \\
\hline
\text{T} & \text{T} & \text{T} & \text{T} \\
\text{T} & \text{T} & \text{T} & \text{T} \\
\text{T} & \text{T} & \text{T} & \text{T} \\
\text{T} & \text{T} & \text{T} & \text{T} \\
\text{T} & \text{T} & \text{T} & \text{T} \\
\text{T} & \text{T} & \text{T} & \text{T} \\
\text{T} & \text{T} & \text{T} & \text{T} \\
\end{array}
\]
Figure 3

A

VPA dose

60

40

20

0

CC  CT  TT

UGT2B7/2 C802T

B

VPA concentration

150

100

50

0

CC  CT  TT

UGT2B7/2 C802T

C

Adjusted VPA concentration

15

10

5

0

CC  CT  TT

UGT2B7/2 C802T