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Research Article

The Genetic Polymorphisms of *CYP3A4*1G* and *CYP3A5*3* in Javanese Indonesian Population

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ABSTRACT

Polymorphisms of *CYP3A4*1G* and *CYP3A5*3* affect the pharmacokinetic profile of various drugs, e.g., fentanyl, tacrolimus, diltiazem, simvastatin. Tetra-primer amplification refractory mutation system-polymerase chain (ARMS-PCR) is a simple and economical method for SNP determination. The polymorphisms in the *CYP3A4*1G* and *CYP3A5*3* genes have not yet been examined using this method in Javanese Indonesian. Our aim was to determine the frequency of polymorphisms in the *CYP3A4*1G* and *CYP3A5*3* genes in Indonesian Javanese using the ARMS-PCR method. Eighty-six patients at the Kalasan Community Health Centre in Yogyakarta, Indonesia, were chosen based on the inclusion criteria, which is Javanese ancestry. They gave their informed consent to blood collection by completing a form. Genetic variants were detected using Tetra-primer amplification refractory mutation system-polymerase chain (ARMS-PCR). The chi-square test was used to determine genotype deviations from Hardy-Weinberg equilibrium, with a significant threshold of 0.05. For homozygous wild types, *CYP3A4 *1/*1* dominated overall among study participants (73.35%), whereas for *CYP3A5*3/*3*, homozygous mutants were more prevalent (83.72%). Hardy-Weinberg equilibrium is consistent with genotype frequencies ($p > 0.005$). One participant carried a homozygous mutation for both *CYP3A4*1G* and *CYP3A5*3*, while the other 49 subjects were heterozygous for *CYP3A4*1G* and homozygous mutant for *CYP3A5*3*, which is the highest number of SNP combinations. The findings of the current investigation demonstrate that the population has the highest proportion of homozygous *CYP3A4*1G* wild-types (*CYP3A4*1/*1*) and homozygous mutants for *CYP3A5*3 (CYP3A5*3/*3)*

Keywords: ARMS-PCR, *CYP3A4*1G*, *CYP3A5*3*, Indonesian, Javanese, Polymorphism

Introduction

Gene variations in the human body are responsible for drug efficacy and safety changes. The CYP3A4 and CYP3A5 genes are two genes responsible for encoding cytochromes that metabolize endogenous and exogenous substances. These genes belong to the cytochrome P450 genes located on chromosome 7q22.1 [1]. The two genes have similar structures that produce overlapping substrates [2].

There are 139 known variants of the CYP3A4 single nucleotide polymorphism (SNP). The wild type of *CYP3A4* is *CYP3A4*1A*, while *CYP3A4*1B* is a -392A>G mutation in the 5'UTR region. Another variant is *CYP3A4*1G*, located in

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intron 10 of the CYP3A4 gene and mostly found in Asian populations [3]. The CYP3A5 gene has 25 allelic variants, and the most common allele found in the population is *CYP3A5*3* [4].

Individuals with *CYP3A4*1G* showed variations in the pharmacokinetic profiles of fentanyl, tacrolimus, diltiazem, sufentanil, and tylerdipine hydrochloride [5-9]. An increased risk of breast cancer occurrence is known to be associated with *CYP3A4*1G* polymorphism [10]. Individuals carrying *CYP3A5*3/*3* have increased simvastatin levels, while studies on tacrolimus therapy found that individuals with *CYP35 *1/*1* and **1/*3* variants take longer to reach steady-state levels than those with *CYP3A5*3/*3* [11],12].

SNP detection methods using sequencing have many advantages, namely precision and reliability, but also limitations, namely high prices. The RFLP method also has an expensive price and can lead to incomplete and/or partial digestion, resulting in false negatives or positives [13]. Tetra-primer amplification refractory mutation system-polymerase chain (ARMS-PCR) is a simple and economical method for SNP determination. This method uses four primers, two outer primers, and two inner primers, followed by conventional PCR and gel electrophoresis. When conditions are optimal, this method has good accuracy and efficiency in detecting SNPs [14].

*CYP3A5*3* allele variations are found in the American (80%) and European (94%) racial populations, while in the ASEAN population 65.7% with variations in the Thai population 37.7%; Vietnam 43.05%, Singapore 57.38% [15]. Based on the literature review conducted by the researchers, no study has been conducted on the variation of *CYP3A5*3* gene in the Indonesian-Javanese population, while for *CYP3A4*, the frequency of polymorphism of the gene in the Javanese population was found as follows: *CYP3A4*1/*1* 0.25; *CYP3A4*1/*1G* 0.55; and *CYP3A4*1G/*1G* 0.20 detected using the RFLP method [16]. This article reports *CYP3A5*3* and *CP3A4*1G* polymorphisms in the Indonesian-Javanese population using ARMs-PCR tetra primer method to detect *CYP3A4*1G rs2242480* and *CYP3A5*3 rs776746.*

Material and Methods

Subjects

Patients from Kalasan Community Health Centre, Yogyakarta, Indonesia, who met the inclusion criteria > 14 years, were of Indonesian-Javanese descent (subjects were surveyed for ethnicity background of three generations ago) and consented to blood collection by signing an informed consent form. The subjects were patients in the Amlodipine Efficacy and Side Effects Study. This study was conducted with ethical approval from the Medical and Health Research Ethics Committee of the Faculty of Medicine, Public Health and Nursing, Gadjah Mada College, under the number KE/FK/0871/ EC /2022.

DNA isolation

A total of 86 peripheral blood samples from patients of Kalasan Community Health Centre, Yogyakarta, Indonesia, were isolated using Favor-Prep Blood Genomic DNA Extraction Mini Kit. DNA isolation was performed according to the instructions on the kit. DNA concentration was measured using a Nanodrop spectrophotometer (TECAN SPARK 20M). Analysis of the quality of the DNA isolates using 1.0% agarose gel and electrophoresis (Sigma-Aldrich®).

Primer design

The *CYP3A4*1G* (rs2242480) variant is located at position chr7:99763843 and has a base C to T change, whereas *CYP3A5*3* (rs776746) is located at position chr7:99672916 with a base T to C change (https://www.ncbi.nlm.nih.gov). The DNA sequences of *CYP3A4*1G* and *CYP3A5*3* were obtained from a database search in NCBI and then used as a reference for primer design (Figures 1 and 2). Two pairs of different primers were designed using Geneious software, consisting of outer forward primers and inner reverse primers to detect single nucleotide polymorphism (SNP), as well as inner forward primers and outer reverse primers that have the function of detecting wild type. Illustrations of the primer design are provided in Figures 1 and 2. The results of the primer design are shown in Table 1 for *CYP3A4*1G* and in Table 2 for *CYP3A5*3*. The specificity of the outer primers in detecting *CYP3A4*1G* and *CYP3A5*3* was tested using the program NCBI BLAST (http://www.ncbi.nlm.nih.gov/blast).

*Identification of CYP3A4*1G SNP using the tetra-primer ARMS PCR method*

The identification of *CYP3A4*1G* SNP was performed in several steps, namely optimization of the tetra-primer ARMS PCR by determining the

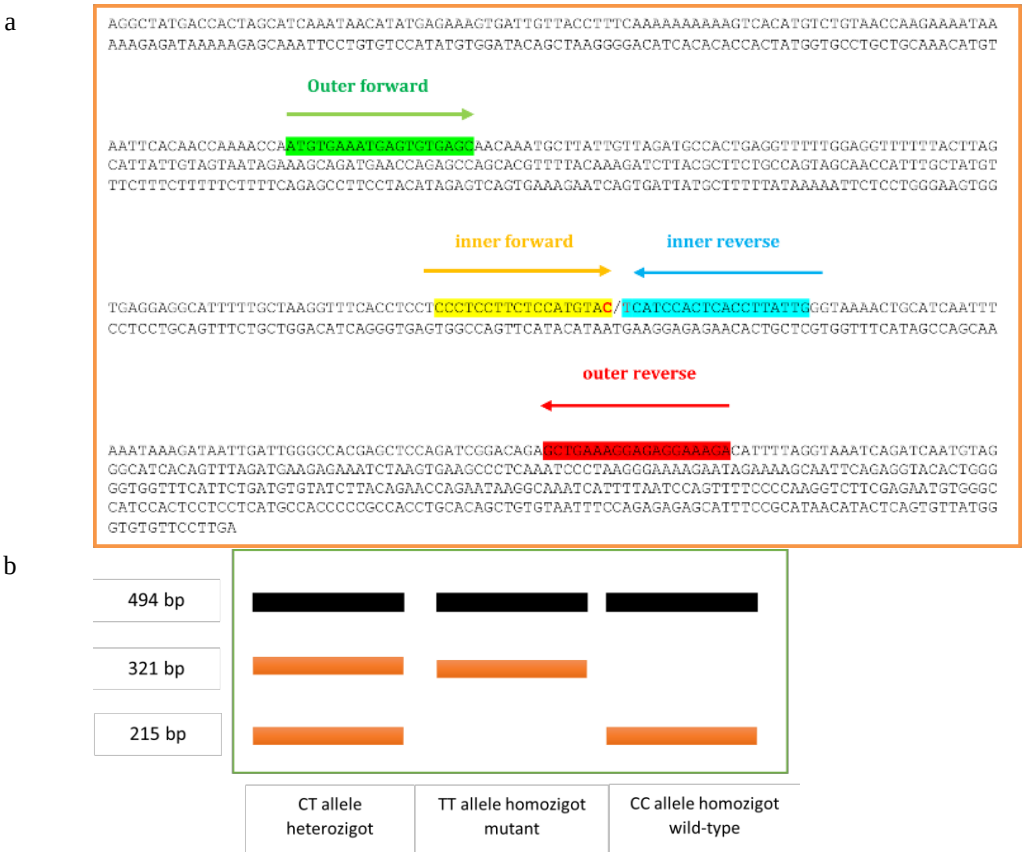

Figure 1. (a) Location of primer designs used in the Tetra-Primer ARMS PCR. T and C symbolize the change from cytosine to thymine. The DNA sequence used as a reference for the primer design is from the genomic scaffold of Homo sapiens chromosome 7, GRCh38.p14 with |ref|NC_000007.14| (https://www.ncbi.nlm.nih.gov/projects/sviewer/?id=NC_000007.14&v=99763843:99763843&mk=9-9763843|rs2242480|008000). The flanking region of *rs2242480* is from NCBI https://www.ncbi.nlm.nih.gov/snp/rs2242480#flanks (b) Illustration of the identification results of *CYP3A4*1G* (rs2242480) by agarose gel electrophoresis.

Table 1. Primers used in the tetra-primer ARMS PCR for detection of *CYP3A4*1G* polymorphism.

Primer set and sequences	**‡Tm (°C)**	**hairpin**	**Product size**
forward outer primer: 5' ATGTGAAATGAG-TGTGAGC 3'	53.3	None	321 bp‡
Reverse inner primer: 5' CAATAAGGTGAGTG-GATGA 3'	51.5	None	
Forward inner primer: 5' CCCTCCTTCTCCATGTAC 3'	53.2	None	215 bp‡
Reverse outer primer: 5' TCTTTCCTCTCCTTTCAGC 3'	54.1	None	

Noted: ‡Tm: Melting Temperature; bp: base pair

optimal annealing temperature and subsequent amplification with primers designed with a touch-down PCR system to avoid smear and prevent mis-priming [17]. The PCR kit used was the Pow-erPol 2x PCR Mix, which consists of DNA poly-merase, dNTPs, MGCl2, KCl, and other stabi-lizers mixed with outer and inner primers. The primer concentration used in this analysis was 40 pmol/microliter. In one PCR tube contained five microliters of DNA isolate, four primers with a volume of 1 microliter each, 12.5 microliters of Taq polymerase, and 3.5 microliters of nuclease-free water. Amplification was performed using a PCR instrument (Perkin Elmer thermal cycler

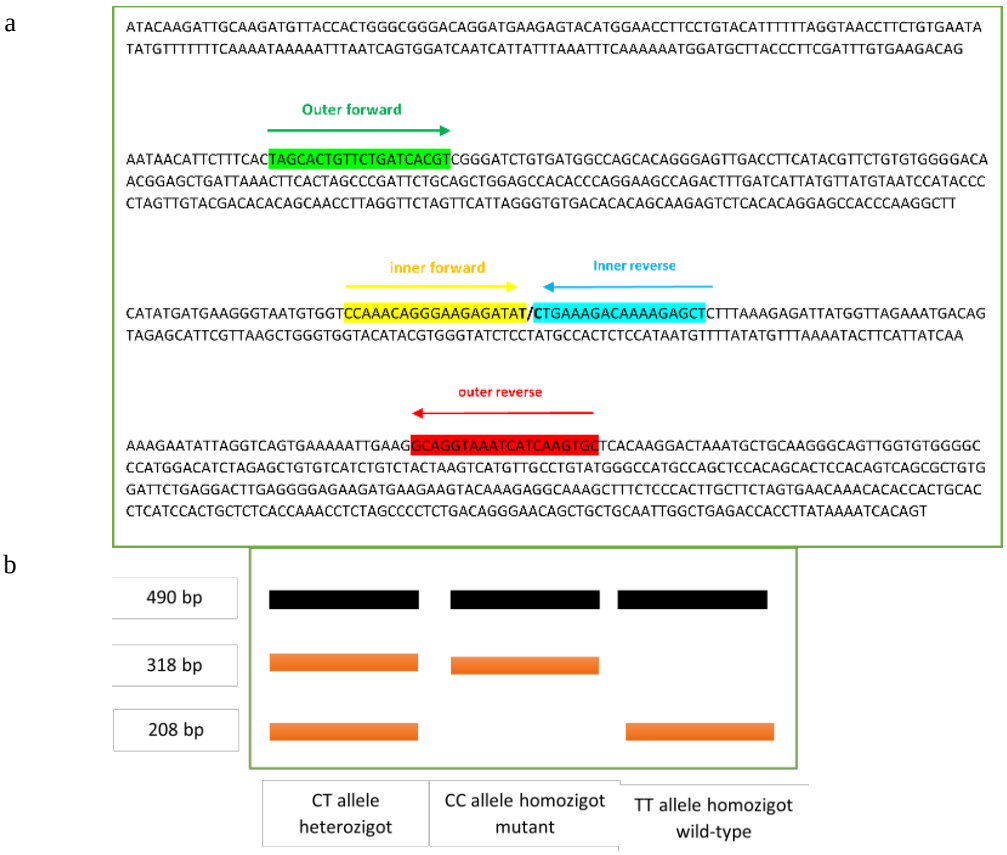

Figure 2. (a) DNA sequence used as a reference for primer design, taken from the genomic scaffold of Homo sapiens chromosome 7, GRCh38.p14 with |ref|NC_000007.14|(https://www.ncbi.nlm.nih.gov/projects/sviewer/?id=NC_000007.14&v=99672916:99672916&mk=99672916|rs776746|008000). The flanking region rs776746 was obtained from NCBI https://www.ncbi.nlm.nih.gov/snp/rs776746-#flanks . (b) Representation of the identification results of *CYP3A5*3 rs776746* by agarose gel electrophoresis.

Table 2. Primers used in the tetra-primer ARMS PCR for detection of *CYP3A5*3* polymorphisms.

Primer set and sequences	‡Tm (°C)	hairpin	Product size
forward outer primer: 5' CTAGCAC-TGTTCTGATCAC 3'	52.8	None	318 bp‡
Reverse inner primer: 5' GAGCTCTTTT-GTCTTTCAG 3'	51.4	None	
Forward inner primer: 5' CCAAACAGGGAAGA-GATAT 3'	51.1	None	208 bp‡
Reverse outer primer: 5' CACTTGATGATTTAC-CTGC 3'	51.5	None	

2400). The PCR conditions used are listed in Table 3.

All PCR products are quantitatively tested using 1.5% agarose gel electrophoresis and compared with the 50 bp DNA ladder (ExcelBand™). Electrophoresis results were performed at 110 volts for 30 minutes. Electrophoresis results were viewed with a UV transilluminator lamp and documented with a DSLR camera.

*Identification of CYP3A5*3 SNPs by the tetra-primer method ARMS PCR*

The identification of *CYP3A5*3* SNPs was performed in several steps, namely the optimization of the tetra-primer ARMS PCR by determining the optimal annealing temperature. The PCR kit used is the PowerPol 2x PCR Mix, which consists of DNA polymerase, dNTPs, MGCl2, KCl, and other stabilizers mixed with outer and inner

primers. Primer concentrations used in this analysis were five ρmol/microliter for outer forward and reverse primers and 30 ρmol/microliter for inner forward and reverse primers. The choice of primer concentration is determined by the concentration optimization method using primer comparison referring to previous research [18,19]. In one PCR tube, five microliters of DNA isolate were mixed with four primers (1 microliter each), Taq polymerase (12.5 microliters), nuclease-free water (3.5 microliters), and PCR was performed with a temperature setting of pre-denaturation at 98°C for 45 seconds, denaturation at 98°C for 10 seconds, annealing at 56.90°C for 15 seconds, elongation at 72°C for 30 seconds, and final elongation at 72°C for 5 minutes with 35 cycles. PCR products were quantitatively tested by gel electrophoresis.

Statistical calculations

Deviations of genotypes from Hardy-Weinberg equilibrium were calculated by chi-square test with a significance level of 0.05 using Hardy-Weinberg Equilibrium Gene-Calc. Differences in genotype distribution between sexes were analysed by chi-square test with statistically significant differences at a P value < 0.05.

Results and Discussion

ARMS PCR method for each single nucleotide polymorphism (SNP) is shown in Figures 3 and 4. Figure 3 shows that samples with *CYP3A4*1/*1G* variations formed 321 bp and 215 bp products (a, b), whereas *CYP3A4*1/*1* variations formed 215 bp products (c). Product 321 bp (d) for *CYP3A4*1G/*1G* variation. The *CYP3A5*3/*3* SNP variation is indicated by the presence of a 318 bp product (a, b, d, f), and *CYP3A5*1/*3* formed 318 bp and 208 bp products (c, g, e).

The genotype and allele frequencies of *CYP3A4*1G* and *CYP3A5*3* polymorphisms in the Indonesian-Javanese population are shown in Tables 4 and 5. The results of this study show that genotype frequencies are consistent with Hardy-Weinberg equilibrium (p>0.005). Homozygous wild types *CYP3A4*1/*1* dominated in the study participants overall (73.35%), while for *CYP3A5*3/*3*, homozygous mutants had the highest number (83.72%), and homozygous wild types were not found in the same group of subjects. The combined variation of SNPs in both genes within a subject is shown in Table 6. Forty-nine subjects were heterozygous for *CYP3A4*1G* and homozygous mutant for *CYP3A5*3*, and only one subject had a homozygous mutation for *CYP3A4*1G* and *CYP3A5*3*. Chi-square analysis showed no difference in the distribution of the number of genotypes between the sexes (Table 4).

In this study, the frequency of the *CYP3A4*1/*1* genotype was higher (73.25%) than in the study by Sutrisna *et al*., [16] who investigated the same SNP in the Indonesian population but specifically in Javanese tribes. In addition, the genotypes of *CYP3A4*1/*1G* and *CYP3A4*1G/*1G* were lower in this study than in the study by Sutrisna *et al.* [16]. However, this study and Sutrina's research showed that the genotype frequency was still in Hardy-Weinberg equilibrium. Sutrisna's research used the RFLP method, which is different from the detection strategy employed in this study. The RFLP approach is costly and has the potential to result in incomplete and/or partial digestion, leading to false negative or positive [13].

The allele frequency in this study showed 86% for *1 and 14% for *1G. In other Asian populations, the allele frequency of *1G is slightly higher than the Indonesian-Javanese population, 24.01% for Chinese-Han, 22.80% for Japanese, and 20.5% for Korean. However, the number is almost the same as Chinese-Uyghur (11.5%) [20]. It is well known that the presence of the *CYP3A4*1G* allele causes changes in the pharmacokinetic profile of some drugs. For example, a study by Zhou et al [7]. showed that the concentrations of diltiazem and its primary metabolites were strongly influenced by the *CYP3A4*1G* allele [7]. The average area under the plasma concentration-time curve of atorvastatin was 36% and 25% lower in *CYP3A4*1G/*1G* than in the wild-type or *1/*1G genotype, respectively [21]. In other cases, the *CYP3A4*1G* polymorphism significantly affects the pharmacokinetics of ticagrelor [22].

*CYP3A4*1G* and *CYP3A5*3* are known to have strong linkage disequilibrium. No linkage disequilibrium studies were performed for these two variations in this study. The researchers calculated the linkage disequilibrium between the two alleles using LDlink (https://analysistools.cancer.gov/LDlink/?tab=ldpair) by selecting the entire Asian population (East and South Asia) and obtained r2 = 0.5714, indicating a moderate level of linkage disequilibrium. In addition to the calculation results, it was discovered that the *rs2242480* (C) allele was correlated with the

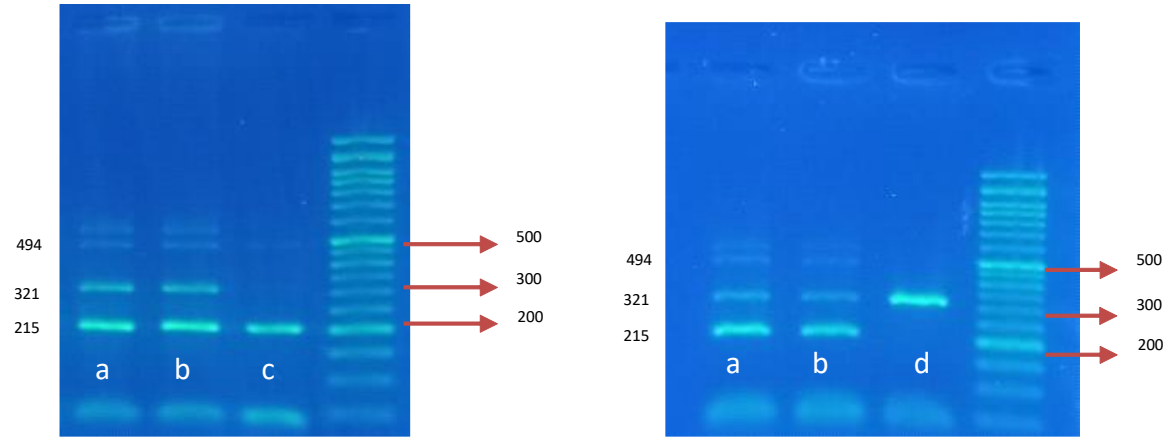

Figure 3. Gel electrophoresis results of *CYP3A4*1G* polymorphism detection using Tetra-primer ARMS PCR. (a), (b) show heterozygotes with the appearance of 2 bands at 321 bp and 215 bp. (c) shows homozygous wild type with the occurrence of 1 band at 215 bp. (d) shows homozygous mutant with the occurrence of 1 band at 321 bp.

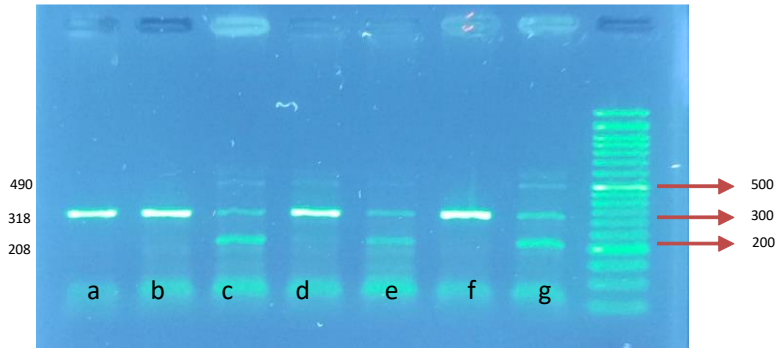

Figure 4. Gel electrophoresis results of *CYP3A5*3* polymorphism detection using Tetra- primer ARMS PCR. (a), (b), (d), (f) show the homozygous mutant with the appearance of 1 band at 318 bp. (c), (e), (g) shows the heterozygous with the occurrence of 2 bands at 318 bp and 208 bp.

Table 4. Gene characteristics by sex

genotype	***CYP3A4*1/*1***	***CYP3A4*1/*1G***	***CYP3A4*1G/*1G***	**p***
Sex	**(n)**	**(n)**	**(n)**	
Male	7	25	0	0.597
Female	15	38	1	
genotype	***CYP3A5*1/*1***	***CYP3A5*1/ *3***	***CYP3A4*3/*3***	
Sex	**(n)**	**(n)**	**(n)**	
Male	0	8	46	0,633
Female	0	6	26	

*Chi-square test ($p < 0.05$)

rs776746 (C) allele, and the *rs 2242480* (T) allele was also found to be correlated with the *rs776746* (T) allele. The study by Zhou et al [7]. showed a strong linkage disequilibrium between *CYP3A4*1G* and *CYP3A5*3* in the Chinese population. However, another study demonstrated a moderate level of LD between *CYP3A4*1/*1G* and *CYP3A5*1/*3* ($r^2 = 0.502$) [23]. The presence of linkage disequilibrium is a challenge in the study of drug metabolism. The primary reason is because the explanation of the activity of the drug-metabolizing enzyme in individuals is influenced not only by one gene locus but also by other alleles located at different sites on the same chromosome.

Table 5. Genotype and allele frequencies in test subjects

subject	Genotype frequency	Allele frequency	Hardy-Weinberg equilibrium
CYP3A4			
- *CYP3A4 *1/*1*	63 (73.25%)		The χ^2 value obtained is 0.361. This value is smaller than the chi-square value of 3.841.
- *CYP3A4 *1/*1G*	22 (25.58%)		
- *CYP3A4 *1G/*1G*	1 (1.16 %)		
- Allele C (*1)		0.86	
- Allele T (*1G)		0.14	
CYP3A5			The χ^2 value obtained is 0.684. This value is smaller than the chi-square value of 3.841.
- *CYP3A5 *1/*1*	0 (0 %)		
- *CYP3A5 *1/*3*	14 (16.27 %)		
- *CYP3A5 *3/*3*	72 (83.72 %)		
- Allele T (*1)		0.08	
- Allele C (*3)		0.92	

‡ C: Cytosine; T: Timine

Table 6. Genotype frequencies of *CYP3A4*1G* and *CYP3A5*3* polymorphisms in the same individual

Combinations of genotypes in one individual	Total
*CYP3A4*1/*1* and *CYP3A5*3/*3*	22 (25.58%)
*CYP3A4*1/*1G* and *CYP3A5*1/*3*	14 (16.28%)
*CYP3A4*1/*1G* and *CYP3A5*3/*3*	49 (56.98%)
*CYP3A4*1G/*1G* and *CYP3A5 *3/*3*	1 (1.16%)

The study by Chan et al [24], for example, showed that *CYP3A5* expressors (patients carrying at least one *CYP3A5*1*) with a combination of *CYP3A4*1/*1G* had a significantly lower midazolam AUC than those who carry *CYP3A4*1/*1*. This suggests that the linkage disequilibrium between *CYP3A5*1* and *CYP3A4*1G* affects the action of metabolizing enzymes, whereby the *CYP3A4*1G* polymorphism may increase CYP3A activity in populations with *CYP3A5* expressors.

This study identified that the most prevalent variant of the *CYP3A5* gene was *CYP3A5*3/*3* (83.72%) with a C allele frequency of 0.92. *CYP3A5*3/*3* is also the most prevalent genotype in several other Asian populations, including China (54.90%), Japan (58.40%), Korea (62.85%), and Taiwan (47.25%). However, compared to the population of Singapore of Malaysian and Indian descent, the percentage of *CYP3A5*1/*3* is higher, at 50% and 46.46%, respectively [15]. Carriers of the *CYP3A5*3* gene are more likely to develop clopidogrel resistance [25]. In another study, *CYP3A5* expressors (*1/*1 and *1/*3) correlated positively with carbamazepine serum concentrations and carbamazepine dose requirements [26]. The *CYP3A5* genotype also influences oral clearance of nifedipine in pregnant women [27]. This study also demonstrated that the distribution of each genotype did not differ significantly between genders. This result was also observed in studies with more participants [28].

This study is limited by the small sample size, but it does provide an overview of the *CYP3A4*1G* and *CYP3A5*3* genotype variations in the Indonesian-Javanese population. In conclusion, the incidence of *CYP3A4*1G* in the Indonesian-Javanese population was 73.25% for *CYP3A4*1/*1*, 25.58% for *CYP3A4*1/*1G*, and 1.16% for *CYP3A4 *1G/*1G*. Meanwhile, the variance of *CYP3A5*3* genotypes was 16.27% for *CYP3A5 *1/*3* and 83.72% for *CYP3A5*3/*3*.

Conclusion

The results of the current study show that the population has the highest proportion of homozy

gous *CYP3A4*1G* wild type and homozygous mutants of *CYP3A5*3*. One subject had a homozygous mutation for both *CYP3A4*1G* and *CYP3A5*3*, while the other 49 subjects were heterozygous for *CYP3A4*1G* and homozygous mutant for *CYP3A5*3*. Chi-square analysis showed no difference in the distribution of the number of genotypes between the sexes.

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