Original Article

Genetic Polymorphism of Cytochrome P450 2A6 Allele *4 and *9: Study on Glycohemoglobine Level Among Javanese Indonesian Smokers

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ABSTRACT

Nicotine, the active compound in cigarettes, was considered as the risk factor for type 2 diabetes mellitus (T2DM). In the human body, nicotine would be metabolized by the enzyme cytochrome P450 2A6 (CYP2A6). CYP2A6 was known to be highly polymorphic. The active form of this gene was CYP2A6 *1, while the CYP2A6 *4 and CYP2A *9 alleles were the inactive alleles. The presence of this inactive allele caused the decreased activity of CYP2A6 so that it would affect the level of nicotine in the blood and would eventually cause an increased blood sugar levels. This study aimed to determine the effect of CYP2A6 polymorphism on glycohemoglobine levels among Javanese smokers. The blood sugar levels were measured by hemoglobin A1c (HbA1c). In this study, 33 active smokers involved in the study were identified as slow metabolizers, by which 63.9% of all test participants had CYP2A6 *1/*4 genotype and as many as 6.1% of the test participants had the CYP2A6 *1/*4/*9. The HbA1c levels among the participants have been analyzed, 28 participants were in normal range (4.83-5.56%); 4 participants were identified in prediabetes condition (5.70% - 5.97%) and 1 participant was in diabetes with HbA1c level was 7.16%. This condition indicates that the presence of CYP2A6 *4 and *9 alleles will affect HbA1c levels which can eventually lead to T2DM disease.

*Keywords: polymorphism; CYP2A6*4; CYP2A6 *9; glycohemoglobine*

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INTRODUCTION

Diabetes, one of the underdiagnosed diseases, has been a serious threat to public health. The uncontrolled blood sugar could have been associated with cardiovascular diseases and blood pressure, development of renal (kidney) complications, nephropathy, retinopathy and foot ulcer. In some cases, by the time diabetes was diagnosed, the complications of diabetes might already be present (Buell et al., 2007). IDF Diabetes Atlas 8th Edition (International Diabetes Federation, 2017) was estimated that 82 million adults aged 20-79 years were living with diabetes in the South East Asia Region in 2017, representing a regional prevalence of 8.5%. About 45.8% of these diabetes cases were undiagnosed.

As of 2017, Indonesia was the world's top ten countries suffering from diabetes including China, India, United State, Brazil and Mexico. Approximately, 10 million people had diabetes in this country (International Diabetes Federation, 2017). Close to half (48.8%) of all adults with diabetes in the region live in urban areas. More than 1 in 4 of them were not aware they had the disease. It was estimated that there are 50% of people with undiagnosed diabetes in Indonesia. Despite being largely preventable, type 2 diabetes (T2DM) accounts for more than 90% of all diabetes cases (Soelistijo et al., 2015).

In 2010, American Diabetes Association (ADA) had recommended the use of the Hemoglobin (HbA1c) test to diagnose diabetes (American Diabetes Association, 2011). HbA1c tests measure average blood glucose over the past two to three months. The HbA1c test is being used for diagnosis of T2DM and prediabetes. A normal HbA1c level is 5.6 percent or below and a level of 5.7 to 6.4 percent indicates prediabetes, while people with diabetes have an HbA1c level at 6.5 percent or above (American Diabetes Association, 2010; The International Expert Committee, 2009).

T2DM is associated with modifiable lifestyle risk factors. Smoking had raised levels of HbA1 in smokers which had been known as the factor causing T2DM. Smokers are 30-40% more likely to develop T2DM than nonsmokers (Nilsson et al., 2004; Vlassopoulos et al., 2013; Xie et al., 2009). Nicotine, the main compound of cigarettes that causes addiction, had been proposed to be involved in the development of T2DM (Bajaj, 2012; Borowitz & Isom, 2008; Xie et al., 2009). It had been proven that nicotine to be a risk factor for insulin resistance and also induced apoptosis of beta cells which caused decreasing insulin in blood and eventually could lead to higher blood glucose levels (Bergman et al., 2012; Houston et al., 2006; Willi et al., 2007; Xu et al., 2012).

Nicotine is primarily metabolized in the liver by cytochrome P450 enzymes (CYP2A6). CYP2A6 shows great interindividual and interethnic variations in its expression levels and conversion activities, which are mainly attributed to CYP2A6 genetic polymorphisms. Genetic variation in the CYP2A6 gene could increase or decrease enzyme activity through altering the protein's expression level or its structure and function (Hukkanen et al., 2005). The CYP2A6*1, a common CYP2A6 allele, was considered as fast metabolizer, while any other alleles, CYP2A6*4 and CYP2A6*9, were associated with slower metabolizers (Raunio & Rahnasto-Rilla, 2012). According to Liu et al. (2011), heavy smoking may increase the risk of T2DM-particularly in smokers with CYP2A6 poor metabolizer genotypes.

This study was the first one of its kinds conducted in Indonesia which aimed to find out the relations between genetic polymorphisms, primarily CYP2A6*4 and CYP2A6*9 alleles, and hemoglobin among Javanese Indonesian smokers. By knowing these predictive relationships that show us how prediabetes occurs, we hopefully can strive for an appropriate and adequate prevention. Besides, we can also determine the needed screening data, to detect prediabetes early before it develops to become diabetes. This research was also expected to support government programs to reduce the number of smokers in Indonesia.

METHODS

The study was performed from August 2016 to July 2017 using a cross-sectional design and was performed in 33 healthy male participants as they were not taking any medications. The participants were recruited from Sanata Dharma University's employees which had three or more Javanese grandparents. Participants were 20 to 45 years of age and were defined as a current regular smoker, who smoked at least one cigarette per day and smoked more than 100 cigarettes during his lifetime at the time of interview. According to the number of cigarettes smoked per day, participants were classified as light smokers (cigarettes a day/CPD < 10), moderate smokers (CPD 11-20) and heavy smokers (CPD > 20). They had been given informed consent sheet prior to participating in this study. Original ethical approval for the Laboratory Research was granted by the Ethics Committees of Medical Research Duta Wacana University No. 424/C.16/FK/2017 (Yogyakarta, Indonesia).

Blood samples were collected from a cubital vein just before genotyping of CYP2A6 and HbA1c analysis. Genomic DNA was prepared using Ron's Blood and Cell DNA Mini Kit (Bioron-GmbH). The genotyping of CYP2A6*1, CYP2A6*4 and CYP2A6*9 alleles was previously assessed using primer-specific Polymerase Chain Reaction in our laboratory (Patramurti et al., 2015; Patramurti, 2017). The primer forward: 2A6-B6 (3'-CCT CAT CAC ACA CAA CTT CCT C-5') and primer reverse: 2A6-UTRAS1 (5'-TGT AAA ATG GGC ATG AAC GCC C-3') were used to identify allele CYP2A6*1 and CYP2A6*4, while the other primer (5'-GATTCCTCTCCCCTGGAAC-3' (primer forward) and 5'-GGCTGGGGTGGTTTGCCTTT A-3' (primer reverse) were used to identify allele CYP2A6*9. A total HbA1c analysis was performed by NORUDIA® N HbA1c in the Clinical Pathology Laboratory, Bethesda Hospital Yogyakarta using the Architect 600 instrument, which was calibrated using Diabetes Control and Complications Trial (DCCT) standards with coefficient of variation < 2.5%.

To describe the study population, we used Microsoft Excel 2016 in which we calculated the mean and standard of deviation of each continuous quantitative variable and the percentage for the genes. The qualitative analysis was used to illustrate the effect of CPY2A6 polymorphism, CYP2A6*1, CYP2A6*4 and CYP2A6*9 alleles, on HbA1c levels.

RESULTS AND DISCUSSION

Various studies reported the associations between smoking and CYP2A6 genotypes in the development of some cancer (Boffetta, 2008; Hecht, 2012; Mallery et al., 2014; Shields, 2002). Other studies suggested that smokers with defective CYP2A6 alleles are at a higher risk of developing T2DM (Liu et al., 2011). Here, we evaluated the influence of CYP2A6 genotypes on T2DM in Javanese Indonesian smokers based on a cross-sectional study, after controlling for age, gender, and family history of diabetes.

There were 33 male Javanese Indonesian smokers participating in this study. The average of the participants' age was 34.3 ± 9.3 years. Three alleles were identified using a PCR method among the participants, they were CYP2A6*1 (an active allele), CYP2A6*4 and CYP2A6*9. The *4 and *9 alleles are more common allele in Asian populations than in people of European ancestry. CYP2A6*4 allele is a gene deletion, leading for most poor metabolizers in Asians (Nakajima et al., 2004; Yusof & Gan, 2009). CYP2A6*9 allele is a single nucleotide polymorphisms (SNPs) which has a −48T to G nucleotide substitution in the TATA box (a sequence of DNA found in the core promoter region of genes) of the 5′-flanking region of the CYP2A6, which is in the TATA box of the CYP2A6 promoter region. The substitution decreases CYP2A6 mRNA expression and subsequently lowers enzyme activity (Pitarque, 2001).

**Table 1. Allele frequencies of CYP2A6*1, CYP2A6*4, CYP2A6*9
and genotype among Javanese smokers**

| Allele	Frequency (n=68)		Total
	CPD < 10 (n=20)	CPD 11-20 (n=13)	
CYP2A6*1	29.4% (20)	19.1% (13)	48.5% (33)
CYP2A6*4	29.4% (20)	19.1% (13)	48.5% (33)
CYP2A6*9	0	3 % (2)	3% (2)
Genotipe	**Frequency (n=33)**		**Total**
CYP2A6*1/*1	0% (0)	0% (0)	0% (0)
CYP2A6*1/*4	60.6% (20)	33.3% (11)	93.9% (31)
CYP2A6*1/*9	0% (0)	0% (0)	0% (0)
CYP2A6*1/*4/*9	0% (0)	6.1% (2)	6.1% (2)
CYP2A6*4/*4	0% (0)	0% (0)	0% (0)
CYP2A6*4/*9	0% (0)	0% (0)	0% (0)
CYP2A6*9/*9	0% (0)	0% (0)	0% (0)

According to genotype analysis, smokers were classified as normal metabolizer (100% CYP2A6 activity), intermediate metabolizer (individuals hypothesized to have approximately 75% of normal activity), and slow metabolizer (individuals hypothesized to have 50% or less of the normal activity). Smokers who did not have any copies of CYP2A6*4 or CYP2A6*9 were classified as 'normal metabolizer' (i.e. *1/*1). Smokers who had only CYP2A6*9 allele were classified as 'intermediate metabolizers' (e.g. *1/*9). The 'slow metabolizer' group refers to smokers who had either CYP2A6*4 allele or two CYP2A6*9 alleles (e.g. *1/*4 or *9/*9) and the 'poor metabolizers' were indicated as smokers who had either CYP2A6*4 and CYP2A6*9 allele or two CYP2A6*4 alleles (e.g. *9/*4, or *4/*4) (Mwenifumbo et al., 2008; Mwenifumbo et al., 2007; Schoedel et al., 2004).

In the present study, we found that all of the 33 participants involved in the study had heterozygous alleles and none of the participant had homozygous CYP2A6*1. There are 93.9% participant had CYP2A6 * 1/*4 genotype and 6.1% had CYP2A6 * 1/*4/*9 genotype (Table 1). Based on this result, all participants were identified as slow metabolizers. According to the number of cigarettes per day, participants were classified into three levels, light smokers (CPD < 10), moderate smokers (CPD 11-20) and heavy smokers (CPD > 20). The participants involved in this study were only classified as light smokers (17) and moderate smokers (15). None of the participants were classified as heavy smokers, so this study was in line with numerous studies that had reported smokers who carry reduced activity or

null CYP2A6 alleles do smoke less (Ando et al., 2003; Fujieda et al., 2004; Liu, David, et al., 2011; Minematsu et al., 2006; Schoedel et al., 2004; Rao et al., 2000).

About 70 to 80% of inhaled nicotine was converted to cotinine by the CYP2A6 enzyme; cotinine was further oxidized into trans-3-hydroxycotinine by this enzyme and all three compounds were excreted in urine (Hukkanen et al., 2005) (Figure 1).

Metabolism of nicotine had associations for addiction
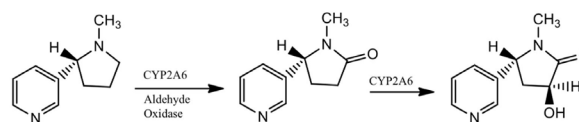
Figure 1. Primary metabolism pathway of nicotine facilitated by CYP2A6

and would have impact on risk of T2DM in smokers. Variation in the CYP2A6 gene could decrease the rate of nicotine metabolic-inactivation, therefore smokers with slow or poor metabolizer genotypes, whose CPD was more than 20, were more likely to saturate this metabolism pathway, resulting to higher nicotine plasma levels. These slow metabolizers would then be more likely to develop T2DM due to their higher level of nicotine in blood (Bajaj, 2012; Borowitz & Isom, 2008; Xie et al., 2009). Some studies suggested that nicotine either could have toxic effect on the pancreatic beta cells, or causing insulin resistance that leading to high risk of T2DM (Bajaj, 2012; Bergman et al., 2012; Xu et al., 2012). Some other studies reported that smoking would increase the HbA1c level (Nilsson et al., 2004; Vlassopoulos et al., 2013).
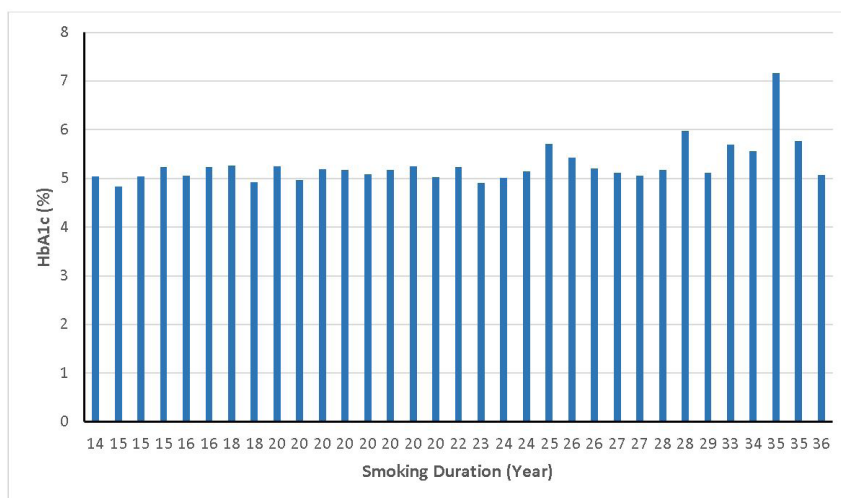
Figure 2. Distribution of HbA1c levels among Javanese Indonesian smokers based on smoking duration (year)

Results of our study showed that HbA1c levels among participants were ranging from 4,83-7,16% with an average of 5.27 and SD of 0.427. Based on our results, almost all participants had a normal HbA1c level, four participants were on prediabetes condition and only one participant was identified suffering diabetes. The prediabetes condition mean that smokers might have a higher chance of getting diabetes, depending on level of hemoglobin A1c and presence of other risk factors, such as obesity and family history (American Diabetes Association, 2010).

None of the participants involved in this study was classified as heavy smoker and all of them were identified as slow metabolizers. This result was consistent with Liu et al. (2011) which demonstrated that the risk of T2DM was higher in heavy smokers who have slow or poor metabolizer genotypes rather than in light or intermediate smokers with slow or normal metabolizers. The participants who had HbA1c level more than 5.7% (prediabetes and diabetic conditions) were observed in individuals who smoke cigarettes for more than 25 years (Figure 2). These results support other studies that reveal chronic smokers with slow and poor metabolizer genotypes would have high risk of T2DM caused by the pancreas exposed to greater circulating levels of nicotine that might contribute to apoptosis of islet β-cells and furthermore decreasing insulin secretion (Jyothirmayi et al., 2013).

Any other compounds, pre-carcinogens, found in tobacco smoke, such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL); N-nitrosodiethylamine (NDEA), could be activated to carcinogens by CYP2A6 in liver or CYP2E1 expressed in pancreatic islets (Anttila et al., 2011; Brown et al., 2007; Hecht, 2003; Wang et al.,

2012). Therefore, smokers with CYP2A6 slow or poor metabolizing genotype, the hepatic first-pass clearance of these compounds might be decreased lead to increasing systemic levels of these pre-carcinogens and more exposure of other organs, such as pancreas. Ultimately, the higher levels of nitrosamines in pancreatic islet cells will cause metabolic activation by CYP2E1, which lead to pancreatic inflammation and apoptosis of islet β-cells, followed by the reducing insulin secretion and consequently the increasing risk of incident T2DM.

Most previous experimental and clinical studies described that nicotine decreased insulin sensitivity. Bergman et al. (2009) revealed that chronic cigarette smokers were less insulin sensitive compared with control subjects that did not smoke. Furthermore, Bergman et al. (2012) had found that nicotine could increase mTOR/p70S6 K activity in cultured L6 myotubes in relation with increased IRS-1 Ser 636 phosphorylation and induced insulin resistance in skeletal muscle which subsequently reduced insulin-stimulated glucose uptake.

This study supports the hypothesis that the blood glucose levels might be increased in smokers, who may have high risk to diabetes mellitus, if smoking is not controlled. Furthermore, our study also supports many previous studies that CYP2A6 polymorphism, primarily caused by defective alleles (*4 and *9), would be a factor of high risk on T2DM. These findings suggest that chronic smokers whose genotype as slow metabolizer would enhance the development of T2DM. It induces the reduction of insulin secretion caused by the toxic effect of nicotine or nitrosamine compound to the pancreas as well as the occurrence of insulin resistance. Finally, the results expand the understanding to the possibility effects of cigarette smoking on T2DM. It may have major public health consequences for tobacco control

and efforts to prevent the development of diabetes in Indonesia, especially to the Javanese smokers.

CONCLUSION

Diabetes mellitus is one of serious problem on public health that many people are unaware of suffering this disease. Diabetes in Indonesia, mostly T2DM caused by lifestyle, is growing precipitously. Smoking, another public health problem encountered in Indonesia, is one of lifestyle factors that causes diabetes. Chronic smokers, either light or intermediate smokers, whose genotypes were classified as slow and poor metabolizers, would have high risk of suffering T2DM.

The most important limitations of this study that other factors have not been investigated. They might be confounding to our results, for example obesity, physical activity, dietary factors and biochemical variation such as plasma nicotine levels or total urinary nicotine equivalents.

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