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CYP2A6 Gene Polymorphism Allele *4 Study in Hypertensive Patients with a History of Smoking

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A B S T R A C T

Nicotine is a specific CYP2A6 substrate found in cigarettes. Nicotine levels in the blood are affected by the metabolic rate of the CYP2A6 enzyme, which is known to have a high level of polymorphism. High levels of nicotine in the blood can result in an increased risk of cardiovascular disease through the mechanism of increasing lipolysis and causing an increase in triglyceride levels in the blood. This study is an observational study conducted on 31 hypertensive men with a history of smoking. The purpose of this study was to determine the frequency of the CYP2A6 allele *4 genes in hypertensive patients with a history of smoking. Result analysis was performed on PCR products using electrophoresis, then the frequency of each allele was determined. The results showed that there was a CYP2A6 allele *4 gene in hypertensive patients with a history of smoking in Yogyakarta with an allele frequency of 62.90%, and only 2 respondents (6.45%) had the CYP2A6*1/*1 genotype or wildtype.

1. Introduction

Cardiovascular disease is the biggest cause of death in the world and accounts for 35% of total deaths in Indonesia.[1–3] Active and passive smokers will be more at risk of developing cardiovascular disease.[4,5] Research shows that Indonesia has 32.8% of smokers aged over 15 years, and the proportion of tobacco consumption among men and women is 62.9% and 4.8%.[6] Nicotine is one of the substances contained in cigarettes that can lead to an increased risk of cardiovascular disease through activation of the sympathetic nerves and increased lipolysis.[7–9] Increased lipolysis results in an increase in free fatty acids in the systemic circulation. This excess of free fatty acids will trigger an increase in triglyceride synthesis by the liver and further result in an increase in LDL levels.[9,10] Triglycerides are an early marker of increasing remnant *cholesterol,* which triggers the formation of atherosclerosis which is a manifestation of cardiovascular disease.[11]

Nicotine activity is influenced by nicotine metabolizing enzymes, namely CYP2A6. The CYP2A6 gene is part of the cytochrome P450 gene of the CYP2A subfamily, which is located on chromosome 19q.13.2. The CYP2A6 gene is a gene that encodes the CYP2A6 enzyme.[12] The CYP2A6 gene is one of the genes with high polymorphism and influences the activity of the enzyme in the password. According to Nakajima and Yokoi (2006), the allele forms of inactive CYP2A6 in Asian populations are CYP2A6*4 (11-20%), CYP2A6*7 (4-7%), CYP2A6*9 (20%). The CYP2A6*4 allele form is the inactive form.[13] In the Javanese in Indonesia, the frequency of CYP2A6*4 alleles was 52.51%, with CYP2A6*1/*4 genotypes of 95% (Patramurti et al., 2015). The method used in this study, namely polymerase chain reaction (PCR)

to identify the presence of the CYP2A6*4 allele, which was read using the electrophoretic method. The results obtained were used to see the frequency of the CYP2A6 allele *4 gene.

2. Methods

Tools and materials

Blood sample collected into a tube containing EDTA; forward primer CYP2A6*1 5' CAG GCC ATA ATA TTC CAC CC 3'; reverse primer CYP2A6*1 5' AGT CTT AGC TGC GCC CCT CT 3'; forward primer CYP2A6*4 5' CGG AAG AGG CGG GTA TAA GAA 3'; reverse primer CYP2A6*4 5' GTT TCC TTC CTC TCA TCC CA 3'; sterile aqua bidestilata; Promega Taq Green Master Mix (contains Taq DNA polymerase, 16dNTPs, MgCl2, and buffers); Favor Prep™ Genomic DNA Mini Kit (Blood/Cultured Cell); 10X Tris-Borate-EDTA (TBE) Buffer pH 8,3 Ultra pure grade; agarose; Loading dye; Gel Red™ Nucleic acid gel stain; VC 100bp Plus DNA marker; ethanol 70%; white tip; blue tip; ice cubes; and LipidPro™.

Data retrieval

The research was conducted at the Kalasan Health Center in August-September 2022 with the inclusion criteria of smokers/smoking history diagnosed with hypertension at the Kalasan Health Center, aged between 30-65 years, consuming clove cigarettes/filters and willing to fill informed *consent*. Exclusion criteria from this study were patients who were taking regular medication or were undergoing treatment to stop smoking in the last month, patients with diseases that required total rest for ≥ 10 days in the last month, not taking blood thinners, and patients with cardiovascular disease. Data collection was carried out after obtaining permission from the ethics commission of the Faculty of Medicine, Duta Wacana.

Blood collection and analysis

Blood sampling was carried out by Kalasan Health Center Laboratory staff. Blood was collected in a K2 vacutainer tube containing EDTA (1.8 mg/mL blood) and then stored at ± 4^{O}C before identification of the CYP2A6 allele. Allele identification was carried out in the tissue culture laboratory, Faculty of Pharmacy, Universitas Sanata Dharma. DNA isolation in this study was carried out by the salting-out method using FavorPrep™ Genomic DNA mini kit (blood/cultured cell). The procedure was carried out following the protocol listed, with the blood sample used as much as 300 µL. Amplification of DNA isolates by duplex PCR was carried out using Promega Taq Green Master Mix (containing Taq DNA polymerase, dNTPs, MgCl2, and buffer) with a final mixed volume of 25.0 µL, consisting of 12.50 µL reagent, 1 primer each. .0 µL (4 µL total), 2.0 µL DNA isolates, and 8.5 µL nuclease-free water. PCR was carried out at an initial denaturation temperature of 95^{O}C, denatured 95^{O}C, annealing 57,9^{O}C, extension 72^{O}C, and the final extension 72^{O}C, with a cycle of 35x. DNA isolates were identified using electrophoresis with 1.5% agarose stationary phase, which had been added with Gel Red™ Nucleic acid gel stain. Electrophoresis was carried out at 110V for 30 minutes. Results were read using a UV lamp.

3. Results and Discussion

Characteristics of 31 hypertensive male respondents aged 30-65 years are shown in Table 1. DNA isolates were analyzed, and the allele frequencies for CYP2A6*1 and CYP2A6*4 and their genotypes were obtained, as shown in Table 2. There were 39 (62.90%) CYP2A6 genes with allele *4, and from 31 respondents, 10 respondents (32, 26%) were homozygous alleles with the CYP2A6*4/*4 genotype.

Characteristics of respondents

The results of the study on 31 male smoking hypertension patients in the age range of 30-65 years showed an average age of 57.58 ± 6.93 years and 96.77% aged ≥45 years. Research shows aged≥45 years have a higher risk of experiencing cardiovascular disease due to degenerative factors.[15] As many as 80.65% of respondents were diagnosed with hypertension at the age of ≥45 years.

Table 1. Characteristics of respondents.

Characteristics	Total (n)	Percentage (%)
Age		
Mean ± SD (years)	57,58 ± 6,93	
<45 years	1	3,23
≥45 years	30	96,77
Age of onset of hypertension		
Mean ± SD (years)	51,29 ± 10,34	
<45 years	6	19,35
≥45 years	25	80,65
Age started smoking		
Mean ± SD (years)	20,13 ± 6,88	
≤15 years	6	19,35
>15 years	25	80,65
Type of cigarette		
Cigarette	23	74,19
White cigarette	8	25,81
Number of cigarettes/day		
≤10 cigarettes	16	51,61
11-20 cigarettes	11	35,48
21-30 cigarettes	4	12,91

Table 2. Frequency of CYP2A6 *1 and *4 alleles, and respondent genotypes.

Allele	Total (n=62)	Percentage
CYP2A6*1	23	37,10
CYP2A6*4	39	62,90
Genotype	**Total (n=31)**	**Percentage**
CYP2A6*1/*1	2	6,45
CYP2A6*1/*4	19	61,29
CYP2A6*4/*4	10	32,26

This is in accordance with the 2018 Riskesdas, which shows an increase in the prevalence of hypertension diagnosed by a doctor at the age of 45-54 years when compared to the age range of 35-44 years.[16] The average age of starting smoking was 20.13 ± 6.88 years, and 19.35% started smoking at the age of ≤15 years. This is in line with the 2020 Indonesian Tobacco Atlas, which states that smokers aged 10-14 years in Indonesia are 23.1%.[6] This study showed that 23 respondents (74.19%) consumed kretek cigarettes, and the rest consumed white cigarettes. Riskesdas (2018) shows that the consumption of kretek cigarettes in Indonesia is 67.8%, followed by white cigarettes and hand-rolled cigarettes.[16]

CYP2A6 allele frequency

DNA isolation was carried out by duplex PCR method. The isolates obtained were analyzed using electrophoresis and viewed under a UV lamp, with an example of the results shown in Figure 1.

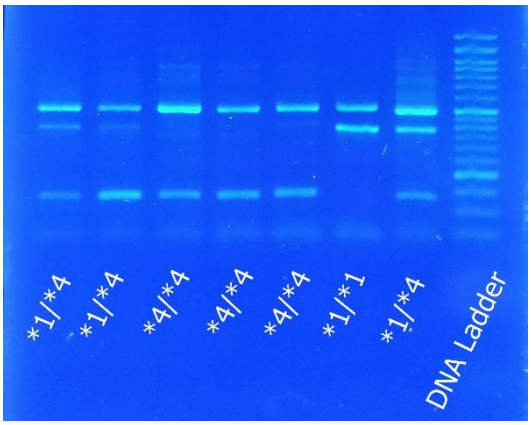

Figure 1. DNA isolation results.

DNA Ladder, which is used a ladder for 50 bp. The product length for CYP2A6*4 is 136 bp, so it can be seen from the readings that it is in the range of 150 bp. CYP2A6*1 is 387 bp long and can be seen at around 400 bp in the DNA sequence ladder. There is an additional 1band detected at a length of 500 bp which is a combined product between primers forward CYP2A6*1 with primer reverse CYP2A6*4 and is characteristic of duplex PCR analysis. The results of DNA analysis (Table 2) found 39 (62.90%) CYP2A6 genes with *4 alleles, and of the 31 respondents, 10 of them (32.26%) were homozygous alleles with the CYP2A6*4/*4 genotype. The results obtained are in accordance with previous studies, which show a high frequency of the CYP2A6 allele *4 gene in Indonesia, especially in the Javanese.[14,17] The CYP2A6*1/*1 genotypes fall into the category of normal metabolizer, i.e., having 100% enzyme activity. Genotype CYP2A6*1/*4 is a slow metabolizer, has an enzyme activity of 50%, and CYP2A6*4/*4 is the form of a poor metabolizer, i.e., has enzyme activity <25%.[18]

Individuals who fall into the category of slow metabolizer and poor metabolizer will have a high risk of cardiovascular disease compared to individuals who have a normal metabolic rate. This can occur due to nicotine levels in individuals with slow metabolizer and poor metabolizer higher than in individuals with normal metabolic rates.[19] The existence of an inactive allele form (CYP2A6*4) results in a decrease in the rate of nicotine metabolism, which means a higher risk of experiencing cardiovascular disease.

Nicotine can trigger cardiovascular disease through sympathetic nerve activation, increased lipolysis, and insulin resistance, which can lead to atherosclerosis.[7,8] Sympathetic nerve activation occurs through binding with nicotinic acetylcholine receptor (nAChR), which results in increased heart rate, blood pressure, and myocardial contractility.[8] Increased lipolysis is triggered by adrenoceptor activation and increased catecholamine hormones resulting in an increase in free fatty acids.[9] Excessive levels of free fatty acids trigger an increase in triglyceride synthesis and the release of VLDL, which will be converted into LDL, and insulin resistance, which in turn plays a role in the formation of atherosclerosis and an increased risk of cardiovascular disease.[20,21]

The speed of nicotine metabolism will also affect the level of a person's dependence on nicotine. The higher the CYP2A6 enzyme activity, the higher the level of a person's dependence on nicotine.[22] The CYP2A6*4 gene is a deletion gene, resulting in a decrease in enzyme activity.[23] Individuals who have the gene CYP2A6*4 will tend to have low dependence on nicotine.[24]

4. Conclusion

There is a gene CYP2A6 allele *4 in hypertensive patients with a history of smoking in Yogyakarta with an allele frequency of 62.90%. Of the 31 respondents

who agreed, only 2 respondents (6.45%) had the CYP2A6*1/*1 genotype.

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