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Research Article

Allele Frequency Distribution of UGT1A6 rs6759892 T>G Valproic Acid Metabolic Enzyme-Encoding Gene among Healthy Javanese Population in Indonesia

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ABSTRACT

Polymorphisms in the gene that encodes the metabolic enzyme of valproic acid are one of the important factors associated with interindividual variability in the effective dose and concentration of the drug. UGT1A6 as the encoding gene of glucuronidase enzyme is responsible for valproic acid metabolism, and polymorphisms can therefore influence the drug effectiveness and plasma concentration. This study aimed to analyze the allele frequency distribution of UGT1A6 rs6759892 T>G gene that encodes the metabolic enzyme of VPA among healthy respondents of Javanese as the largest ethnic group in Indonesia. This study used stored biological specimens in the form of DNA isolates from 100 healthy adult respondents who met the inclusion criteria. Genotyping of UGT1A6 rs6759892 gene was performed using PCR-RFLP method with 5'-CTGACACGGCCATAGTTGGT-3' forward primer and 5'-CCAGCAGCTTGTCACCTACA-3' reverse primer. The results showed that the frequencies of T allele and G allele of UGT1A6 rs6759892 among Javanese population in Indonesia were 0.86 and 0.14, respectively. The frequency of G allele in Javanese-Indonesian ethnic population is similar to that found in a study involving Chinese, Caucasian, and African populations. This study recommends further analysis regarding the influence of such SNP on the pharmacokinetic variability of VPA and its clinical response. Analysis of such correlation as a risk factor for cancer is also required as an effort to seek an early and effective preventive therapy.

Keywords: *Javanese-Indonesian population, rs6759892 T>G, UGT1A6, valproic acid*

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INTRODUCTION

Epilepsy is the most common chronic neurological disorder affecting approximately 50 million people worldwide. This neurological disease is characterized by unexpected and periodic seizure incidence (Löscher *et al*, 2009). In developing countries such as Indonesia, the prevalence is higher compared to developed countries, ranging from 5 to 74 per 1000 people. To date, anti-epileptic drugs (AEDs) remain a popular choice of therapy (PERDOSSI, 2014).

Valproic acid (VPA; 2-propylpentanoic acid) is one of the most widely used anti-epileptic drugs to control various types of seizure, including myoclonic, absent, general, and partial seizures in both adult and pediatric patients since they have the lowest adverse effects with a broad therapeutic index (Zhu *et al*, 2017). VPA is also known as a therapy for bipolar disorder, migraine, neuropathic pain, and cancer (Luís *et al*, 2011). However, interindividual variability in its dose and plasma concentration still occurs. A study of 208 adult patients with epilepsy showed an average variability of the required VPA dose of up to 10-fold (Blanco-Serrano *et al*, 1999). In addition, the incidence of life-threatening adverse drug reactions (ADR), such as hepatotoxicity, teratogenic effects, and pancreatitis which correlate with the drug concentration in the blood, reinforces the importance of pharmacogenetic testing along with TDM services in VPA therapy (Gervasini *et al*, 2010). It is acknowledged that one of the factors influencing the variability is the genetic polymorphisms in the metabolic enzyme-encoding gene that has been proven to correlate with its pharmacodynamic and pharmacokinetic variability [Zhu *et al*, 2017; Nagar *et al*, 2004).

An understanding of the mechanism of VPA metabolism can explain the influence of genetic polymorphisms on the changes in plasma concentration and their clearance with an implication for the effective dose. VPA metabolism has three main routes including 2 metabolic pathways of phase I, namely β-oxidation in the liver mitochondria as well as desaturation and hydroxylation catalyzed by cytochrome

enzymes (CYP) in the liver endoplasmic reticulum. The third metabolic pathway is phase II metabolism through the formation of glucuronate conjugates by glucuronidase, an enzyme encoded by UGT1A6 gene and one of the most commonly-found enzymes compared to other UGT1A isoforms (Owens *et al*, 2005) in the liver endoplasmic reticulum. This third pathway becomes the major route of valproic acid, accounting for 30-50% dose (Hung et, 2011; Balestrini and Sisodiya, 2017). Such process of conjugation reaction takes place through the transfer of UDPG1cA to VPA aglycones to form β-glucuronide with a high polarity structure that facilitates its renal excretion (Guillemette, 2003; Goey *et al*, 2016). A previous study found 3 SNPs in UGT1A6 gene, including rs6759892, rs2070959, and rs1105879 with the highest mutant allele frequencies in rs6759892 among Chinese population, and these three alleles correlated with lower VPA concentration in the blood, thus requiring a higher dose (Guo *et al*, 2012).

This present research was part of the pharmacogenetic studies of VPA use among Indonesian population suffering from epilepsy. Analysis of genetic variants in target genes that encode VPA metabolism enzymes is essential to provide information on the profile of genetic variations in Indonesian population of which implications for individual therapeutic dose and response to the use of VPA as the first line antiepileptic drug for several types of seizure can be further studied (Zhu *et al*, 2017). This study therefore aimed to determine the allele frequency distribution of UGT1A6 rs6759892 T>G gene in Javanese population as the largest ethnic group in Indonesia.

MATERIALS AND METHODS

Research Subjects: This study used the stored biological samples of DNA isolated from 3 ml of the blood of 100 healthy respondents from a previous study. Respondents were categorized as originated in Javanese ethnic group determined by their 3 previous generations.

Ethical considerations: This study has passed the ethical review from the Ethics Committee of the Faculty of Medicine of Universitas Islam Indonesia with the protocol No. 43/Ka.Kom.Et/70/KE/XI/2018.

Genotyping of *UGT1A6* rs6759892 T>G: Genotype analysis of UGT1A6 rs6759892 T>G was performed using the PCR method with 5'-CTGACACGGCCATAGTTGGT-3' as the forward primer and 5'-CCAGCAGCTTGTCACCTACA-3' as the reverse primer prior to the process of Restriction Length Fragment Polymorphism (RLFP). The conditions for PCR amplification were initial denaturation at 94°C for 45 seconds, 34 cycles of denaturation at 94°C for 30 seconds, annealing at 57°C for 30 seconds, and extension at 72°C for 50 seconds as well as final extension at 72°C for 3 minutes. The amplification products (305 bp) were then identified in 1.5% agarose gel electrophoresis for 30 minutes with a voltage of 100 V followed by restriction digestion using *Hha1* enzyme and incubation at 37ºC for 1 hour. *Hha1* enzyme cut the sequence of GCG|C into 233 DNA templates. GG genotype was recognized and digested by *Hha1* enzyme. The digestion products were then analyzed using 3% agarose gel electrophoresis for 90 minutes with a voltage of 70 V. The digestion of amplification products resulted in 305 bp fragments of TT genotype (wild type) and 233 bp and 72 bp fragments of GG genotype (mutant) with 305 bp, 233 bp, and 72 bp for heterozygotes (TG).

Univariate data analysis was then used to determine the allele frequencies of UGT1A6 rs6759892 T>G gene. Allele frequencies were calculated using the Hardy-Weinberg equation as in the previous study with the following formula Oussalah *et al*, 2015).

$$\text{T Allele} = \frac{(\text{TT Genotype x 2}) + \text{TG Genotype}}{\text{2 x number of samples}}$$

$$\text{G Allele} = \frac{(\text{GG Genotiype x 2}) + \text{TG Genotype}}{\text{2 x number of samples}}$$

RESULTS

There were 100 healthy Javanese-Indonesian respondents involved in the genotyping of UGT1A6 rs6759892 T>G with equal numbers of male and female respondents. The characteristics of the research subjects are presented in Table 1. The electrophoretic display of digestion by the enzyme that detected the target polymorphisms in this study is presented in Figure 1.

Table 1.
Characteristics of the subjects involved in the genotyping of UGT1A6 rs6759892 gene

Patient characteristic	Male (n=50)	Female (n=50)
Average age (years)	21.26±1.21	21.14±1.43
Average BMI (kg/m^2)	22.734.0±9	21.49±3.41
Genotype of *UGT1A6* rs6759892 T>G		
TT	41	36
TG	7	12
GG	2	2

In addition, to identify the allele frequencies of *UGT1A6* rs6759892 T>G in both male and female groups, a descriptive analysis was performed, and the results are displayed in Table 2.

Table 2.
Allele frequencies of *UGT1A6* rs6759892 based on gender

Allele Variant	Male (%)	Female (%)
T Allele	0.89	0.84
G Allele	0.11	0.16

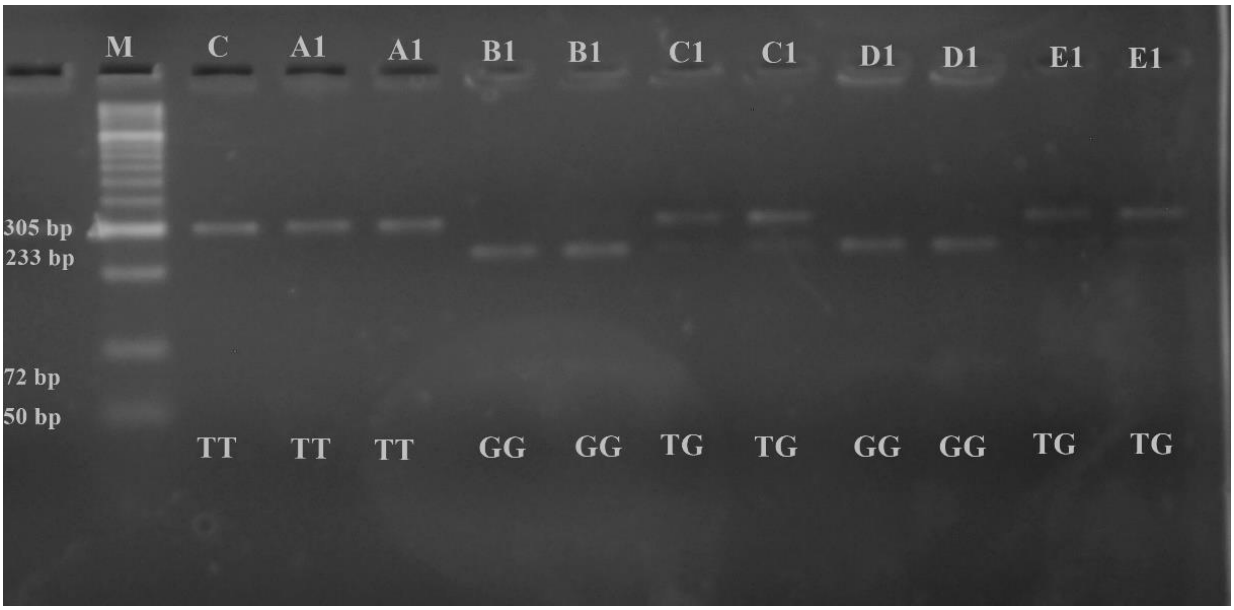

Figure 1.
Region cutting by Hha1 restriction enzyme as the detector of polymorphisms in *UGT1A6* rs6759892 T>G gene
Note: lane M = marker/ladder of 50 bp; lane C = negative control/undigested products/PCR products; lane A1 = samples with wild type (TT genotype); lane C1 and E1 = samples with heterozygote (TG genotype); lane B1 and D1 = samples with mutant/homozygote (GG genotype)

DISCUSSION

It is acknowledged that one of the main enzymes for valproic acid metabolism typically expressed in the liver is the glucuronidase enzyme with an amino acid length of 531, encoded by UGT1A6 gene. This enzyme exists not only in the liver but also in various organs, such as the bile, colon, stomach, and brain, and it becomes a catalyst in glucuronidation reactions, including in both endogenous substrates such as steroids and exogenous substrates including a number of drugs like valproic acid (Nagar *et al*, 2004) UGT1A6 is a major isoform of UGT enzyme. Interindividual variability in a glucuronidase process mediated by this enzyme can have essential pharmacological and therapeutic consequences (Chatzistefanidis *et al* 2012).

Genotypic analysis of glucuronidase enzyme-encoding gene can provide important basic information for the development and implementation of genetic screening in therapeutic decision making and become the basis for "individualized medication" particularly when calculating the need for effective individual dose in VPA therapy.

The lowest frequency of GG variant in both male and female respondents was found to be identical in this study (2%). The lowest proportion of homozygotes was also found in a study involving 94 Chinese patients with epilepsy (6.4%) (Guo *et al*, 2012). Similarly, some studies involving 162 healthy adult Chinese volunteers, 190 healthy Taiwanese subjects, and 162 adult Taiwanese patients with epilepsy revealed that their frequencies of GG genetic variant were 0.62%, 4.74%, and 6.79%, respectively (Hung *et al*, 2011; Kua *et al*, 2012). A percentage of less than 10% was also found among Greek pediatric and adult patients with epilepsy (5.22% vs 7.46%) (Chatzistefanidis *et al*, 2016). Meanwhile, 3 other studies of healthy subjects involving German, Indian population and case-control studies involving German-Finnish-Swedish population each showed a higher frequency of GG genetic variant of 17.20%, 16.3%, and 19.7%, respectively (Jain *et al*, 2015; Justenhoven *et al*, 2013; MARIE-GENICA, 2012). As it was expected, the findings of this study confirmed previous research related to similar frequencies of genetic variations between Javanese-Indonesian population and Chinese population as opposed to other populations (Ningrum *et al*, 2018; 2019). This underpins the significance of genetic testing to be considered in drug selection as well as in determination of safe and effective dose among races-populations that have almost never been involved in clinical trials of new FDA-approved drugs, such as among Indonesian population.

The highest proportion of alleles in UGT1A6 rs6759892 in this study is T allele (> 80%) in both male and female patients. A study using human liver bank as the modeling system showed that gender differences did not correlate with the level of UGT gene expressions, but using enzyme inducer drugs, smoking, and drinking alcohol were proved to be the factors associated with increased UGT activity [24]. Therefore, differences in gender proportions in a pharmacogenomic study analyzing the influence of polymorphisms on proteins that encode UGT enzymes can be ignored, or no matching techniques are required by these factors in the data analysis.

Several studies have been carried out to analyze the influence of genetic variation of UGT1A6 gene, an isoform with the second most variability of activities after UGT1A1 (Court 2010)), on interindividual pharmacokinetics of VPA

therapy. A study involving 98 pediatric patients with epilepsy who received VPA showed that polymorphisms in UGT1A6 affected its biotransformation, leading to lower plasma concentration of VPA, and implied the need for dose increase (Hung *et al*, 2016; Guo *et al*, 2012).

It is acknowledged that the type of genetic variation in UGT1A6 rs6759892 gene is single nucleotide polymorphisms (SNP) with two variations in the form of intron variant polymorphism and variant of amino acid coding sequences (exon). The variant that changes the activity of glucuronidase enzyme is the polymorphisms in amino acid coding sequences that transform thymine nucleotide base (T) into guanine (G) which change the formation of codons from TCA (encoding the serine amino acid - Ser) into GCA (encoding the alanine amino acid - Ala). Serine is located in the 7th amino acid of the allosteric site of glucuronidase enzyme, while the active site of the enzyme is in the amino acids Glu451, Tyr504, and Asn450 (Nagar *et al*, 2004). Changes in the structure of amino acid sequences in glucuronidase enzyme from serine into alanine are assumed to result in 2-fold increase in enzyme activity, thus leading to a higher level of catalyst function in the metabolic process (Krishnaswamy *et al*, 2005).

A study involving 162 Chinese patients (36.23 ± 1.02 years old) with epilepsy found that among the 6 gene candidates studied, polymorphisms in UGT1A6 gene significantly correlated with changes in valproic acid plasma concentration (LnCDR/ln concentration to dose ratio). Patients with UGT1A6 rs6759892 allele variant tended to have lower LnCDR (3.88 ± 0.41; 3.70 ± 0.47 days/ml) compared to the other groups (4.22 ± 0.45 days/ml). In addition, patients with homozygous variants also had lower LnCDR (3.70 ± 0.47 days/ml) compared to those in the heterozygous variant group (3.88 ± 0.41 days/ml). These results indicate that the polymorphisms in UGT1A6 rs6759892 gene correlate with the decreased plasma concentration of VPA and thus influence the dose to generate the expected biological response (Hung *et al*, 2011). In addition, a study of 98 Chinese patients with epilepsy (7.8 ± 7.5 years old) found a significantly higher plasma concentration of VPA in the group of patients with double heterozygotes on UGT1A6 (Guo *et al* 2012).

This study is part of the pharmacogenomic study of anti-epileptic drug therapy for Indonesian patients, in which a study of patients with epilepsy is performed to provide an approach to the individual therapeutic dose of anti-epileptic drugs, including VPA. Not only related to the variability of VPA metabolism, polymorphisms in UGT1A6 rs6759892 T>G gene are also proven to be one of the risk factors for cancer and other pathological conditions. Two population-based case-control studies revealed that subjects with GG genotype correlated with an increased risk of breast cancer with 1.17 OR (MARIE-GENICA, 2012). This was also confirmed through the pooled analysis of a study involving 7148 subjects with cancer and 8720 control subjects from Finland, Germany, and Sweden with 1.09 OR (Justenhoven *et al*, 2013). Although UGT1A6 has a low activity towards estrogen and its carcinogenic metabolites, it is suspected that the polymorphisms of such gene are also related to its activity to metabolize exogenous carcinogens (Hu *et al* 2014). In addition, the UGT1A6 rs6759892 Ser7.Ala variant showed an increased risk of gallstone-related cholecystectomy with 3.88 OR among geriatric patients involved in a cohort study with 773 Italian patients (Oussalah *et al* 2015). This is likely because UGT1A6 polymorphisms disrupt the process of inactivation in several endogenous and exogenous molecules, including bilirubin. It is necessary to acknowledge that there was a contradictory report about changes in UGT enzyme activity due to UGT1A6 polymorphisms. In addition to changes in enzyme activity, mRNA degradation might be involved in the variability of its activity (Kua *et al*, 2012).

Meanwhile, there was a contradictory study related to how UGT1A6 rs6759892 polymorphisms were associated with VPA biotransformation and its plasma concentration in 134 patients (43.3% of whom were pediatric patients) (Chatzistefanidis *et al*, 2016) and the difference between non-optimum UGT expression in infants and children as opposed to that of adult patients probably became the reason for this contradictive finding. Transition of VPA metabolic pathways particularly through mitochondria and CYP in children increased the risk of VPA toxicity due to higher concentration of 4-ene-VPA metabolites (Anderson, 2002; Ghodke-Puranik 2013). Therefore, studies of the influence of polymorphisms on UGT encoding genes involving pediatric patients are more appropriately associated with variability in the prevalence of VPA toxicity instead of variations in dose requirement which is probably more suitable with the use of polymorphism-free variables in CYP metabolic pathway or mitochondrial β-oxidation.

In conclusion, the research on the allele frequency of UGT1A6 gene involving Javanese-Indonesian population has a novelty value in the preliminary pharmacogenetic study of the use of valproic acid since it has never been performed in previous studies. The frequency of G allele of UGT1A6 rs6759892 among Javanese population in Indonesia was 0.14 (4%). Therefore, this research recommends further studies regarding the influence of these polymorphisms on the pharmacokinetic profile of VPA as well as the clinical response to valproic acid among Indonesian patients with epilepsy. Further studies related to its influence on the risk of breast cancer, the most common type of cancer among Indonesian women, are also suggested to encourage earlier health promotion and prevention programs.

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