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ABCC2 rs2273697 is associated with valproic acid concentrations in patients with epilepsy on valproic acid monotherapy

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Valproic acid (VPA), a widely used antiepileptic drug, is characterized by intensive inter-individual variability in concentration. Both efflux and influx transporters are reported to play important roles in the disposition of VPA, however, no comprehensive investigation into the association of the single nucleotide polymorphism (SNP) in ABC/SLC families with VPA concentration are reported. In the present study, we investigated the association of 12 SNPs in *ABCC2, ABCC4, ABCG2, MCT1, MCT2*, and *OATP2B1* in 187 Chinese patients with epilepsy on VPA monotherapy with the trough concentrations of VPA. The data showed that VPA concentration in patients with ABCC2 rs2273697 AA genotype was significantly higher than that in those with GA+GG genotypes (p=0.000). The findings of the present study suggest that ABCC2 polymorphisms influence VPA concentrations in patients with epilepsy on VPA monotherapy, which may affect the treatment outcomes.

1. Introduction

Valproic acid (VPA) is an effective broad-spectrum antiepileptic drug used in the treatment of primary generalized tonic-clonic, absence, and partial seizures (Perucca 2002). However, large inter-individual variability in VPA efficacy has been observed in our and other researchers' study (Haerian et al. 2012; Huang et al. 2015). Even tenfold differences in mean dose of VPA in adults were reported (Blanco-Serrano et al. 1999). To maintain VPA concentration within the therapeutic range, therapeutic drug monitoring (TDM) is commonly used (Neels et al. 2004).

Compared to other antiepileptic drugs (AEDs) such as carbamazepine and phenytoin, fewer pharmacogenomics studies on VPA have been reported. Moreover, most of them concentrated on well-known genetic polymorphisms in the metabolic enzymes such as UDP glycosyltransferase (UGT) and Cytochrome P450 (CYP) (Ghodke-Puranik et al. 2013). However, drug transporters also play key roles in the absorption, distribution and excretion of therapeutic agents, suggesting that, to comprehensively understand the mechanisms underlying the inter-individual variability in VPA pharmacokinetics, studies on the effects of single nucleotide polymorphisms (SNPs) in transporters genes involved in VPA transportation are needed.

ATP binding cassette (ABC) family, one of the major efflux transporters superfamily, play an important role in the excretion of drugs from body as drug efflux transporters, thus ABC family expression should be considered as an important factor in the variability in PK/PD of AEDs. Some members in ABC superfamily have been identified participating in the process of VPA transportation, including ABCC2 (also named MRP2, multidrug resistance protein-2) (Yi et al. 2013). ABCC4 (also named MRP4, multidrug resistance protein-4), and ABCG2 (also named BCRP, Breast cancer resistance proteins) (Kim et al. 2015).

Solute-linked carriers (SLC), the other major transporter superfamily, play an important role in the influx transport of therapeutic agents, especially organic anions, of which monocarboxylate transporters (MCT) 1(SLC16A) and 2 (SLC16B), organic anion transporting polypeptides (OATP/SLCO) 2B1 (SLCO2B1, SLC21A9) have been shown to mediate the transport of endogenous short chain monocarboxylates and exogenous drugs such as salicylic acid, valproic acid, and simvastatin acid (Vijay and Morris 2014). It is reported that coordination of the related members of SLC family and ABC transporters allow an efficient vectorial transport of VPA across the endothelial cells (Kusuhara and Sugiyama 2005).

Genetic variants such as SNPs could lead to the change of gene expression, mRNA degradation and translation, as well as protein structures which may affect the biological functions of proteins (Shastry 2009). Considering the critical role of transporters in the disposition of xenobiotics, in the present study, we selected SNPs with function or with the minor allele frequency higher than 10% in ABCC2, ABCC4, ABCG2, MCT1, MCT2 and OATP2B1 to comprehensively identify the genetic impacts on VPA concentration in Chinese patients with epilepsy who received VPA monotherapy.

2. Investigations and results

2.1. Patient characteristics and genotype analysis

In total, 187 patients with epilepsy were included, their demographic characteristics are presented in Table 1. The genotype distribution of the SNPs detected in this study were consistent with the Hardy-Weinberg equilibrium ($p > 0.05$) (Table 2). Except for rs1693614 (the data of which in other populations are absent) and rs2273697 (the minor allele frequencies of which are similar among populations), the other SNPs showed significant difference

in the minor allele frequency among populations (for details see Table 3). No difference in age, sex, seizure types and etiology was found between VPA nonresponsive group and VPA responsive group. Significant linkage disequilibrium was detected among MCT2 rs2711655 and rs3763980 (D' = 0.983, r^2 =0.881), which agreed with a previous report (Moncrieffe et al. 2010).

Table 1: Demographic characteristics of the patients with epilepsy included in the study (n=187)

Male/Female 79/108
Age(year) 19.9±11.7
Weight(kg) 47.96±18.22
Daily VPA dose(mg/kg) 679.14(500-1000)
Plasma VPA concentration(μg/mL) 61.27(44.74-75.41)
VPA CDR(μg•mL^{-1}/mg•kg^{-1}) 4.42(3.06-5.27)

Data are presented as M(median) with P_{25}-P_{75}(percentile, 25%-75%) or mean standard deviation or amount

Table 2: Comparison of MAF difference of 12 SNPs among various populations

Gene SNP	Chinese epilepsy patients	Normal Chinese population	General Japanese population	General Caucasian population	General Sub-Saharan African popuation
ABCC2 rs2273697	0.092	0.078	0.128	0.243**	0.221**
ABCC2 rs3740066	0.245	0.267	0.284	0.342	0.275
ABCG2 rs2231137	0.363	0.289	0.193**	0.017**	0.050**
ABCC4 rs899494	0.196	0.232	0.244	0.164	0.221
MCT1 rs60844753	0.114	NA	NA	NA	NA
MCT1 rs7169	0.372	0.337	0.327	0.398	0.080**
MCT2 rs10784000	0.229	0.244	0.198	0.288	0.115**
MCT2 rs10877333	0.111	0.151	0.110	0.201**	0.031**
MCT2 rs1693614	0.317	NA	NA	NA	NA
MCT2 rs2711655	0.310	0.378	0.378	0.408	0.720**
MCT2 rs3763980	0.291	0.367	0.329	0.299	0.194*
OATP2B1 rs2306168	0.212	0.267	0.405**	0.04**	0.402**

P values were calculated by χ^2 test (*P<0.05, **P<0.01).

Table 3: Genotype and allele frequency for SNPs studied in 187 Chinese epilepsy patients

SNP	Genotype frequency n(%)	Allele frequency n(%)	H-Wp
ABCC2 rs2273697 G>A	GG 134 (71.7%) GA 46 (24.6%) AA 7 (3.7%)	G (84.0%) A (16.0%)	0.23
ABCC2 rs3740066 C>T	CC 105 (56.1%) TC 70 (37.4%) TT 12 (6.4%)	C (74.9%) T (25.1%)	0.94
ABCG2 rs2231137 G>A	GG 76 (40.6%) GA 89 (47.6%) AA 22 (11.8%)	G (64.4%) A (35.6%)	0.59
ABCC4 rs899494 A>G	AA 6 (3.2%) GA 63 (33.7%) GG 118 (63.1%)	A (20.1%) G (79.9%)	0.48
MCT1 rs60844753 G>C	GG 148 (79.1%) CG 39 (20.9%)	G (89.5%) C (10.5%)	0.11
MCT1 rs7169 G>A	GG 19 (10.2%) GA 92 (49.2%) AA 76 (40.6%)	G (37.8%) A (65.2%)	0.24
MCT2 rs10784000 G>A	GG 6 (3.2%) GA 76 (40.6%) AA 105 (56.1%)	G (23.5%) A (76.5%)	0.08
MCT2 rs10877333 T>G	TT 143 (76.5%) GT 38 (20.3%) GG 6 (3.2%)	T (86.6%) G (13.4%)	0.93
MCT2 rs1693614 C>T	CC 18 (9.6%) TC 82 (43.9%) TT 87 (46.5%)	C (31.6%) T (68.4%)	0.83
MCT2 rs2711655 A>G	AA 86 (46.0%) GA 85 (45.5%) GG 16 (8.5%)	A (68.7%) G (31.3%)	0.43
MCT2 rs3763980 A>T	AA 13 (7.0%) AT 88 (47.0%) TT 86 (46.0%)	A (30.5%) T (69.5%)	0.13
OATP2B1 rs2306168 C>T	CC 114 (61.0%) CT 65 (34.8%) TT 8 (4.2%)	C (78.3%) T (21.7%)	0.73

2.2. Ethnic difference of the candidate SNPs

MAF (mutant allele frequency) difference of 12 candidate SNPs among various populations (http://www.ncbi.nlm.nih.gov/SNP) are listed in Table 2. The MAF in ABCC2 rs2273697 is significantly lower in the Asian population (around 10%) than that in the Caucasian and in General Sub-Saharan African population (> 20%). For ABCG2 rs2231137, the frequency in the Chinese population is around 30%, significantly higher not only than that in Caucasian and Sub-Saharan African population, but also than that in the Japanese population with 19.3 %. Ethnic and regional differences among Chinese, Japanese, sub-Saharan African population and Caucasian people in the frequency of OATP2B1 rs2306168 was significant (21% *vs* 40% *vs* 40% *vs* 4%). As for the candidate SNPs in MCT1 and MCT2, significant MAF differences was found in all the studied 5 SNPs between Sub-Saharan African population and the other five population groups, except MCT1 rs60844753 and MCT2 rs1693614 whose MAF were reported only in Chinese patients with epilepsy so far.

2.3. Influence of the genetic polymorphisms on VPA concentrations

The present study showed that VPA concentration varied greatly with CDR (concentration/dose) from 1.02 to 17.02 (μg•mL^{-1}/mg•kg^{-1}). To determine the influence of genetic polymorphisms in the candidate transporters on VPA pharmacokinetics, the association of ABCC2, ABCG2, ABCC4, MCT1, MCT2, and OATP2B1 SNPs with VPA CDR were analyzed in 187 patients with epilepsy. Among the SNPs detected, only ABCC2 rs2273697 showed a significant influence on VPA CDR (P = 0.000) (Table 4). Homozygous ABCC2 rs2273697 AA genotype was associated with a higher CDR value in comparison with that observed in GA and GG genotypes (AA vs. GA + GG [median with P25-P75]: 9.45[7.49~9.76] vs. 4.22[3.05~5.15] (μg•mL^{-1}/mg•kg^{-1}), p=0.000, Fig.) in the dominant model. There was no significant intergroup difference in VPA daily dose (mg/kg) distribution among the different ABCC2 rs2273697 genotypes.

ABCC2 rs3740066 exhibited no association with VPA concentration (Table 4). In addition, any significant association of SNPs in ABCG2, ABCC4, MCT1, MCT2 and OATP2B1 with VPA CDR in dominant model, recessive model and additive model (data not shown) was observed.

Multiple linear regression analysis was applied to evaluate the effect of ABCC2 rs2273697 on VPA plasma concentration under the adjustment of cofactors, such as VPA dose, gender, weight and age. It was shown that, by stepwise method, VPA dosage

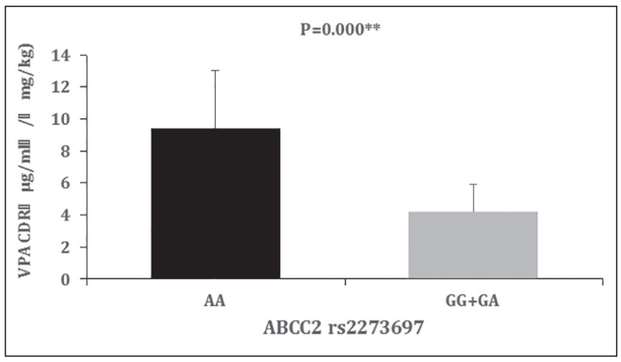

Fig. 1: VPA concentration/dose normalized by body weight (CDR) in patients with ABCC2 rs2273697 genotypes, compared by the Mann-Whitney test.

Table 4: Comparison CDR (concentration/dose) of VPA in epilepsy patients according to *ABCC2 ABCG2, ABCC4, ABCB1, MCT1 MCT2 OATP2B1* SNPs genotype

SNP	Genotype	n	C/D(μg·mL^{-1}/mg·kg^{-1})	P
ABCC2	GG	134	4.30(3.00~5.26)	0.000[a]
rs2273697	GA	46	4.00(3.13~4.67)	
	AA	7	9.45(7.49~9.76)	
ABCC2	CC	105	4.40(3.04~5.13)	0.69
rs3740066	TC	70	4.39(3.37~5.37)	
	TT	12	4.65(2.66~5.90)	
ABCG2	GG	76	4.29(3.05~5.26)	0.87
rs2231137	GA	89	4.37(3.04~5.11)	
	AA	22	5.04(3.67~6.36)	
ABCC4	AA	6	4.00(1.88~5.93)	0.72
rs899494	GA	63	4.46(3.32~5.38)	
	GG	118	4.41(3.04~5.16)	
MCT1	GG	148	4.43(3.13~5.24)	0.69
rs60844753	CG	39	4.38(2.73~5.29)	
MCT1	GG	19	4.26(3.55~4.82)	0.63
rs7169	GA	92	4.22(2.94~5.23)	
	AA	76	4.69(3.13~5.64)	
MCT2	GG	6	4.59(3.49~4.77)	0.99
rs10784000	GA	76	4.45(2.84~5.50)	
	AA	105	4.38(3.17~5.23)	
MCT2	TT	143	4.36(3.10~5.26)	0.15
rs10877333	GT	38	4.55(3.35~5.39)	
	GG	6	4.88(2.40~2.95)	
MCT2	CC	18	4.96(3.48~5.51)	0.50
rs1693614	TC	82	4.26(2.90~5.23)	
	TT	87	4.44(3.06~5.28)	
MCT2	AA	86	4.38(2.90~5.64)	0.89
rs2711655	GA	85	4.42(3.09~5.14)	
	GG	16	4.60(3.35~5.16)	
MCT2	AA	13	4.73(3.38~5.73)	0.70
rs3763980	AT	88	4.33(3.25~5.13)	
	TT	86	4.46(3.00~5.52)	
OATP2B1	CC	114	4.41(3.01~5.27)	0.98
rs2306168	CT	65	4.08(3.34~5.23)	
	TT	8	5.57(2.17~6.69)	

Data was presented as M (median) and P_{25}-P_{75} (percentile: 25%-75%).
a: P values were calculated by Kruskal-Wallis test.

was the most important factor influencing VPA concentration (P =0.000, Table 5). Overall, the most fitted model indicated that ABCC2 rs2273697 together with VPA daily dose can explain approximately 23.3% variation of VPA concentrations in VPA monotherapy patients (Table 5).

Table 5: Results of multiple linear regression analysis

Variable	Unstandardized coefficients		Standardized coefficients	t	Sig
	β	Std.Error	Beta		
Constant	72.09	2.35		30.72	0.000
Gender	2.94	0.757	0.060	3.88	0.000
Weight	-0.594	0.032	-0.347	-18.826	0.000
VPA dose	0.051	0.002	0.545	32.204	0.000
ABCC2 rs2273697 R^2=0.233	-7.335	0.777	-0.14	-9.443	0.000

Multiple linear regression analysis of the combined effects of positive SNPs on VPA concentration under adjustment of cofactors: VPA dose, gender, age and weight.

3. Discussion

VPA has an extensive inter-individual pharmacokinetic variability (Johannessen Landmark et al. 2017; Smith et al. 2016), which is partly ascribed to the genetic polymorphisms in drug transporters. In the present study, the association of 12 SNPs in related transporters possibly participating in VPA disposition including ABCC2, ABCG2, ABCC4, MCT1, MCT2 and OATP2B1 with the VPA blood concentration in Chinese patients with epilepsy on VPA monotherapy were comprehensively investigated. The findings showed that the ABCC2 rs2273697 G allele was significantly correlated with lower VPA concentration than A allele (P=0.000) in dominant model. Furthermore, a significant ethnic difference was found in the frequency of ABCC2 rs2273697 in different populations (Table 2), which indicated that research in other populations is warranted. This is the first investigation into the association of genetic polymorphisms in the transporters possibly involved in VPA disposition with the VPA concentration.

ABCC2 (MRP2) is one of multidrug resistance proteins (MRPs, ABCCs) which serve as efflux carriers in organs with excretory function (e.g. liver, intestine, kidney) or cells with barrier function (e.g. endothelial cells of brain capillaries, syncytiotrophoblasts of the placenta) (Jedlitschky et al. 2006). ABCC2 rs2273697 G>A is a nonsynonymous variation that causes substitution of a valine with an isoleucine at position 417, it was reported that the mutation lead to reduced carbamazepine (CBZ) transportation, and resulted in higher frequency of central CNS ADRs (Kim et al. 2010). Similar results were published that in children with epilepsy, allele A is associated with increased probability of AEDs response compared to allele G (Ufer et al. 2011). These previous studies suggested that A allele was proposed to be loss-of-function mutation and lead to lower activity of the efflux transporter. Consistently, the present study found that the mutant homozygous AA was significantly correlated with higher VPA concentration compared to the genotype GG and GA (Table 4 and Fig. 1). However, it was found that in patients with epilepsy on AEDs treatment, patients with an AA genotype may have an increased likelihood of resistance to treatment as compared to patients with the GG genotype (Ufer et al. 2011), and some other studies found that the allele A is not associated with the responses to AEDs as compared to allele G (Hilger et al. 2012; Hung et al. 2012; Kwan et al. 2011; Qu et al. 2012; Radisch et al. 2014; Sporis et al. 2013). This discrepancy was partially attributed to different sample size, population ethnicity, patients with different seizure type and medicine applications. Therefore, further studies are warranted in different ethnic groups to investigate the effects of the ABCC2 variants on VPA concentration and to perform stratified analysis based on different phenotypic covariates.

To the best of our knowledge, the present study is the first comprehensive report focusing on the association of SNPs in two transporter superfamilies of ABC and SLC with VPA concentration in patients with epilepsy on monotherapy. Additionally, significant differences in the frequencies of these SNPs in different ethnics were found in the present study. Further independent studies with larger sample sizes to confirm these results are warranted.

In summary, the present study demonstrated that the ABCC2 rs2273697 AA genotype exhibits a significant association with VPA concentration, which might contribute to the interpatient variability VPA pharmacokinetics.

4. Experimental

4.1. Study subjects

A total of 187 patients were enrolled at the Department of Neurology at the First Affiliated Hospital of Sun Yat-sen University. All patients had been diagnosed with epilepsy, with normal liver and kidney functions based on results from electroencephalograms and biochemical laboratory tests. The following exclusion criteria were considered: poor compliance, severe adverse drug reactions (ADRs), history of alcohol or drug abuse, hepatic/renal failure, unstable medical conditions that required treatment (HIV, hepatitis C, thyroid disorder, or diabetes mellitus). All patients were treated with sodium valproate (Deparkin; Sanofi-Synthelabo Minsheng Pharmaceutical, Hangzhou, China) monotherapy. Blood sampling was performed after 1 month of stable dosing regimen at steady-state of VPA pharmacokinetics. CDR value was calculated by dividing the steady state VPA plasma concentration by the weight adjusted VPA daily dose (mg/kg). The study was performed according to the Declaration of Helsinki. All subjects gave their written informed consent by verbal and written information. This study was approved by the ethics committee of the First Affiliated Hospital of Sun Yat-sen University (Serial number: [2012] 269, Guangzhou, China).

4.2. Determination of VPA concentration

Steady-state trough plasma concentrations of VPA were determined by the high-performance liquid chromatography ultraviolet (HPLC-UV) method (Chen et al. 2012). The calibration curves ranged from 5.0–200 μg/mL. The accuracy and precision data for the inter- and intra-batch variations were <15%. This method was applied in routine TDM.

4.3. Genotyping

Genomic DNA was extracted by phenol-chloroform extraction and ethanol precipitation, as previously prescribed (Loparev et al. 1991). The single nucleotide polymorphisms (SNPs) investigated in the present research are as follows: ABCC2 rs2273697 and rs3740066; ABCG2 rs2231137; ABCC4 rs899494; MCT1 rs60844753 and rs7169; MCT2 rs10784000, rs10877333, rs1693614, rs2711655 and rs3763980; OATP2B1 rs2306168. Polymorphisms were genotyped using MALDI TOF mass spectrometry (MassARRAY®, Sequenom, CA, USA) at the Institute of Clinical Pharmacology, Sun Yat-sen University. The DNA absorbance ratio (A260/A280) was greater than 1.8 to ensure high quality, and the concentrations were determined by NanoDrop 2000 (Thermo). For data acquisition and analyses, the MassArray Typer 4.0 software was used. Inspection of the clusters was carried out to ensure a clear cluster separation with satisfactory signal-to-noise cutoff. SpectroChip results with less than 99.5% concordance in duplicate checks along with more than a 10% call rate in a blank check were repeated.

4.4. Statistical analysis

Statistical analysis was carried out using IBM SPSS statistics 21.0 software (IBM). The Hardy-Weinberg equilibrium (HWE) test was performed using an appropriate χ^2 test, $P < 0.05$ indicated a lack of agreement with HWE. Linkage disequilibrium (LD) between SNP pairs was estimated by SHEsis (Shi and He 2005). As VPA CDRs were not normally distributed according to Kolmogorov-Smirnov test, statistical differences in VPA CDR among various groups classified by genotypes were analyzed by nonparametric methods (Kruskal-Wallis and Mann-Whitney test for multiple comparisons). Two-sided P-values less than 0.05 were considered statistically significant. Bonferroni's corrections were used for multiple comparisons. Multiple linear regression analysis was applied to evaluate the effect of SNPs on VPA plasma concentration under the adjustment of cofactors, such as VPA dose, gender, weight and age.

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The trial registry name is "Exploration of Genotype Based Personalized Prescription of Valproate Sodium in Anti-epileptic Treatment (EGBPPVPA)" and the ClinicalTrials.gov Identifier is NCT01172626 (http://clinicaltrials.gov/show/NCT01172626).

Conflict of interest: None of the authors has any conflicts of interest.

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