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Research Article

POLYMORPHISM OF CYTOCHROME P450 2A6 (CYP2A6*1 AND CYP2A6*4) AMONG JAVANESE INDONESIAN SMOKER AND NON SMOKER

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ABSTRACT

Cytochrome P450 2A6 (CYP2A6) is the principal enzyme involved in the metabolic activation of tobacco-specific nitrosamines to their ultimate carcinogenic forms and metabolism of nicotine. The present study was developed to investigate the genetic polymorphism of CYP2A6 in Javanese Indonesian subjects carrying the CYP2A6*1 allele and the CYP2A6*4. The whole gene deletion of CYP2A6 (CYP2A6*4) may inhibit smokers from giving up smoking, but appears to function as a protective factor against some cancer. However, the investigation of these allele, a major functional polymorphisms common in Asian populations, have not been reported among Javanese Indonesian population. A single polymerase chain reaction-restriction fragment length polymorphism was used to resolve the genotypes into CYP2A6*1 (wild type) and CYP2A6*4 (CYP2A6*del*). The sample studied consisted of 100 healthy subject that consist of 50 non smokers and 50 smoker from Javanese Indonesian population. The allele frequencies of *1 (wild type) and *4, were 47.5 and 52.5%, respectively. When the two allel were considered simultaneously, among the non-smokers, 45% were genotyped for CYP2A6*1/*4 and 5% were genotyped for CYP2A6*4/*4; on the other hand all of the smoker were genotyped for CYP2A6*1/*4 and there was no homozygote of wild type. Based on the data collected, it could be concluded that the polymorphism of CYP2A6 were detected in among Javanese population sample study and the allele frequencies of CYP2A6*4 were high.

Key word: Polymorphism, CYP2A6*1, CYP2A6*4, Javanese Indonesian

INTRODUCTION

The cytochrome P450 2A6 (CYP2A6) gene is expressed at a high level in liver and at lower levels in nasal mucosa and respiratory tract (Fernandez-Salguero *et al*., 1995). CYP2A6 plays an important role in the activation of many procarcinogens, including aflatoxin B1, N-nitrosodiethylamine, and 1,3-butadiene and some tobacco-related nitrosamines, as well as in the clearance of some pharmaceuticals (Kushida *et al*., 2000; Oscarson, 2001; Raunio *et al*., 2008; Yoshida *et al*., 2002). In addition, CYP2A6 is involved in the metabolism of nicotine, the primary compound in tobacco that establishes and maintains tobacco dependence (Benowitz *et al*., 2011; Gullstén, 2000; Hukkanen *et al*., 2005; Messina *et al*., 1997; Oscarson, 2001).

A genetic polymorphism of CYP2A6 was recognized as one of the causes for the interindividual differences in the metabolism of nicotine (Heravi *et al*., 2010; Johnstone *et al*., 2006; Kwon *et al*., 2001; Lea *et al*., 2005; Nagano *et al*., 2010; Nakajima *et al*., 2006; Yang *et al*., 2001). There are 80 numbered CYP2A6 allelic variants identified to date, however, not all have been functionally characterized (www.cypalleles.ki.se/cyp2a6.htm). Among 80 CYP2A6 allelic variants, CYP2A6*4 presents a whole gene deletion (Oscarson *et al*., 1999), that accounts for the majority of poor metabolizer (PM) in Asian populations (Nakajima and Yokoi, 2005; Oscarson, 2001).

The CYP2A6*4 allele is of great importance in correlation studies, for example, smoking behaviour, pre-carcinogen activation

or drug metabolism to the CYP2A6 genotype, in particular when oriental populations are investigated (Oscarson, 2001). Subjects who are homozygotes for CYP2A6*4 are completely deficient in nicotine formation and polymorphism may inhibit smokers from giving up smoking (Ando *et al*., 2003; Ariyoshi *et al*., 2002; Fujieda *et al*., 2004; Minematsu *et al*., 2006; Rao *et al*., 2000). However, since the subjects lacking the CYP2A6 gene cannot activate N-nitrosamines such as NNK and N'-nitrosonornicotine, which are contained in tobacco smoke as nicotine-derived carcinogens, it indicates that smokers carrying the CYP2A6*4 allele might have less risk of tobacco-related cancers (Ariyoshi *et al*., 2002; Fujieda *et al*., 2004; Liu *et al*., 2013; Minematsu *et al*., 2003; Nowell *et al*., 2002; Topcu *et al*., 2002; Wang *et al*., 2013).

A high frequency of CYP2A6*4 among Malaysian Malay population was reported (Yusof and Gan, 2009), but in Indonesian population the allele frequency of CYP2A6*4 have not been reported yet. The present study was developed to investigate the genetic polymorphism of CYP2A6 in Javanese Indonesian subjects carrying the CYP2A6*1 allele and the CYP2A6*4.

MATERIAL AND METHODS

Subjects and data collection

Individuals involved in this study had to have Javanese parents and grandparents. Subjects were healthy male volunteers, between 18 and 50 years-old, took no concurrent medications, and had no illnesses requiring investigation or treatment and have signed a statement of informed consent. This study was approved by the Ethics Committees of Medical Research Gadjah Mada University (Yogyakarta, Indonesia).

The sample population involved 100 adult subjects (50 non smokers and 50 smokers) that were recruited from students and staffs of Sanata Dharma University. The non smokers were those who had never smoked in their life. Smokers were considered current smokers if they smoked up to 1 year before the date of the interview, not currently planning to stop smoking, smoke between 1 and 30 cigarettes daily. Information was collected on the amount of cigarettes smoked per day (CPD), the age at which the subject started smoking, the nicotine content of the cigarettes, and time to smoke the first cigarette of the day, which is generally accepted as a clinical index for nicotine dependence (Kubota *et al*., 2006).

CYP2A6 genotyping

Blood samples were collected from venous blood samples into EDTA containing tubes and genomic DNA was extracted by the salting-out method using Ron's Blood and Cell DNA Mini Kit (Bioron-GmbH). The genotyping of CYP2A6*4 was based on polymerase chain reaction (PCR)/restriction fragment length polymorphism and was performed by a previously described method (Ariyoshi *et al*., 2000; Muroi *et al*., 2012) with minor modifications. Briefly, a novel forward primer named 2A6-B6 (5'-CCT CAT CAC ACA CAA CTT CCT C-3') and a reverse primer named 2A6-UTRAS1 (5'-TGT AAA ATG GGC ATG AAC GCC C-3') were used to amplify the common regions of CYP2A6*1 and CYP2A6*4. The PCR reaction was perform using KAPA HiFi HotStart PCR Kit (Kapa Biosystems) that contained 1 x Kapa GC Rich HiFi Buffer (contain 2mM $MgCl_2$), 0.3mM Kapa dNTP Mix, 0.3µM 2A6-B6, 0.3µM 2A6-UTRAS1, 1 U Kapa HiFi HotStart DNA Polymerase, and approximately 50ng of genomic DNA in a final volume of 25µL. PCR was carried out under the following conditions: initial denaturation at 95°C for 5min; followed by 30 cycles of denaturation at 98°C for 20s, annealing at 64°C for 15s and extension at 72°C for 30s; and subsequently a final extension at 72°C for 5min. The PCR product patterns were analyzed by electrophoresis with 1% agarose gel. The PCR products consisted of 1358-bp fragments from the CYP2A6*1 allele and 1356-bp fragments from the CYP2A6*4 allele; these fragments were digested with Eco81 I (Thermo Scientific). The digestion patterns were analyzed by electrophoresis with 1% agarose gel. Fragments of 824 bp and 728 bp were derived from the CYP2A6*1 and CYP2A6*4 alleles, respectively.

Data analysis

Two CYP2A6 alleles (CYP2A6*1 (wild type allele), and CYP2A6*4 were identified in the Javanese Indonesian subjects used

in the study. According to the genotypes, subjects were divided into three groups: normal metabolizers, low metabolizers and poor metabolizers. In brief, normal metabolizers were defined as having allele *1/*1; slow metabolizers had allele *1/*4 which was associated with 50% of the activity of normal metabolizers; and poor metabolizers had allele *4/*4 which was associated with less than 25% of the activity of normal metabolizers (Mwenifumbo *et al*., 2008; Schoedel *et al*., 2004). The frequencies of CYP2A6 genotypes for each allele were assessed using Hardy Weinberg distribution. Differences in allele frequencies and genotype among smokers and non smokers were assessed using the chi-square test.

RESULT AND DISCUSSION

The population heterogenity in Indonesia consists of ethnic classifications, based on continental origin, racial background or physical appearance. The main aim of the study was to investigate the distribution of cytochrome P450 2A6 (CYP2A6) among Javanese Indonesian people. Two CYP2A6 alleles (CYP2A6*1 (wild type allele) and CYP2A6*4 were identified in the Javanese subjects used in the study. The selection of variant alleles for genotyping was based on functional importance and occurance frequency in Asian (Nakajima and Yokoi, 2005; Oscarson, 2001).

We examined the frequency of the variant alleles in Javanese Indonesian that consist of 50 smokers and 50 nonsmoker with a mean age of 33 years (Table I). The smokers were selected according to their smoking habits which were categorized into three levels: light smokers (CPD: 1-10), intermediate smokers (CPD: 11-20) and heavy smokers (CPD: 21-30) (Yang *et al*., 2001). According to B-Rao (2001), the minimum sample size in genetic polymorphism studies with two allele detected is 50. In these sample size both alleles will be detected in high probability.

In this study, the amplification of the CYP2A6*1 and CYP2A6*4 alleles were successfully carried out using polymerase chain reaction (PCR)/restriction fragment length polymorphism (Muroi *et al*., 2012). The primers 2A6-B6 were used to amplify exon 8 (Nunoya *et al*., 1999) and the primers 2A6-UTRAS1 were used to amplify regions from exon 1 to the 3'-untranslated region of the CYP2A6 gene. In this reaction, primers were specifically designed for amplification of either the CYP2A6*1 or the CYP2A6*4 allele, allowing for the convenient detection of heterozygous or homozygous carriers of the CYP2A6*4 allele. The CYP2A6*4 allele genotyped in this study was the CYP2A6*4A variant, which is identical to CYP2A6*4C (Ariyoshi *et al*., 2000; Muroi *et al*., 2012). The CYP2A6*4C allele is suggested to have arisen due to an unequal crossing-over event with CYP2A7 (Nunoya *et al*., 1999). Studies on the CYP2A6 gene have been rather problematic, because the highly (94%) homologous CYP2A7 gene is located just 25kb upstream of the CYP2A6 gene (Rautio, 2003).

In vitro DNA amplification of the CYP2A6 gene using these specific primers resulted in a 1358-bp fragments from the CYP2A6*1 allele and 1356-bp fragments from the CYP2A6*4 allele. Further, to differentiate between CYP2A6*1 allele and CYP2A6*4 allele, the PCR products were digested using Eco801 I. The 824 bp and 728 bp were derived from the CYP2A6*1 and CYP2A6*4 alleles, respectively (Ariyoshi *et al*., 2000; Muroi *et al*., 2012) (Figure 1).

A PCR product was detected in all (100%) samples. Among 50 non smokers, 45% were genotype for CYP2A6*1/*4 and 5% were genotype for CYP2A6*4/*4; on the other hand all of the smoker were genotype for CYP2A6*1/*4. These data suggested that the distribution of CYP2A6*1/*4 genotype frequency was not different significantly (χ^2=3.841; P=0.022) between smokers and non smokers in this study population (Table II).

The allele frequencies of CYP2A6*4 are highly variable among races. The reported frequencies of CYP2A6*4 allele is 0.5% in Spaniards (n=100), 15.1% in Chinese (n=96) (Oscarson *et al*., 1999), 1,2% in Caucasian (n=296) (Rao *et al.,* 2000), 11% in Korean (Kwon *et al.,* 2001), 20.1% in Japanese (n=92) (Nakajima *et al.,* 2001), and 16.7% in Malaysians (n=24) (Yusof and Gan, 2009). Among these studies, Asian showed to have a relatively high frequency of CYP2A6*4 allele. In this study, the allele frequencies of CYP2A6*1, and CYP2A6*4 in the Javanese Indonesian (n=100) were

Table I. Demographic characteristics and CYP2A6 genotypes of subjects according to smoking status

Characteristics	Smoking status			
	Non smokers	Smokers		
		Light	Intermediete	Heavy
Number of subjects	50	20	17	13
Age				
Mean ± SD	33±8	33.6±9.1	34.94±9.56	36.07±8.78
Range	18–45	18–45	18–46	22–47
Number of CPD				
Mean ± SD	–	8.15±1.17	13.06±1.03	22.69±0.95
Range		6–10	12–14	21–24

47.5% and 52.5%, respectively. A high frequencies of CYP2A6*4 in the Javanese were consistent with the finding of the other results above.

In these study, no CYP2A6*1 homozygous were found both in non smokers and smokers, these data imply that there is no difference in the frequency of CYP2A6*4 alleles among smokers when compared with non-smokers. It is worth noting that the gene deletion mutation CYP2A6*4 as being homozygous, was detected in only five out of the 50 non smokers tested.

CYP2A6 deletion alleles (CYP2A6*4) are of great importance in studies to aimed at correlating smoking behaviour, pre-carcinogen activation or drug metabolism with the CYP2A6 genotype, especially in Oriental populations. A number of studies have found that smokers who inherit CYP2A6 alleles associated with slower nicotine metabolism presumably need to smoke fewer cigarettes to achieve the same pharmacological effect experienced by smokers who inherit CYP2A6 alleles associated with faster nicotine metabolism. The study by Ariyoshi *et al.* (2002) exhibits a tendency of the subjects with the CYP2A6*1B/*1B genotype to smoke a higher number of cigarettes per day (average number 20–40 cigarettes) than the subjects with the CYP2A6*1A/*1A genotype (average 20–30 cigarettes). Ando *et al.* (2003) found the lack of a significant impact of heterozygous CYP2A6 deletion on smoking behavior. Fujieda *et al.* (2004) reported that subjects carrying CYP2A6*4, CYP2A6*7, CYP2A6*9 and CYP2A6*10 alleles smoked significantly less than subjects with the CYP2A6*1/*1 genotype (combining CYP2A6*1A and CYP2A6*1B). Slow metabolizers smoked fewer cigarettes per day and had an earlier age of first smoking (Schoedel *et al.*, 2004). According to Minematsu *et al.* (2006), homozygous mutants and compound heterozygotes (CYP2A6*4, CYP2A6*7 and CYP2A6*9) smoked fewer cigarettes daily than heterozygotes and homozygous wild-type individuals (CYP2A6*1). CYP2A6 poor metabolizer genotypes were associated with lighter smoking, a later age of initiation and a shorter duration of smoking (Liu *et al.*, 2011). However, these observations remain conflicting. Other studies have failed to detect an association between genetically low CYP2A6 activity and the number of cigarettes smoked. Genetic variation in CYP2A6 did not correlate with the ability to quit smoking (Kwon *et al.*, 2001) and there is no significant difference in the smoking status was observed according to the CYP2A6 genotype (Gambier *et al.*, 2005; Tan *et al.*, 2001). This discrepancy may be partly explained by the different frequencies of inactive allele.

Regarding the basis for the classification of the CYP2A6 genotypes into three groups, we recently analyzed that all of the smoker were classified as slow metabolizers as having CYP2A6*1/*4 genotype (Mwenifumbo *et al.*, 2008; Schoedel *et al.*, 2004). The results indicate that the CYP2A6 genotype does not have any notable effect on the number of smoked cigarettes among smokers.

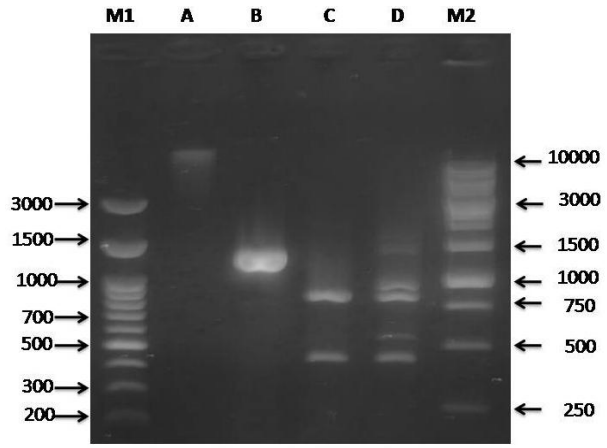

Figure 1. Electrophoregrame PCR product and Digested product identification of CYP2A6*1 and CYP2A6*4 allele. M1: Marker DNA Ladder, A: Isolated DNA, B: PCR Product (1358/1356bp), C: Digested Product (728bp) and D: Digested Product (824bp and 728bp), M2: Marker DNA ladder.

Table II. CYP2A6 genotype and allele frequencies in the Javanese Indonesian according to smoking status

Genotypes	Observed Frequency				Total
	Number of Subject (n=100)				
	Nonsmoker (50)	Smoker (50)			
		Light	Intermediate	Heavy	
CYP2A6*1/CYP2A6*1	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
CYP2A6*1/CYP2A6*4	45.0% (45)	20.0% (20)	17.0% (17)	13.0% (13)	95.0% (95)
CYP2A6*4/CYP2A6*4	5.0% (5)	0.0% (0)	0.0% (0)	0.0% (0)	5.0% (5)
Allele	**Number of Allele (n=200)**				
CYP2A6*1	22.5% (45)	10.0% (20)	8.5% (17%)	6.5% (13)	47.5% (95)
CYP2A6*4	27.5% (55)	10.0% (20)	8.5% (17%)	6.5% (13)	52.5% (105)

Futhermore we did not find any significant association between CYP2A6 gene defect and tendency to tobacco smoker among Javanese participants. These observations are consistent with previous findings that impaired function of CYP2A6 could not reduce cigarette consumption. The similar frequency of the CYP2A6*4 genotype in non smokers and smokers may be important (Table II) in understanding the essential role of CYP2A6 genotypes in smoking related diseases, because the CYP2A6*4 allele does not protect subjects from becoming habitual smokers. It is clear that CYP2A6 genetic variations are only part of the genetic basis of addiction to nicotine, smoking depends on many genetic and situational factors.

CYP2A6 plays a major role in the metabolism of nicotine and coumarin, and involveds in the clearance of certain pharmaceuticals and the activation of some tobacco-related nitrosamines (Oscarson *et al.*, 1998; Pelkonen *et al.*, 1999; Raunio *et al.*, 2001). It is important to know whether the different alleles are producing active enzyme able to metabolize drugs and other toxic or carcinogenic chemicals. Daigo *et al.* (2002) found that when giving tegafur (an anticancer drug) to gastric cancer patients with a CYP2A6 poor metabolizer status, the patient could not produce high enough concentrations of the active metabolite to have a beneficial effect of the drug treatment. It is possible that in the future, when physicians prescribe medication to

their patients, they need information on the patient's CYP status. Other studies reported that the frequency of the CYP2A6*4C was significantly lower in the lung cancer patients than healthy volunteers, suggesting that the subjects carrying the CYP2A6*4C alleles are resistant to carcinogenesis caused by N-nitrosamines because of the poor metabolic activation capacity (Islam *et al*., 2013; Kamataki *et al*., 2002; Muroi *et al*., 2012; Tan *et al*., 2001; Wang *et al*., 2013). It could be speculated that individuals who have CYP2A6*4 allele may also be less efficient at bioactivating tobacco smoke procarcinogens to carcinogens.

CONCLUSION

Based on the data collected, it could be concluded that the polymorphism of CYP2A6 were detected in among Javanese population sample study and the allele frequencies of CYP2A6*4 were high. The findings of this study also provide evidence that among these sample study are more likely to be genetically slower nicotine metabolizers. However, to evaluate the real effect of CYP2A6 gene defect on addiction to tobacco smoke, further studies that more precisely address smoking addictive behavior, tobacco consumption and current smoke intake via measurement of valid biomarkers such as CO-hemoglobin, cotinine and 3-hydroxycotinine/cotinine ratio are necessary.

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