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[Original Article]

EFFECT OF *CYP2A6*4* GENETIC POLYMORPHISMS ON SMOKING BEHAVIORS AND NICOTINE DEPENDENCE IN A GENERAL POPULATION OF JAPANESE MEN

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Abstract Objectives : Nicotine in cigarettes is metabolized primarily by CYP2A6-catalyzed oxidation. The *CYP2A6*4* allele, in which *CYP2A6* is a homozygous whole-deletion variant, completely lacks enzyme activity. The aim of this study was to examine the effects of *CYP2A6*4* genetic polymorphism on smoking behavior and nicotine dependence in a general population of Japanese men.

Methods : The subjects were 124 healthy Japanese men who gave informed consent to give saliva samples. The survey items included general information, smoking behaviors and nicotine dependence. The polymerase chain reaction restriction fragment length polymorphism method was used to analyze the genetic polymorphisms of *CYP2A6*. The subjects were classified into two groups : Group W (*CYP2A6*4* absence : **1A/*1A*, **1A/*1B* and **1B/*1B*) and Group D (*CYP2A6*4* presence : **1B/*4A*, **4A/*4A*, **1A/*4A* or **1B/*4D*, and **1A/*4D*). We analyzed the differences in the survey items between the two groups.

Results : There were no significant differences in smoking behaviors between the two groups. However, Group D tended to have less difficulty in refraining from smoking after waking in the morning compared to Group W ($p=0.051$).

Conclusions : *CYP2A6*4* genetic polymorphisms may not strongly affect smoking behavior but may possibly have an effect on nicotine dependence.

Key words : CYP2A6, genetic polymorphism, smoking behavior, nicotine dependence, Japanese adult men

INTRODUCTION

Smoking is known to cause adverse health effects and remarkably increases the risk of major causes of death including cancer, heart disease, and cerebrovascular disease[1)]. However, such causes are believed to be preventable[2,3)]. Cessation of smoking is important to reduce associated risks, but is difficult to achieve. This is because the nicotine contained in cigarettes develops dependence, causing addiction. It is now well recognized that smoking is a disease of nicotine-dependence rather than a personal choice[1)].

Recently, it was reported that genetic factors concerning nicotine metabolism may influence differences in the ease of smoking cessation[4)]. Genetic polymorphisms exist in almost all drug-metabolizing enzymes and cause the individual differences

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in drug metabolism[5]. Furthermore, the genetic polymorphisms of CYP2A6, a member of the cytochrome P450, have received much attention with regard to being a metabolic enzyme that can influence smoking behavior. Nicotine in cigarettes is metabolized primarily by CYP2A6-catalyzed oxidation[6], and several variants of *CYP2A6* genetic polymorphisms are known. The *CYP2A6*4* allele, in which *CYP2A6* is a homozygous whole-deletion variant, completely lacks enzyme activity[7]. That *CYP2A6*4* is associated with smoking behavior in the Japanese population has been reported ; for instance, the number of cigarettes smoked per day by smokers with *CYP2A6*4* was relatively less than those without the allele[8]. In addition, individuals with *CYP2A6*4* were at a low risk of cancer due to smoking[9]. These reports suggest that deletion of *CYP2A6* causes nicotine metabolism inhibition, reduces the number of cigarettes smoked and consequently aids smoking cessation. However, most previous studies were performed in outpatients who visited hospitals due to pulmonary diseases[9,10], which may have caused bias in both the genetic polymorphism of the subjects and the difference in smoking behavior. Furthermore, blood samples were used in these studies, which were invasive to collect. In contrast, saliva is a non-invasive resource for evaluating physiological and pathological conditions in humans, and thus it is suitable for a general population, the subjects of our study. Furthermore, saliva samples provide a similar amount of human DNA as compared to the amount obtained from blood[11].

The smoking rates of Japanese men and women aged ≥20 years in 2012 were 32.7% and 10.4%, respectively[12], and differences in the rate of achievement of smoking cessation and continuation between men and women have been reported to exist[13]. Another study reported that men were more likely to quit and maintain abstinence than women, and the gender of the patient was a primary predictor of success in smoking cessation[14]. Currently, in outpatient smoking-cessation clinics, medical services are provided to patients in accordance with the "Standard Manual for Smoking Cessation Therapy, 5th Edition" in Japan[15]. Because medical insurance covers smoking cessation treatment for outpatients, the number of patients, especially male patients visiting smoking-cessation clinics, is gradually increasing[16]. If smoking cessation education using a genetic polymorphism is established, a more effective measure suitable for each person can be provided especially to male smokers.

In this study, we used saliva samples, as they could be easily collected noninvasively, to analyze the genetic polymorphisms of *CYP2A6* and examined the effect of *CYP2A6*4* genetic polymorphisms on smoking behaviors and nicotine dependence in a general population of Japanese men.

METHODS

Subjects and survey items

In this study, the investigators conducted a face-to-face survey of 2,000 subjects who were randomly selected from the "Basic Resident Registries" of municipalities all over Japan between 2009 and 2010[17]. Of these, 124 healthy adult men, who provided written informed consent to give saliva samples, were selected as the subjects of this study. Individuals with the genetic polymorphisms **1A/*1A*, **1A/*1B* and **1B/*1B* were categorized as Group W (*CYP2A6*4* absence), and those with **1B/*4A*, **4A/*4A*, **1A/*4A* or **1B/*4D*, and **1A/*4D* were categorized as Group D (*CYP2A6*4* presence).

The survey items were aimed at examining smoking behavior and nicotine dependence. Age and current smoking status were asked for general information. As for smoking behavior, smoking history, change in smoking frequency between the present and when the subject started smoking, change in smoking behavior after becoming aware that smoking may cause health problems, and change in smoking behavior after becoming aware that smoking may cause mental problems were asked. Finally, with regard to nicotine dependence, the number of cigarettes smoked per day, whether the subjects can refrain from smoking in a non-smoking area, and whether they have the hardest time to refrain from smoking soon after waking in the morning.

Saliva sampling

The Oragene-DNA Collection Kit (Kyodo International, Inc., Kawasaki, Japan) was used for saliva sampling. The subjects were instructed not to drink, eat (including chewing gum), brush their teeth, gargle, and smoke for 30 min prior to saliva sampling. Saliva was put to the position of the fill line of the container and poured into a tube. Saliva sampling was completed within 30 min. After each tube was inverted for 5 sec to mix the solution, the samples were stored at room temperature (15-30°C) until further use.

Purification of DNA

The stored sample tubes were inverted, mixed for several seconds, and then incubated at 50°C for 2 h. The 500 μL sample solution was transferred into a 1.5 mL tube. Oragene-DNA purification solution (20 μL) was added to the sample solution, and the tube was vortexed and stirred for several sec. The tube was cooled on an ice bath for 10 min, kept at room temperature for several minutes, and was centrifuged at 13,000 rpm for 5 min also at room temperature. The supernatant was transferred to a fresh tube and the precipitate was discarded. Ethanol (500 μL) was added to the supernatant, and the tube was inverted and mixed 10 times. The tube was left at room temperature for 10 min to allow complete DNA precipitation, and was then centrifuged at 13,000 rpm for 2 min at room temperature. The supernatant was removed, and 250 μL of 70% ethanol was gently added to the DNA precipitate. The tube was left for 1 min at room temperature. The DNA was dissolved in 100 μL of Tris-ethylenediaminetetraacetic acid buffer, and the mixture was vortexed and stirred for 5 sec.

Analysis of CYP2A6 genetic polymorphisms in saliva

CYP2A6 in the saliva samples was analyzed by Riken Genesis Co., Ltd. (Yokohama, Japan). The polymerase chain reaction (PCR)-restriction fragment length polymorphism method was used to analyze the genetic polymorphism of *CYP2A6*[18]. The concentration of the sample DNA was measured by using the NanoDrop Spectrophotometer (Thermo Fisher Scientific K.K., Yokohama, Japan). The sequence of the PCR primer was 2Aint7F : 5´-TTTGTGTCAGGAGAATCAAAC-3´ and 2A6R2 : 5´-AAAATGGGCATGAACGCCC-3´. A reaction solution of 20 μL containing Taq DNA polymerase (2.18 U), PCR primer (0.68 μM) and deoxynucleotide triphosphates (1.25 mM) were aliquoted into a 96-well PCR plate. The prepared sample was added to this plate for the subsequent PCR analysis carried out under the following conditions : after 94°C for 3 min, 30 cycles of 94°C for 30 sec, 53°C for 30 sec and 72°C for 2 min were performed, and 72°C was kept for more 5 min. Then 3 μL of the amplified PCR product was identified by electrophoresis on 1% agarose gel. The PCR product (15 μL) was digested with the restriction endonucleases AccII, StuI and Eco81I in a thermal cycler at 37°C overnight and confirmed by electrophoresis on a 3% agarose gel to analyze the genetic polymorphisms of *CYP2A6*.

Statistical analysis

The SPSS Statistics software (version 17.0 ; IBM Japan, Ltd., Tokyo, Japan) was used for the statistical analysis. Chi-Square test or Fisher test was used for all statistical analyses.

Approval of ethical committee

This study was approved by the ethical committee of Fukushima Medical University (No. 1166).

RESULTS

Table 1 shows the distribution of genetic polymorphisms. **1A/*1A*, **1A/*1B* and **1B/*1B* in Group W were 20.2%, 27.4% and 17.0%, respectively. **1B/*4A*, **4A/*4A*, **1A/*4A* or **1B/*4D*, and **1A/*4D* in Group D were 12.1%, 5.6%, 17.7% and 0%, respectively.

Table 2 shows the general information of the subjects in both groups. The number of participants aged ≥60 years was highest in both groups. No significant differences were observed in age and current smoking status between the two groups.

Table 3 shows a comparison of smoking behavior between Groups W and D. There were no significant differences in smoking behaviors between the two groups.

Table 4 shows a comparison of nicotine dependence between Groups W and D. There were no significant differences between the two groups. The Group D subjects, however, had a tendency to have less difficulty with refraining from smoking soon after waking in the morning compared to Group W ($p=0.051$).

Table 1. Distribution of genetic polymorphisms

Genotypes	Number (%)
Group W	
**1A / *1A*	25 (20.2)
**1A / *1B*	34 (27.4)
**1B / *1B*	21 (17.0)
Total	80 (64.5)
Group D	
**1B / *4A*	15 (12.1)
**4A / *4A*	7 (5.6)
**1A / *4A or *1B / *4D*	22 (17.7)
**1A / *4D*	0 (0.0)
Total	44 (35.5)

Table 2. General information of the subjects

All subjects	Group W (N=80) Number (%)	Group D (N=44) Number (%)	p value
Age			0.622
20–39	22 (27.5)	14 (31.8)	
40–59	18 (22.5)	12 (27.3)	
≥60	40 (50.0)	18 (40.9)	
Current smoking status			0.907
Smokers	28 (35.0)	17 (38.6)	
Quitters	30 (37.5)	15 (34.1)	
Non-smokers	22 (27.5)	12 (27.3)	

Table 3. Comparison of smoking behavior between the two groups

Subjects : Smokers and quitters	Group W (N=58) Number (%)	Group D (N=32) Number (%)	p value
Length of the smoking history (Years)			0.173
≤14	13 (22.8)	10 (31.3)	
15–29	16 (28.1)	10 (31.3)	
30–44	19 (33.3)	4 (12.5)	
45≤	9 (15.8)	8 (25.0)	
Unknown	1	0	
Change in smoking frequency between the present and when the subject started smoking			0.380
Increase	23 (39.7)	16 (50.0)	
Same or decrease	35 (60.3)	16 (50.0)	
Change in smoking behavior after becoming aware that smoking may cause health problems			0.631
Yes	18 (31.0)	8 (25.0)	
No	40 (69.0)	24 (75.0)	
Change in smoking behavior after becoming aware that smoking may cause mental problems			1.000
Yes	19 (32.8)	10 (31.3)	
No	39 (67.2)	22 (68.8)	

Table 4. Comparison of nicotine dependence between the two groups

Subjects : Smokers	Group W (N=28) Number (%)	Group D (N=17) Number (%)	p value
Number of cigarettes smoked per day			0.186
≤10	10 (35.7)	9 (56.3)	
11≤	18 (64.3)	7 (43.8)	
Unknown	0	1	
Whether the subjects can refrain from smoking in a non-smoking area			1.000
Yes	5 (17.9)	3 (17.6)	
No	23 (82.1)	14 (82.4)	
Whether the subjects have the hardest time to refrain from smoking soon after waking			0.051
Yes	13 (46.4)	3 (17.6)	
No	15 (53.6)	14 (82.4)	

DISCUSSION

CYP2A6 has recently received attention for the purpose of understanding smoking behavior. The presence or absence of *CYP2A6*4* is suggested to be involved in smoking behavior in the Japanese population[4]. However, most of the subjects in these studies were outpatients who visited hospitals for respiratory diseases. It is important to investigate the distribution of *CYP2A6* genetic polymorphisms and the meaning of these differences in smoking behaviors in the general population. In our study, **1A/*1B* was the most common genetic polymorphism of *CYP2A6*, and the distribution was almost the same as that of a previous report on hospital outpatients in Japan[9,19].

There were no significant differences in smoking behaviors between the two groups in the current study. Ando *et al.* suggested that the *CYP2A6* polymorphism had only limited impact on public health because *CYP2A6* genotypes were correlated with neither the number of cigarettes smoked per day nor the age of smoking onset. In their study, however, all subjects homozygous for the gene deletion had no smoking habits, and after adjustment for sex and age, the homozygous deletion genotype had a tendency to correlate with active smoking status[19]. In our study, Group D subjects tended to have less difficulty with refraining from smoking soon after waking in the morning compared to Group W subjects. Therefore, *CYP2A6*4* genetic polymorphisms may not strongly affect smoking behavior but possibly nicotine dependence. A previous study reported that, for an individual with higher nicotine dependence, smoking soon after waking in the morning is the most satisfying smoke of the day[20]. In addition, another study reported that a smoker's dependence on nicotine can be assessed from the duration of smoking history, the number of cigarettes smoked daily, and how soon the smoker needs to smoke after waking in the morning[21]. These previous reports are in agreement with our results.

There are several limitations in our study. First, because the number of the subjects was not sufficient, significant differences in smoking behaviors and nicotine dependence were not observed between those with and without *CYP2A6*4*. More detailed results can be expected with a larger sample size. Second, we paid attention to only *CYP2A6*4* genetic polymorphisms in this study. *CYP2A6*7* and **9* are also major functional polymorphisms common in Asian populations[22]. The effects of other polymorphisms of *CYP2A6* on smoking habits should be examined. Third, in this study, only male subjects were recruited. According to a past study, in subjects who attempted to quit smoking themselves, the continuance rate of smoking cessation at one year after quitting was 9% in the male subjects and 0% in the female subjects[23]. A survey among adolescent smokers found that nicotinic dependence was higher in female smokers than in male smokers and that the state of depression and the presence of withdrawal symptoms tended to be stronger in female smokers[24]. Thus, several studies indicated that smoking cessation is more difficult in women. It is therefore necessary to investigate the association between genetic polymorphism and smoking behavior in female subjects in the future.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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