Nicotine Metabolism and Genetic Polymorphism Cyp2a13 in Male Smokers in Indonesia

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Research Article

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Abstract

BACKGROUND: The rate of nicotine metabolism described the presence of nicotine metabolites, which are the negative impact of cigarette smoke. CYP2A13 is one of the main enzymes responsible for nicotine metabolism and xenobiotic activity in tobacco smoking-related lung cancer.

AIM: This study aims to analyze the correlation between CYP2A13 and nicotine metabolism in male smokers in Indonesia.

METHODS: This was a cross-sectional study with consecutive sampling to 100 male smokers aged between 20 and 65 years old meeting inclusion and exclusion criteria. The CYP2A13 polymorphism was examined by restriction fragment length polymorphism (RFLP) PCR through venous blood extraction. The levels of nicotine metabolites in the urine samples were assessed by high-performance liquid chromatography (HPLC). The associations between nicotine metabolism and genetic polymorphism of the CYP2A13 gene in male smokers in Indonesia was determined by logistics regression test using Epi Info-7 software.

RESULTS: There were 100 participants involved in this study, in which 78 subjects were with the first stage of CYP2A13 polymorphism. There was no significant correlation between CYP2A13 genotype and nicotine metabolism (p value > 0.05). The CC genotype was more frequently found in the population in this study. Most research subjects had rapid metabolizers.

CONCLUSION: There is no significant relationship between CYP2A13 polymorphism and nicotine metabolism in male smokers in Indonesia. Therefore, it is important to carry out further studies to investigate genetic polymorphisms and nicotine metabolism in different population.

Introduction

Smoking remains the world's most serious problem, with a significant mortality and morbidity rate due to its negative impact on human health. According to the World Health Organization (WHO), there were over 8 million deaths due to smoking in 2021, with 7 million being active smokers and 1.2 million being secondhand smokers. Around 80% of the active smokers resided in the low-middle-income countries including Indonesia[1]. There are many theories proposed to explain the smoking habit in the Indonesian population. However, low self-awareness among the people and public perceptions about smoking as normal and death from smoking as a non-serious matter are the most prominent causes[2,3]. Cigarette smoke, on the contrary, contains many carcinogenic substances that contribute to lung carcinogenesis and other pulmonary and systemic diseases. Seventy-three substances have been identified as factors contribute to the development of cancer, with nicotine being the most influential substance to cause smoking addiction[4]

Nicotine metabolism activity has an important impact on nicotine dependency, tumor growth, and inflammation, making it an interesting concern for clinicians around the world [5–8]. CYP2A6, CYP2A13,

and CYP2A7 are the main enzymes in nicotine metabolism activities. The polymorphism of these enzymes contributes to the rate of nicotine metabolism[9]. The mutation of CYP2A13 is considered as a slow metabolizer activity causing the change of nicotine to cotinine and cotinine to 3-Hydroxycotinine (3HC) to occur slowly, resulting in lower accumulation of nicotine metabolite [10]. According to recent literature, nicotine is not a carcinogen, but the product of nicotine metabolism may alter DNA synthesis and cause inflammations, metabolic change, and gene promotor methylations [4,11].

There is very few studies that discuss the impact of genetic polymorphism in nicotine metabolism, which later correlates with certain diseases, particularly lung cancer [10,12–17]. This study aims to observe the correlation between the genetic polymorphism of CYP2A13 and nicotine metabolism in healthy smokers in Medan, North Sumatera, Indonesia. This is the first study to examine nicotine metabolism in healthy subjects in Indonesia.

Methods

Study Design

This research used cross-sectional design study with consecutive sampling method. There were two basic examinations used in this study, such as polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) to assess the genotype polymorphism of CYP2A13 through venous blood extraction and high-performance liquid chromatography (HPLC) to determine nicotine metabolite in the urine samples. This research received ethical approval from the Ethics Committee of Faculty of Medicine, Universitas Sumatera Utara

Subject Characteristics

There were 100 subjects, who met the inclusion and exclusion criteria, participated in this study. The inclusion criteria were male, age between 20 and 65 years old, status as an active smoker with a minimal lifetime consumption of 100 cigarettes, were not consuming any regular medications, and with no history of renal and pulmonary diseases. The exclusion criteria were subjects who did not follow all the examinations or subjects whose urine samples were damaged during sampling and analysis processes. The research subjects signed the informed consent after receiving explanation about the study procedure. Next, they filled out a smoking habit questionnaire composed of a few questions about smoking history (smoking durations and cigarette consumptions per day) and biomass exposure history (firewood, charcoal use, mosquito repellant), as well as Fagerstrom nicotine dependence questionnaire.

DNA extraction

A peripheral blood sample was obtained by venipuncture about 2mL. The sample was placed in a sterile tube containing EDTA and stored at -80°C. The DNA was extracted by using Isolation Kit DNA Puregene (Pro Mega). The genotypes were analyzed by PCR-based methods.

Genotyping CYP2A13

The identification of the genetic polymorphism of CYP2A13 was carried out by polymerase chain reaction with restriction fragment length polymorphism (RFLP-PCR). Exon 5 of the CYP2A13 gene was amplified by using a forward primer 5'-CCTGGACAGATGCCTTTAACTCCG-3' paired with a reverse primer 5'-TGGCT-TTGCACCTGCCTGCACT-3'. PCR amplification was performed in Bio-Rad DNA Model T-100 Thermal Cycler in a total volume of 25 µl containing approximately 200 ng genomic DNA, 2.5 µl x PCR buffer 2 mmol/l MgCl2, 0.2 mmol/l of each dNTP, 0.28 µmol/l of each primer, and 2 U of Taq DNA polymerase. The PCR conditions involved an initial denaturation at 95^{o}C for 3 minutes, followed by 35 cycles of denaturation at 95^{o}C for 30 seconds, annealing at 63^{o}C for 45 seconds, and extension at 72^{o}C for 30 seconds, with a final extension at 72^{o}C for 5 minutes. After the amplification, the PCR products (332 bp) were digested by HhaI restriction endonuclease at 37^{o}C for at least 4 hours. The digested products were analyzed by electrophoresis on a 2% agarose gel in the presence of ethidium bromide.[1218]

Nicotine metabolism rate using High-Performance Liquid Chromatography (HPLC)

Urine sample preparation

Two weeks before the study, the research subjects were prohibited from taking any medication or drinking alcoholic beverages. The urine as the sample was excreted right before the test. The urine sample was stored at -20°C before the analysis. First, 1.0 mL of urine sample was added with 1 g/mL of standard solution, then the sample was extracted by 2 mL chloroform/methanol (9:1, v/v) solvent for 5 times. The organic phase was collected and heated at 50°C to remove the solvent until approximately 2 mL solution remained (Organic phase A). Next, the extraction of the organic phase obtained was carried out by 1 mL 0.1 N KOH solution for 2 times. The aqueous phase was collected and re-extracted by 2 mL chloroform/methanol (9:1,v/v) solvent for 2 times. This organic phase was collected and combined with organic phase A, then all the organic phases were evaporated until the dry. Then, the residue was dissolved in 1.0 mL of 30% methanol and then filtered by using a Millipore filter and air-conditioned by using an ultrasonicator for 15 minutes. This solution was then ready to be injected into the HPLC system for further analysis [19].

Nicotine Metabolism Test

Nicotine metabolism test analysis was performed by using Premier C8 column at room temperature and a UV detector at 260 nm on the HPLC apparatus. The analyte separation was carried out with various compositions of the mobile phase and optimized ratio composition of methanol: acetic acid 5mM, such as 9:1; 7:3; 6:4, and 5:5. Another optimized parameter controlled was the flow rate of the mobile phase, such as at 0.5; 0.8; and 1.0 mL/min. The injection volume of the analyte solution was 20 L.

Preparation of the Internal Standard and Standard Solutions (SI)

The internal standard used in the analysis was acetanilide. Standard stock solutions, such as COT, 3-HCOT, and 1 mg/mL internal standard were prepared with 30% methanol as the solvent to enable storage at 4°C. The working standard solution was prepared by diluting the stock standard solution in 30% methanol. The concentration of the 3-HCOT standard solution was 1.0; 2.0; 3.0; 4.0 and 5.0 g/mL; while for COT as the working standard solution was 2.0; 4.0; 6.0; 8.0 and 10.0 g/mL. The concentration of the SI solution used was 1 g/mL. Then each of the solution was filtered by using a Millipore filter and air-conditioned by using the ultrasonicator for 15 minutes. After this step, the solution was ready to be injected into the HPLC system.

The Optimization of COT and 3-HCOT Separation Using HPLC

First, 20.0 L of each standard solution was injected into the HPLC system following the composition for mobile phase. Peaks and retention time (tR) from the three analytes, i.e COT, 3-HCOT, and 1 mg/mL internal standard, were observed from the produced chromatogram based on the values of tailing factor (tf) and Rs between the two compounds adjacent to each other. HPLC tool system condition was considered optimum if the chromatogram had a tf value of 1.5 and a tR below 10 minutes.

Statistical analysis

Data analysis was performed by using Epi Info-7™ software. Conditional logistic regression test was used to determine the role of CYP2A13 genetic polymorphism with gender, age, and smoking history of the research subjects.

Results

Demographic characteristics

Although most subjects were with mild Brinkmann index, their nicotine dependence status was at high dependency (Table 1). CYP2A13 genotype analysis showed that the majority of research subjects had wildtype CYP2A13, while only 1 subject was with TT genotype (Table 1). Nicotine metabolism analysis revealed that rapid metabolism was dominating. However, there was no correlation between the genotype of CYP2A13 and nicotine metabolism. Similarly, the allele of CYP2A13 did not correlate with nicotine metabolism either (Table 2 and Table 3).

Table 1
Demographic Characteristics

Characteristics		N	%
Age	< 40 yo	77	77
	40–49 yo	9	9
	50–59 yo	12	12
	>= 60 yo	2	2
Occupation	University Student	26	26
	Officer	25	25
	Entrepreneur	49	49
Brinkmann 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
Genotype of CYP2A13	CC	73	73
	CT	26	26
	TT	1	1
Nicotine Metabolism	Rapid Metabolizer	76	97.4
	Slow Metabolizer	2	2.6
Total		100	100

Nicotine metabolism was just assessed in 786 subjects because not all research subjects, from their urine samples, showed both cotinine (COT) and trans-3'–hydroxycotinine (3HC). This could be the case because of the low levels of nicotine metabolites in the sample, which was below the minimum standard that could be detected by the HPLC tool.

Table 2
The correlation between CYP2A13 genotype and nicotine metabolism among smokers

		Rapid Metabolizer		*Slow Metabolizer*		OR	95%CI	p-value
		n	%	n	%			
CYP2A13	**CC**	**57**	**75.0**	**2**	**100**	**1**	**1**	**0.416**
	CT	**19**	**25.0**	**0**	**0**	**0.96**	**0.99–1.08**	
Total		76	100.0	2	100			
Logistic Regression Test								

Table 3
The correlation between CYP2A13 allele and nicotine metabolism among smokers

		Rapid Metabolizer		Slow Metabolizer		OR	95%CI	p-value
		n	%	n	%			
CYP2A13	**C**	**133**	**87.5**	**4**	**100**	**1**	**1**	**0.45**
	T	**19**	**12.5**	**0**	**0**	**N/A**	**N/A**	
Total		152	100.0	4	100			
Logistic Regression Test								

The activities of nicotine metabolism were depicted in the levels of nicotine, cotinine (COT), and 3HC (OH-Cotinine) in the urine samples. Figure 1 showed the activities of nicotine metabolism calculated based on 3HC/COT ratio value, where Fig. 1a represent rapid metabolizer and Fig. 1b represent slow metabolizer.

Discussions

There is a downward trend in the smoking prevalence among young people globally. But, the number remains high in a few countries in Europe, Southeast Asia, Latin America, North Africa, and Central Asia [20]. This fact is in line with the data this study where 73% of the research subjects were under 40 years old. Based on the data from the Basic Health Research (RISKESDAS) 2018 in Indonesia, most active smokers were in the age range of 25–44 years old[21]. The prevalence of smokers aged 15 years and above increased from 26.9% in 1995 to 31.5% in 2001. This was associated with the increase in the prevalence of male from 53.4–62.2% during that period, while no significant change in number for female smokers. The WHO data stated that 59% of Indonesian men and 3.7% of the women were smokers[22]. The research subject demography in this study was similar to the data collected in the United States in 2019,

which reported the highest number of active smokers in the United States was between 25–44 years old[23].

This study observed the correlation between genetic polymorphisms of the CYP2A13 gene and nicotine metabolism in male smokers in Medan City, one of the biggest cities in Indonesia. Nicotine is metabolized in the liver into various forms of its metabolites, such as nicotine N-oxide and cotinine N-oxide [24]. Nicotine is the main psychoactive ingredient in tobacco. Genetic variations can influence the pharmacokinetics and pharmacodynamics of nicotine associated with changes in tobacco consumption and lung cancer risk. The rate of nicotine metabolism was considered to be one of the factors that influenced the number of cigarettes smoked by a smoker. Faster nicotine metabolism causes a greater desire to smoke more cigarettes and result in a higher ratio of cotinine (COT) and trans-3'-hydroxycotinine (3HC) induced by nicotine metabolism. This study showed that the majority (97.4%) of the research subjects had rapid metabolizer activity. Theoretically, individuals with rapid metabolizer activity would inhale more nicotine in cigarettes and the activity of the CYP2A13 enzyme could signal the need for more cigarettes – in order to maintain nicotine levels in the body. As a result, the number of cigarettes consumed would be higher in people with fast nicotine metabolism than those with slow metabolism [25–27]. Furthermore, the rate of nicotine metabolism was affected by genetic polymorphism in the enzymes that metabolize nicotine, particularly CYP2A6 and CYP2A13[4,11,28–30]. Recent studies showed that certain alleles of CYP2A13 might reduce nicotine metabolic activity and lower the intake of cigarettes; consequently lower the risk of developing lung cancer[10].

The rate of nicotine metabolism in the body can be calculated based on nicotine metabolite ratio (NMR) by calculating the ratio value between cotinine (COT) and trans-3'-hydroxycotinine (3HC). Nicotine in tobacco is metabolized to cotinine (COT) and then to trans-3'-hydroxycotinin (3HC) by the enzymes CYP2A6 and CYP2A13[31,32]. In this case, however, there were no relationships between either allele or genotype of CYP2A13 and the rate of nicotine metabolism in male smokers. This might be the case because of the absence of genotype TT, the mutant genotype, analysis in this study in subjects with slow metabolizer activity. Moreover, there was no allele T in the subjects with slow metabolizer activity – hence did not allow statistical analysis to be performed. A recent study in 2019 involving never smokers reported low prevalence of TT genotype and T alleles with no significant relationship between CYP2A13 genotype and the incidence of lung cancer[33]. Another recent study carried out in the Bataknese population as the dominant ethnic in North Sumatra, evaluated CYP2A6 – another substantial enzyme in nicotine metabolism, also found no relationship between genetic polymorphism of CYP2A6 and nicotine metabolism rate [34].

Conclusions

This study showed no significant correlation between the polymorphism of CYP2A13 gene and nicotine metabolic status in male smokers in Indonesia. Therefore, it is important to carry out further studies to investigate genetic polymorphisms and nicotine metabolism in different population.

Declarations

Authors Contributions

1. Conception and design:

All authors

2. Administrative support:

Noni Novisari Soeroso, Chaliza Soliha

3. Provision of study materials or patients:

Noni Novisari Soeroso, Chaliza Soliha

4. Collection and assembly of data:

Noni Novisari Soeroso, Chaliza Soliha, Fannie Rizki Ananda

5. Data analysis and interpretation:

Noni Novisari Soeroso, Fannie Rizki Ananda

6. Manuscript writing: All authors
7. Final approval of manuscript: All authors

Conflict of Interest

There is no conflict of interest in this study

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Figures

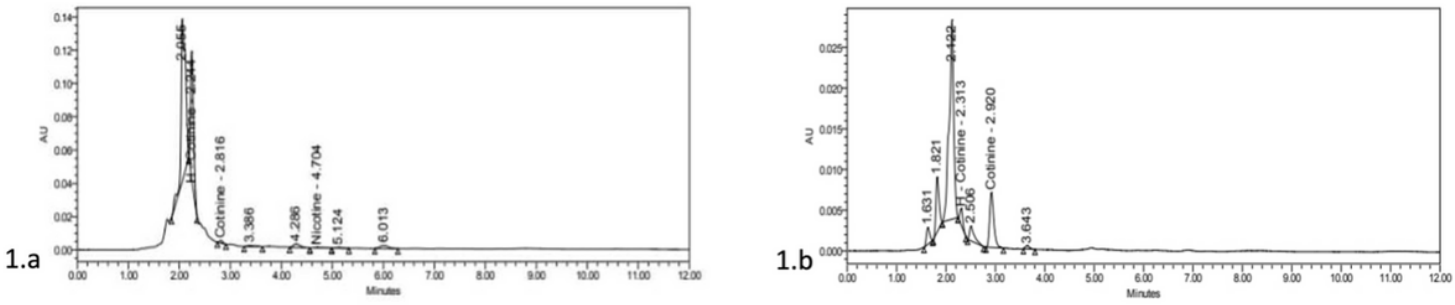

Figure 1

Nicotine metabolism activities based on nicotine, cotinine, and 3HC levels in the urine samples: a) The graph represents rapid metabolizer activities – ratio value of 3HC/COT > 0.5. b) The graph represents showed the slow metabolizer activities – ratio value of 3HC/COT < 0.5.