

Lack of Association between the Serotonin Transporter (*5-HTT*) and Serotonin Receptor (*5-HT2A*) Gene Polymorphisms with Smoking Behavior among Malaysian Malays

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Abstract

An insertion/deletion polymorphism in the promoter region of the serotonin transporter gene (*5-HTTLPR*) and a polymorphism (rs6313) in the serotonin 2A receptor gene (*5-HT2A*) have previously been linked to smoking behavior. The objective of this study was to determine the possible association of the *5-HTTLPR* and *5-HT2A* gene polymorphisms with smoking behavior within a population of Malaysian male smokers (n=248) and non-smokers (n=248). The *5-HTTLPR* genotypes were determined using the polymerase chain reaction (PCR) and were classified as short (S) alleles or long (L) alleles. The *5HT2A* genotypes were determined using PCR–restriction fragment length polymorphisms (PCR-RFLP). No significant differences in the distribution frequencies of the alleles were found between the smokers and the non-smokers for the *5-HTTLPR* polymorphism ($\chi^2 = 0.72$, $P > 0.05$) or the *5HT2A* polymorphism ($\chi^2 = 0.73$, $P > 0.05$). This is the first study conducted on Malaysian Malay males regarding the association of *5-HTTLPR* and *5HT2A* polymorphisms and smoking behavior. However, the genes were not found to be associated with smoking behavior in our population.

Keywords

5-HTTLPR • 5HT_{2A} • Genetic polymorphism • PCR-RFLP • Malaysian Malays male

Introduction

Nicotine is the main addictive substance in cigarettes and is responsible for the development, as well as the maintenance, of the smoking addiction [1]. Cigarette smoking is a major preventable cause of disease. Genetic variables appear to play key roles in every aspect of nicotine addiction [2]. Therefore, genes involved in the pharmacodynamics and pharmacokinetics of nicotine are logical targets of nicotine addiction.

Serotonin (5-hydroxytryptamine, 5-HT) is a neurotransmitter derived from the amino acid tryptophan, and it has a wide range of central nervous system activities [3]. Polymorphisms affecting the serotonergic system, such as those in the serotonin transporter (5-HTT) and 5-HT genes, have been linked to smoking-related behaviors because nicotine increases serotonin release [4]. Therefore, variations in the serotonergic system may influence some aspects of smoking, such as mood variations during nicotine withdrawal [5]. One study showed that serotonin release was increased in the cortical region of the brain in rats treated with nicotine and that nicotine withdrawal seemed to be related to the subsequent decrease in serotonin [4].

5-HTT controls the duration and concentration of serotonin neurotransmission in the synaptic cleft [6]. 5-HTT is encoded by a single gene (SLC6A4) that is located on the long arm of chromosome 17 (17q12) [7]. The 5-HTT gene has been linked to psychological traits, such as anxiety-related personality traits [8] and depression [9] and these traits are also related to nicotine dependence [10, 11]. A 44 bp insertion or deletion polymorphism located in the serotonin transporter-linked polymorphic region (5-HTTLPR) was identified within this gene, resulting in two allelic variants, the long (L) and short (S) alleles, which alter the transcriptional efficiency of the 5-HTT gene [12]. The S allele has been associated with reduced serotonin uptake, leading to the hypothesis that individuals with the S allele are not prone to smoking [8]. This hypothesis is supported by two studies in Japanese and Chinese populations that found that individuals with the homozygous S genotype were less likely to initiate smoking behavior and could more easily stop smoking than others [13, 14]. However, the hypothesized role of the S allele in smoking behavior has not yet been confirmed; other studies that attempted to replicate these findings have obtained contradictory results [15–18], indicating that the influence of these genes may vary from one ethnicity to another.

The 5-HT receptor has been classified into seven groups (5-HT₁₋₇); one of them, subgroup 5-HT₂, can be further divided into three subclasses (2A, 2B, and 2C). The 5-HT_{2A} gene is located on chromosome 13q14–q21 [19]. The 5HT_{2A} gene has been associated with emotional disorders and alcohol dependence, both of which are related to smoking behavior [20]. One of the polymorphisms that have been described in this receptor is T102C, which involves the substitution of a cytosine residue with a thymine residue [19]. This polymorphism has been associated with the continuation of smoking behavior, but is less likely to be involved in the initiation of smoking behavior [21]. A study conducted in a Brazilian population found that individuals with the CC genotype are more likely to be current smokers than individuals with other genotypes. Interestingly, the

opposite finding was obtained in Caucasian Australians; in this study, the occurrence of the TT genotype was associated with current smokers [22]. However, this finding has not been replicated in several other studies in different populations [23–25], indicating that the influence of these genes may vary from one ethnicity to another.

The present study investigated the possible associations between the 5-HTTLPR and 5-HT2A polymorphisms and the smoking behavior of Malaysian Malay males, which has not yet been investigated. To the best of our knowledge, this is the first study conducted on a Malaysian Malay male population regarding the association of 5-HTTLPR and 5-HT2A polymorphisms and smoking behavior. If such an association is established, it may explain why different individuals have different risks for nicotine dependence and why some individuals are able to quit smoking more easily, suggesting that specific interventional approaches should be used for certain individuals based on their genetic background.

Results and Discussion

The average number of cigarettes per day of current smokers is less than 11. The genotype and allele frequencies of the 5-HTTLPR and 5-HT2A polymorphisms in smokers and non-smokers are given in Table 1. The frequencies of the 5-HTT alleles S/S, L/L, and heterozygous S/L in the non-smoker population were 39.1%, 11.3%, and 49.6%, respectively, whereas in the smokers, the frequencies of the S/S, L/L, and heterozygous S/L alleles were 41.1%, 12.9%, and 46.0%, respectively. The genotype frequencies for the 5-HT2A polymorphism in Malay smokers were 10.1% for CC, 46.8% for TT, and 43.1% for CT, whereas in the Malay non-smokers, the frequencies were 8.1% for CC, 46.4% for TT, and 45.6% for CT. No significant differences in the genotype frequencies of the 5-HTTLPR polymorphism ($\chi^2 = 0.73$, $P > 0.05$) and the 5-HT2A polymorphism ($\chi^2 = 0.72$, $P > 0.05$) were observed between the smoking group and the non-smoking group. The samples demonstrated Hardy–Weinberg equilibrium. Population stratification was not likely to be a confounder in this study because the samples were carefully limited to individuals with at least three generations of Malay descent.

Several studies previously examined the role of 5-HTT in smokers, but the results have been contradictory. We established that the 5-HTTLPR polymorphism was not associated with smoking behavior in the Malay population in Malaysia. This finding is consistent with five previous studies in Dutch [17], American [27], Austrian [15], Polish [16], and Greek [18] populations. Another study involving a Thai population also found no significant association between 5-HTTLPR polymorphisms and smoking behavior. However, individuals who carried the L allele were found to have higher-intensity smoking behaviors [25].

An association between 5-HTLLPR and smoking behavior was found in Japanese and Chinese populations. These studies suggested that the L allele was more likely to be carried by smokers than non-smokers, and smokers with homozygous S alleles were able to quit smoking more easily [13, 14]. Additionally, Lerman et al. [29] and Gerra et al. [31] reported that individuals with either homozygous or heterozygous S alleles were more likely to be dependent on nicotine than individuals with homozygous L alleles. The association of the 5-HTTLPR polymorphism with altered transcriptional activity might explain why different individuals exhibit different risks for nicotine dependence and why some individuals are able to quit more easily.

Tab. 1. Genotype and allele frequencies of the 5-HTTLPR and 5-HT2A with smoking behavior in the Malay Male population

Genotypes	Groups (n=496)		Statistics ^a	
	Smokers (n=248)	Non-smokers (n=248)	χ^2	P-value
5-HTTLPR				
SS (%)	102 (41.1)	97 (39.1)	0.734	0.693
LL (%)	32 (12.9)	28 (11.3)		
SL (%)	114 (46.0)	123 (49.6)		
Alleles				
S (%)	318 (64.1)	317 (63.9)		
L (%)	178 (35.9)	179 (36.0)		
5-HT2A				
CC (%)	25 (10.1)	20 (8.1)	0.724	0.696
TT (%)	116 (46.8)	115 (46.4)		
CT (%)	107 (43.1)	113 (45.6)		
Alleles				
C (%)	157 (31.7)	153 (30.8)		
T (%)	339 (68.3)	343 (69.2)		

^a Chi-square likelihood ratio test was used to compare the genotype frequencies.

Similar to the findings for 5-HTTLPR, our findings for the 5-HT2A polymorphism were consistent with several previous studies that found no association between the polymorphisms in the 5HT gene and smoking behavior [23–25]. A study on a Brazilian population found that the 5-HT2A polymorphism was related to smoking behavior; in particular, the CC genotype was more frequently found in current smokers than in former smokers or individuals who had never smoked [21]. However, this finding contradicts a study of Caucasian Australians that found an association between the TT genotype and current cigarette smoking status [22].

Tables 2 and 3 show the different studies that have investigated the association of the serotonin transporter and receptor with smoking behavior. Ethnic differences may contribute to the varied findings for the associations of the 5-HTTLPR and 5-HT2A polymorphisms with smoking behavior, as the distributions of certain alleles largely vary within different races. The L allele of 5-HTTLPR is more common among Polish populations [18], whereas the frequency of the L allele is lower in the Japanese population than in European, American, or African American populations [28]. By contrast, in Malay and Thai populations, the S allele is more common than the L allele. The T allele of the 5-HT2A gene is common in Brazilian and Asian populations, including the Malay, Thai, and Japanese populations. However, Caucasians and South Asians living in the United Kingdom have a higher C allele frequency [24]. This finding may be due to intermarriages between citizens of the United Kingdom that contribute to the inheritance of the C allele in South Asian descendants.

Tab. 2. Association studies of 5-HTTLPR in different ethnic groups with smoking behavior

Region	Sample size	Association with smoking behavior	Reference
Malaysia	248 smokers and 248 non-smokers	No	Current study
USA	268 smokers and 230 non-smokers	No	[27]
Japan	202 smokers, 82 non-smokers, and 103 ex-smokers	Long allele	[13]
USA	185 smokers	Short allele	[29]
Israel	190 smokers and 54 ex-smokers	Long allele	[30]
Italy	107 smokers and 103 non-smokers	Short allele	[31]
Austria	470 smokers and 419 non-smokers	No	[15]
Poland	149 smokers and 158 non-smokers	No	[16]
Denmark	327 smokers, 642 non-smokers, and 396 ex-smokers	No	[17]
China	144 smokers and 135 non-smokers	Long allele	[14]
Sweden	80 smokers and 120 non-smokers	Long allele	[32]
Greece	172 smokers and 254 non-smokers	No	[18]
Thailand	200 smokers and 111 non-smokers	No	[25]
USA	177 smokers, 458 non-smokers, and 124 ex-smokers	Short allele	[33]

Tab. 3. Association studies of 5-HT2A in different ethnic groups with smoking behavior

Region	Sample size	Association with smoking behavior	Reference
Malaysia	248 smokers and 248 non-smokers	No	Current study
Japan	82 smokers and 125 non-smokers	No	[23]
Caucasian	47 smokers and 85 non-smokers	Yes	[22]
Australia	200 smokers and 111 non-smokers	No	[25]
Brazil	193 smokers and 432 non-smokers	Yes	[21]

Additionally, the discrepancies observed in previous studies on the results of the 5-HTTLPR and 5-HT2A polymorphisms may be attributed to gene-gene interactions. These two genes may be linked to other genes that determine smoking behavior. However, the degree of linkage between 5-HTTLPR or 5-HT2A and the putative genes may again vary from ethnicity to ethnicity. This difference may explain why polymorphisms in 5-HTTLPR and 5-HT2A have no association with smoking behavior in the Malaysian population but may be associated in other populations.

Variations in subject recruitment methods may also explain discrepant results between studies. Intermarried ancestors may become confounding factors in these studies; therefore, subjects should be restricted to a single race. Additionally, sample sizes also vary between studies. The study design should clearly define which individuals are

smokers. Some studies may include ex-smokers as smokers [21], whereas other studies may define ex-smokers as non-smokers [16], leading to further variations. However, the correlation between genetics and smoking behavior and the smoking cessation success rate may affect the overall outcome of the correlation.

Our study is subjected to some limitations. First, our study did not focus on the age groups where all subjects between 18 and 50 years old were included which may lead to some variability. In addition, the average age for the non-smokers was in the 20s while that for the smokers was in the 30s. A possible reason for this discrepancy could be attributed to the fact that most non-smokers were recruited from institutes of higher education (Kota Bharu Institute of Teacher Education and Universiti Sains Malaysia). On the other hand, the majority of smokers were recruited from various locations in Kota Bharu. This supports the findings that community and school level variables are predictive of adolescent smoking [34]. Secondly, we did not consider the effects of negative environment and social conditions of the subjects such as stressful life events and schooling level which may also interfere with the prevalence of the 5-HT polymorphism.

The study of the association between genetics and smoking behavior is complex and involves many known and unknown genes, as well as clinical and environmental factors, which may interact with each other. Any finding of an association between gene polymorphisms and smoking behavior may only be true for the population studied. Future research should investigate other possible gene polymorphisms using a larger sample size across cultural and ethnic groups to identify the true universal contributors that influence smoking behavior.

Experimental

Subjects

A total of 496 Malaysian Malay male subjects, both smokers (n=248) and non-smokers (n=248), between 18 and 50 years old were selected. Smoking was defined as having smoked more than 100 cigarettes in a lifetime and being a current smoker at the time of the study. The subjects were confirmed to be of the Malay ethnic group for at least three generations. Subjects having a serious disorder or a previous history of a mental disorder were excluded from this study. Only males were recruited to avoid inter-individual variations that may arise due to sex differences and hormonal changes commonly shown by females. The demographic data of the subjects are shown in Table 4. All participants gave written informed consent before they were enrolled, and the study was approved by the Ethics Committee (ethical no: USMKK/PPP/JEPeM[233.3.(05)]/Amend.01) of the School of Medical Sciences, Universiti Sains Malaysia, which complies with the Declaration of Helsinki.

DNA Extraction

Three milliliters of venous blood were drawn into a sterile tube containing EDTA and stored at -20°C until the isolation of genomic DNA. Genomic DNA was isolated from the blood using the QIAamp DNA Blood Mini Kit (Qiagen, USA). DNA purity and concentration were determined by measuring the absorbance at 280 nm using a Biophotometer Uvette spectrophotometer (Eppendorf, Germany).

Tab. 4. Demographic data subjects

	Smokers	Non-smokers	<i>p</i> value
	(n=248)	(n=248)	
	Mean (SD)	Mean (SD)	
Age (years) ^a	33.50 (19.0) ^c	24.00 (16.0) ^c	0.000 ^a
Height (m) ^b	168.5 (0.06)	168.64 (0.06)	0.307 ^b
Weight (kg) ^b	69.24 (12.45)	69.58 (12.32)	0.760 ^b
Body mass index (kg/m ²) ^b	24.50 (4.05)	24.47 (4.16)	0.945 ^b
Brachial systolic blood pressure (mm/Hg) ^b	126.89 (11.81)	130.27 (13.69)	0.001 ^b
Brachial diastolic blood pressure (mm/Hg) ^b	75.42 (9.91)	75.91 (9.45)	0.578 ^b

^a Mann Whitney test; ^b Independent-samples T-test; ^c Median (IQR).

Molecular Analysis

Genotyping of the 5-HTTLPR gene polymorphism was performed by using the polymerase chain reaction (PCR) as previously described by Heils et al. [12] with some slight modifications. The PCR reaction was performed in a final volume of 25 µl containing 60 ng of genomic DNA, 200 µM dNTP mix, 0.24 µM of each primer (forward 5'-GGCGTTGCCGCTCTGAATGC-3' and reverse 5'-GAGGGACTGAGCTGGACAACCAC-3'), 0.75 mM magnesium chloride (MgCl₂), 1x ammonium sulfate buffer [(NH₄)₂SO₄] (Fermentas, Vilnius, Lithuania), and 1.25 U Taq polymerase (Fermentas, Vilnius, Lithuania). After an initial incubation at 95°C for 2 min, the PCR products were amplified for 35 cycles of denaturation at 95°C for 30 sec, annealing at 62°C for 30 sec, and extension at 72°C for 1 min. The final extension step lasted 7 min at 72°C. The PCR products were then resolved using a 2% agarose gel and were visualized under UV light. Each gel contained one lane of a 100 bp ladder to identify the 528 bp fragment, designated as the L allele, and the 484 bp fragment, designated as the S allele (Fig. 1).

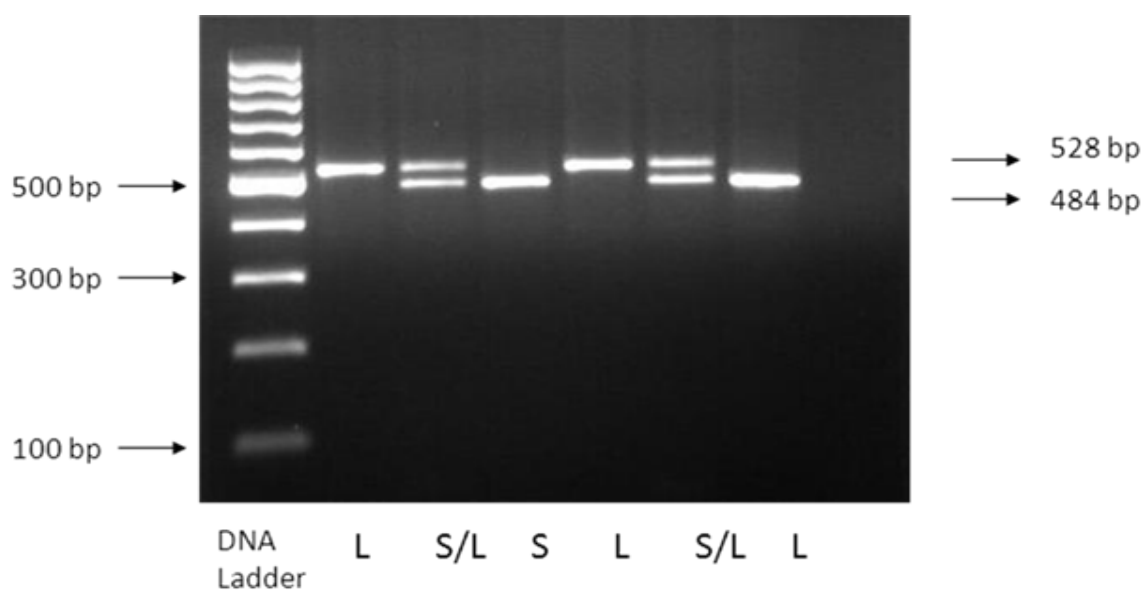


Fig. 1. PCR products for amplification using 5-HTTLPR primers. Lane 1 and 4 show homozygous long allele (L), lane 2 and 5 show heterozygous short-long allele (S/L), while lane 3 and 6 show homozygous short allele (S)

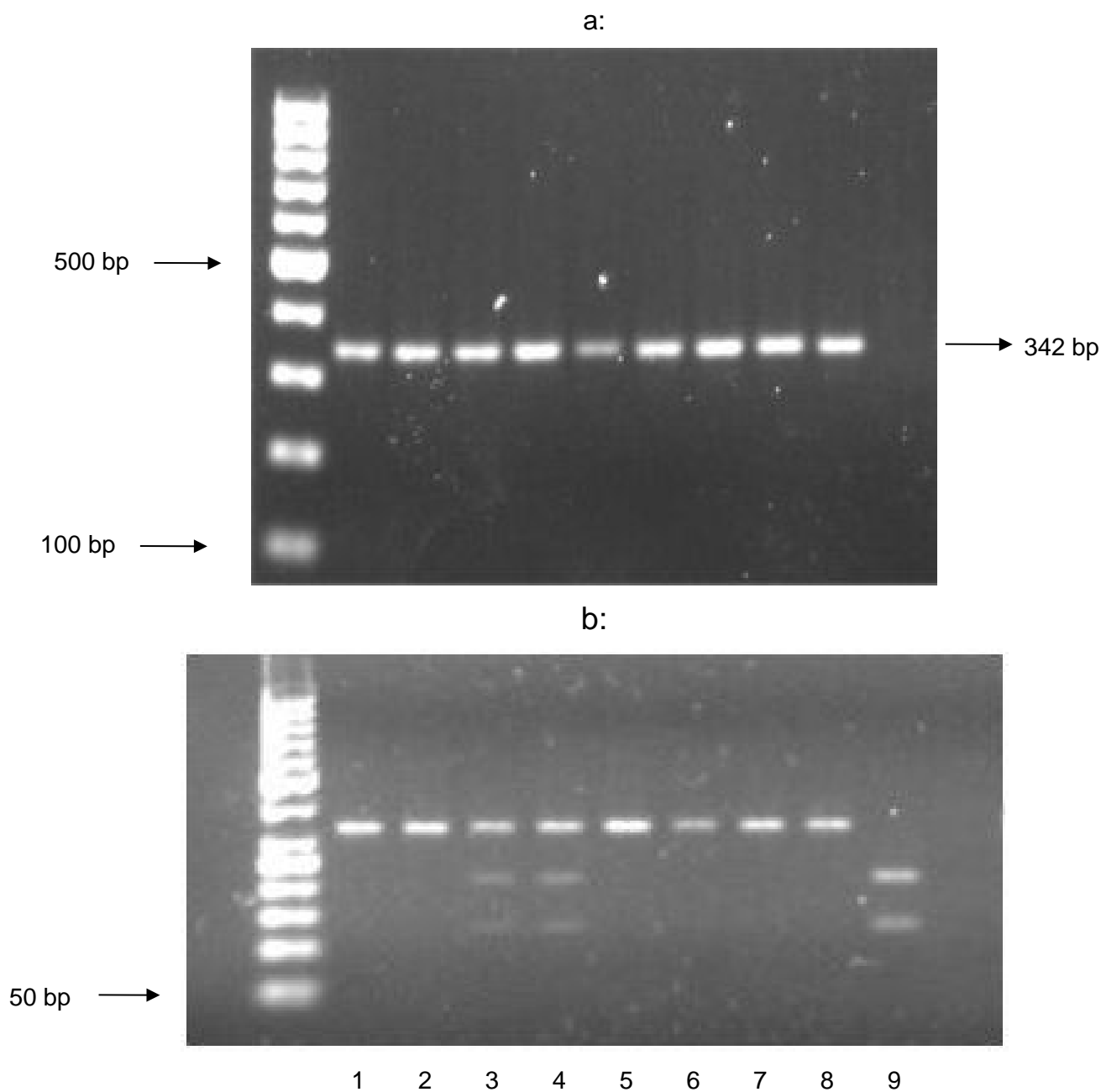


Fig. 2. a: PCR products for amplification using 5-HT_{2A} primers, b: PCR-RFLP result after digestion with *Hpa* II restriction enzyme. Lane 1, 2, 5, 6, 7, 8 show mutant allele with 342 bp. Lane 9 shows wild type allele with 126 and 216 bp fragments and Lane 3 and 4 show wild type-mutant alleles

Genotyping of the 5-HT_{2A} polymorphism (*rs6313*) was performed as previously described [26], with several modifications. PCR was performed in a 25 μ l reaction containing 78 ng of genomic DNA, 100 μ M of each dNTP, 1.5 mM MgCl₂, 0.25 μ M of each primer (forward 5'-TGTGCTACAAGTTCTGGCTT-3' and reverse 5'-GTGCAGTTTTTCTCTAGGG-3'), and 0.75 U DNA Taq polymerase (Fermentas, Vilnius, Lithuania). After an initial denaturation at 95°C for 3 min, the PCR products were amplified for 35 cycles of 1 min at 94°C, 45 s at

53°C, and 1 min at 72°C, followed by a final extension step of 10 min at 72°C. The 342 bp PCR product was digested with Hpa II (New England Biolabs, MA, USA) for 3 h at 37°C. The digestion products were then resolved on a 2% agarose gel containing ethidium bromide and were visualized under UV light. The 102T mutant remained uncut (342 bp), whereas the wild type 102C allele was digested into two bands of 216 and 126 bp; three fragments of 342, 216, and 126 bp were observed for samples containing both the wild type and mutant alleles (Fig. 2).

Statistical Analysis

The allele and genotype frequencies were calculated to equilibrium using the Hardy–Weinberg equation ($p^2 + 2pq + q^2 = 1$). The significance of the allele frequency or the genotype distribution among the volunteers with different smoking habits was examined using a non-parametric chi-square test. A $P < 0.05$ was considered statistically significant. All statistical analyses were performed using the SPSS package version 20.0 (IBM, Armonk, NY).

Conclusion

The 5-HTTLPR and 5-HT2A gene polymorphisms were not found to be associated with smoking behavior in Malaysian Malay males. The study of the association between genetics and smoking behavior is complex and is dependent on many factors, making the association unique to each population.

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Authors' Statements

Competing Interests

The authors declare no conflict of interest.

Informed Consent, Ethical Approvals

The Institutional and National Ethical Guides for Experiments on Human Subjects were followed and informed consent was obtained. Refer to 'Experimental' for details.

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