ORIGINAL ARTICLE

CYP2A6 Genetic Polymorphism and Nicotine Metabolism of Male Smokers in Indonesia

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ABSTRACT

Introduction: One of the main ingredients in cigarettes is nicotine, which has a significant impact on a person's dependence on cigarettes. Nicotine can be detected in a smoker's urine as a sign that his body is processing nicotine. The important enzyme CYP2A6 is involved in nicotine metabolism. This study aimed to determine the relationship between CYP2A6 genetic polymorphisms and nicotine metabolism among male smokers in Indonesia.
Methods: This study included 100 male smokers who met the inclusion criteria in a cross-sectional design using a consecutive sampling between the ages of 20 and 65 years old. Restrictions fragment length polymorphism (RFLP) of the polymerase chain reaction (PCR) was applied to examine the genetic polymorphism of CYP2A6, and nicotine metabolite levels in urine were examined by high-performance liquid chromatography (HPLC) examination.
Results: This study involved one hundred smokers, and 78 tested positive for the CYP2A6 polymorphism. The CYP2A6 genetic polymorphism and nicotine metabolism were not significantly correlated (p-value > 0.05). Allele *1A and genotype 1B/1B were more common in this study population. The majority of study participants had fast metabolic rates.
Conclusion: No correlation was seen between the genetic polymorphisms of CYP2A6 and nicotine metabolism in Indonesian male smokers. Consequently, it is crucial to conduct future research in diverse populations with larger samples.

INTRODUCTION

One of the primary chemicals in cigarettes is nicotine, which significantly causes a smoker to become dependent on cigarettes.[1] Because smoking has a harmful effect on one's health, smoking continues to be one of the most important issues in the world, with significant morbidity and mortality rates. According to the estimation from the World Health Organization (WHO), up to 8 million individuals will pass away from smoking-related diseases in 2021, of which 7 million people will become active smokers and 1.2 million people will become passive smokers.[2] Up to 80% of people who smoke regularly live in low- and middle-income countries, including Indonesia.[2] Many carcinogens found in cigarette smoke cause the development of lung disease and other systemic disorders, as well as lung cancer. Nicotine is the substance most strongly associated with smoking addiction out of the 73 substances known to be connected to the onset of cancer.[3] Several ideas have been put forward to explain the prevalence of smoking in Indonesian society, with the most common factors being a lack of public knowledge, the public's belief that smoking is normal, and the public's assumption that death from smoking is not a serious matter.[4,5]

The main enzymes in nicotine's metabolic action are CYP2A6, CYP2A13, and CYP2A7. The speed at which a smoker's body breaks down nicotine will depend on this enzyme polymorphism.[6] The presence of nicotine in a smoker's urine may be a sign that nicotine is being metabolized in the body.

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CYP2A6 is an important enzyme that converts nicotine to cotinine (COT) and COT to trans-3'-hydroxycotinine (3HC).[1] All these enzymes will metabolize COT to 3HC. Nicotine will be converted into (methylnitrosamino) N'-nitrosonornicotine (NNN), 1-(3-pyridyl)-1-butanone (NNK), and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL).[7] The best enzyme in the CYP2A subfamily involved in nicotine metabolism is CYP2A6. Although all three genes were determined to be transcriptionally active in the liver, CYP2A6 was more common than CYP2A7 and CYP2A13.[8]

Adult smokers who have consumed 100 cigarettes or more during their lifetime and still do so are regarded as active smokers.[9] This CYP2A6 enzyme polymorphism can potentially interfere with nicotine metabolism, which has several implications for its metabolic rate. Nicotine, which has a slow metabolic rate, for instance, in the CYP2A6*4 allele gene variation, has a preventive effect on smokers' risk of developing lung cancer.[7] Thus, this study aimed to determine how the CYP2A6 genetic variant interacts with nicotine metabolism in Indonesian male smokers.

METHODS

Study Design

This cross-sectional study included one hundred male smokers who met the inclusion and exclusion criteria. Their ages varied from 20 to 65 years old. After venous blood collection, the genetic variations of gene CYP2A6 were examined using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). By using high-performance liquid chromatography (HPLC) analysis, it is possible to determine whether urine samples contain nicotine metabolites. Epi Info-7 software was utilized in this study. The Research Ethics Committee of the Faculty of Medicine, Universitas Sumatera Utara, had given ethical approval for this study.

Subject Characteristics

One hundred men between the ages of 20 and 65 years old who had smoked cigarettes regularly for at least 100 cigarettes during their lifetime, did not use prescription medications, and had no history of kidney or lung disease met the inclusion criteria for this study. Exclusion criteria were subjects whose urine samples were altered during collection or analysis and those who refused to undergo any procedures. Subjects gave their informed consent after being briefed about the study protocol. They answered questions regarding their smoking history and behaviors, including how long they had smoked, how many cigarettes they smoked each day, and the brand of cigarettes they used), as well as a quiz about the level of nicotine dependence using the Fagerstrom score.

DNA extraction

Approximately 2 mL of blood was taken during venipuncture to obtain a peripheral blood sample. Samples were put into sterile tubes with ethylenediaminetetraacetic acid (EDTA) and stored at −80°C for storage. Then, the Puregene Deoxyribonucleic Acid (DNA) Isolation Kit (Promega) was used to extract DNA. The genotype was checked using PCR.

Genotyping CYP2A6

In examining the genotypes of CYP2A6*1A, CYP2A6*1B, CYP2A6*4A, and CYP2A6*4D, primers 2A6R2 (5'-AAAAATGGCATGAACGCCC-3') and 2Aex7F (5'GRCCAAGATGCCCTACATG-3') were used. Samples were mixed with PCR mixture (25 l) made up of 1 x PCR buffer (67 mM Tris-Hcl [67 mM Tris–HCl buffer (pH 8.8); 16.6 mM $(NH_4)2\ SO_4$; 0.45% Triton X -100; 0.02% gelatin]; 1.5 mM $MgCl_2$; 0.4 lM per primer; 250 lM dNTPs, and 1 U of Taq DNA polymerase. Following a 1-minute initial denaturation at 95°C, 15 seconds of denaturation and amplification at 95°C, 20 seconds of annealing at 60°C, 35 cycles of extension at 72°C for three minutes, and a final 7-minute extension at 72°C. PCR products were performed with restriction enzymes Eco81I and AccII. The product was analyzed by electrophoresis on 1.5% agarose gel.[10]

Nicotine metabolism rate using HPLC

Urine sample preparation

Research subjects were not allowed to use illegal drugs or alcohol for two weeks before conducting the research. Shortly before the test was performed, a urine sample was taken. Before analysis, urine samples were stored at −20°C. Using 2 mL of chloroform/methanol solvent (9:1, v/v), the urine sample was extracted five times after being initially added to 1.0 mL of a standard solution that had been diluted to 1 g/mL. The solvent was removed after gathering the organic phase and heating it to 50°C until only around 2 mL of solution (organic phase A) remained. Next, one milliliter of 0.1 N KOH solution was used twice to remove the organic phase. Two milliliters of a 9:1 chloroform/methanol solvent were used to recover and extract the aqueous phase twice. After being gathered and combined with organic phase A, this organic phase was dried out by evaporating with the other organic phases. Following a 15-minute ultrasonically air-conditioning session, the residue was filtered through a millipore and dissolved in 1.0 mL of 30% methanol. Lastly, the HPLC system was

prepared to receive this solution for further examination.[11]

Nicotine Metabolism Test Method

An HPLC device with a Premier C8 column and a UV detector set at 260 nm was used for analysis. The analyte separation process was tailored to several mobile phase compositions. The optimal methanol:acetic acid composition ratios were 9:1, 7:3, 6:4, and 5:5. The flow rates for the mobile phase were 0.5, 0.8, and 1.0 mL/minute, was one of the optimal parameters besides the comparison of the composition of the mobile phase. The injection volume of the analyte solution was 20 L.

Preparation of Internal Standards and Standard Solutions

Acetanilide was the internal standard used in testing. With 30% methanol as solvent, standard stock solutions for COT, 3-HCOT, and a 1 mg/mL internal standard were prepared. These solutions could be maintained at 4°C. The stock standard solution was diluted using 30% methanol as a solvent to produce a working standard solution. While the concentrations of the COT working standard solution were 2.0, 4.0, 6.0, 8.0, and 10.0 g/mL, the concentration of the standard solution for 3-HCOT were 1.0, 2.0, 3.0, 4.0, and 5.0 g/mL. The SI solution used had a concentration of 1 g/mL. After that, a millipore filter was used to separate each solution, and an ultrasonicator was used to cool it for 15 minutes. The solution was then prepared for injection into the HPLC apparatus.

HPLC-Based COT and 3-HCOT Separation Optimization

A 20.0 L mobile phase and each prepared standard solution were introduced into the HPLC system. The tailing factor (tf) value, the Rs value between two compounds from the three analytes examined, and the retention time (tR) were used to determine the peak shape of the ensuing chromatogram. If the final chromatogram showed a tf value of 1.5 and a tR of fewer than 10 minutes, then the HPLC instrument system conditions were considered optimal.

Statistical analysis

The data was analyzed using Epi Info-7TM software. The relationship between CYP2A6 genetic variants and the sex, age, smoking history, and nicotine metabolic state of research participants was examined using the conditional logistic regression test.

RESULTS

Demographic characteristics

Although most participants had a low Brinkmann index, most of the study sample was between 40 and 60 years old. They were mostly self-employed, and their nicotine dependence status was high (Table 1). This might be because the sample had recently consumed cigarettes. CYP26 genotype analysis showed that most subjects had the 1B/1B gene, while only 1 had the 1A/4A genotype (Table 1). Only 78 study participants were able to measure levels of COT and 3HC in their urine. This might have occurred due to the low concentration of nicotine metabolites in the samples and the fact that they were below the minimum standard that HPLC could detect. Thus, it was not possible to determine the nicotine metabolite ratio (NMR) (Table 2). Analysis of nicotine metabolism showed that fast metabolism predominates. However, there was no association between nicotine metabolism and the CYP2A6 genotype (Table 3 and 4).

Table 1. Demographic Characteristics of Research Subjects

Characteristics		n	%
Age	<40 years old	77	77
	40-49 years old	9	9
	50-59 years old	12	12
	≥60 years old	2	2
Occupation	University student	26	26
	Officer	25	25
	Entrepreneur	49	49
Brinkman Index	Mild	64	64
	Moderate	23	23
	Severe	12	12
Nicotine Dependence	Very low	1	1
	Low	20	20
	Moderate	27	27
	High	43	43
	Very high	9	9
BMI	*Underweight*	5	5
	Normal	77	77
	Overweight	13	13
	Obesity	5	5
CYP2A6 Genotype	1A/1A	27	27
	1A/1B	32	32
	1B/1B	40	40
	1A/4A	1	1
CYP2A6 Allele	*1A	61	61
	*1B	39	39
Total		**100**	**100**

Table 2. Nicotine Metabolism of Research Subjects

Nicotine Metabolism	n	%
Rapid Metabolizer	76	97.4%
Slow Metabolizer	2	2.6%
Total	**78**	**100%**

Table 3. The Correlation between CYP2A6 Genotype and Nicotine Metabolism among Smokers

		Rapid Metabolizer		*Slow Metabolizer*		OR	95%CI	p-value
		n	%	n	%			
CYP2A6	1A/1A	22	28.9	1	50	1	1	0.601
	1A/1B	25	32.9	0	0	N/A	N/A	
	1B/1B	29	38.2	1	50	0.75	0.45 - 12.8	
	Total	**76**	**100.0**	**2**	**100**			

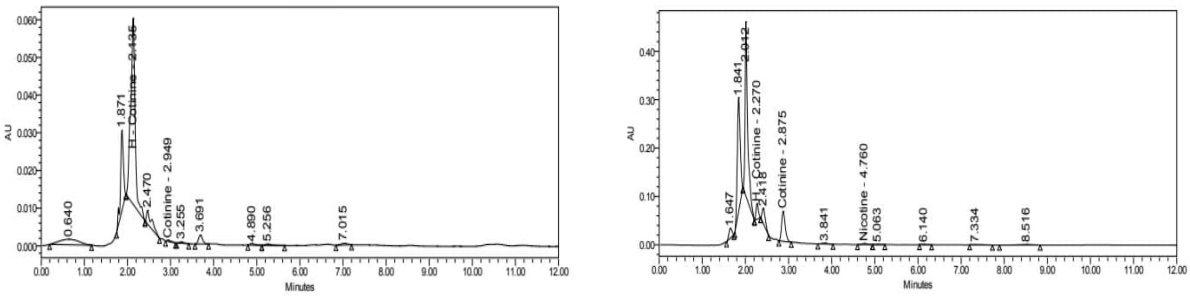
*Logistic regression test

Table 4. The Correlation Between CYP2A6 Allele and Nicotine Metabolism Among Smokers

		Rapid Metabolizer		*Slow Metabolizer*		OR	95%CI	p-value
		n	%	n	%			
CYP2A6	1A	69	45.4	2	50	1	1	0.85
	1B	83	54.6	2	50	1.2	0.16 - 8.77	
Total		**152**	**100.0**	**4**	**100**			

*Logistic regression test

NMR determined based on the 3HC/COT ratio values were used to represent nicotine metabolic activity regarding nicotine, COT, and 3HC (OH-COT) levels in urine samples. Figure 1 describes the nicotine metabolism process in study participants, with Figure 1a and 1b indicating rapid and slow metabolism, respectively.



1a **1b**

Figure 1. Based on the levels of nicotine, COT, and 3HC in urine samples, nicotine metabolic activity was identified as follows: a) The graph describes fast metabolic activity based on a 3HC/COT ratio > 0.5; b) Based on the 3HC/COT ratio value of 0.5, the graph shows the action of slow metabolic substances.

DISCUSSION

Seventy-three percent of the male smokers in this study were under 40 years old. According to 2018 National Data, the 25–44 age group was found to be the most active smokers in Indonesia.[12] The Centers for Disease Control and Prevention (CDC) administered the National Health Interview Survey (NHIS) in 2020 to estimate the use of commercial tobacco products among the United States (US) population aged 18 years and older. In the US, an estimated 47.1 million people (19.0%) had used commercial tobacco products in 2020. This figure included users of cigarettes (12.5%), smokeless tobacco (2.3%), cigars (3.5%), pipes (1.1%), and e-cigarettes (3.7%). The usage of combustible tobacco products, e-cigarettes, and two or more tobacco products together declined in the prevalence of smoking between 2019 and 2020. A total of 79.6% of those who admitted to using combustible items (such as cigarettes, cigars, or pipes) and 17.3% of people who admitted to using two or more tobacco products. Adult men under 65 years old are more likely to consume commercial tobacco products than women in this age group. Based on WHO data, around 3.7% of women and 59% of men in Indonesia smoke.[13] The findings of this study are also consistent with data collected in the US in 2019, where statistical data from the CDC revealed that the most active smokers in that country were in the 25 to 44-year-old age range.[14]

The maximum level of nicotine dependence determined based on the Nicotine Dependency Test (Fagerstrom Test) in this study found a high level of dependence with a percentage of 44.9%. However, the highest level of Brinkman index was found to be mild. The low Brinkman index was due to the smoking period of smokers measured this year was still relatively short.

This study examined the relationship between nicotine metabolism in Indonesian male smokers and CYP2A6 genetic variations. The liver breaks down nicotine into metabolites, such as cotinine N-oxide and nicotinic N-oxide. The primary psychotropic component of tobacco is nicotine. The existence of genetic variations can have an impact on the pharmacokinetics

and pharmacodynamics of nicotine, which will be related to tobacco use and the potential for lung cancer.

Nicotine metabolism rate is one of the variables known to influence the number of cigarettes a smoker smokes. Someone with a slow metabolism and who smokes more cigarettes will typically have a fast nicotine metabolism. Most nicotine in humans is converted to COT by the CYP2A6 enzyme. Then, COT is converted to 3HC via CYP2A6. NNN and 4-(methylnitrosamino)-NNK are tobacco-specific nitrosamines (TSNAs) that structurally resemble nicotine but do not cause cancer. Urinary biomarkers can be used to detect the ingestion and metabolism of procarcinogens. NNK is impossible to detect in urine because the human body rapidly degrades it. One frequent biomarker of NNK is NNAL, a reductive metabolite of NNK. Urinary NNAL levels are highly specific for evaluating tobacco exposure, are more stable, and have a longer half-life.[15]

The liver produces the CYP450 family of enzymes, Cytochrome P2A6, which significantly impacts how quickly nicotine is converted to COT. Tobacco's unique metabolic activation of nitrosamines is also believed to be related to the CYP2A6 enzyme. The CYP2A6 gene shows deletions and polymorphisms in Caucasians, although the frequency of these variations differs between races. It is estimated that 15-20% of the variation exists in the Asian race. Several important drugs, including toxins, procarcinogens, nicotine, and nicotine metabolites, are metabolized by this enzyme. In this regard, it is believed that CYP2A6 expression increases the risk of patients developing nicotine addiction as well as cancer due to tobacco use.[16]

Individual variations will impact how active a particular enzyme is during the metabolic process, as it will be responsible for most reactions. Enzymes called cytochrome P450 are crucial in this.[17] NMR is determined by dividing COT by 3HC, which is used to estimate the metabolic rate of nicotine in the body. As is known, the CYP2A6 enzyme breaks down nicotine in tobacco into COT and 3HC.[18–20]

Several studies have shown that CYP2A6 genetic variants and nicotine metabolism are related, which aligns with the previously mentioned reasons.[21] A study by Nakajima, *et al*. (2000) also found that CYP2A6*1 had greater enzyme activity, indicating a faster rate of nicotine metabolism compared to the slower nicotine metabolism of CYP2A6*4.[22] The majority of CYP2A6 genotypes in this study were *1B/*1B, representing 40 people (40%), and *1A/*1B, representing 32 people (32%), with *1A being the most common allele, presented in 61 people (61%). These findings align with previous studies that found the *1A allele to be a wild-type allele. Yoshida, *et al*. (2002) found that the CYP2A6 allele frequency distribution in Japanese and Koreans was 42.4% for *1A, compared with *1B (27.7%) and *4 (20.1%) in the Japanese population and 45.9% for *1A, *1B, and *4 respectively in the Korean population.[23]

Additionally, the results of this study demonstrated that the rate of nicotine metabolism was unaffected by genotypic polymorphisms in CYP2A6 and CYP2A13 ($p > 0.05$). These findings are consistent with a previous study conducted by Soeroso, *et al*. (2018), which found no relationship between the metabolic state and the CYP2A6 and CYP2A13 genotypes in Bataknese smokers who developed lung cancer.[24]

Faster nicotine metabolism can increase the desire to smoke more, thereby increasing the ratio of COT to 3HC as a result of the stimulation of nicotine metabolism.[25] In this study, 76 subjects (97.4%) smokers had a fast metabolic condition. Presumably, people with a fast metabolism will inhale more nicotine from cigarettes. Furthermore, increased CYP2A6 enzyme activity may suggest that smokers require more cigarettes to keep their bodies' nicotine levels stable. On the other hand, the results of this study showed that CYP2A6 polymorphisms and the condition of nicotine metabolism in male smokers from Indonesia were unrelated. Therefore, it is crucial to conduct more research to examine genetic variations and nicotine metabolism in different populations.

CONCLUSION

This study did not find a correlation between CYP2A6 genetic variations and the state of nicotine metabolism in Indonesian male smokers. Thus, further research is required to investigate the connection between genetic polymorphisms and nicotine metabolism across various populations.

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Conflict of Interest

The authors declared there is no conflict of interest.

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Authors' Contributions

Collecting data, drafting manuscript, concepting and designing the manuscript: CS, NNS, and FRA.

Processing data and interpreting: RZH, SB, DWTL. The data collection, processing, and interpretation were assisted by CS, RZH and SB. DWTL All authors contributed and approved the final version of the manuscript.

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