α-4 subunit of nicotinic acetylcholine receptor polymorphisms exhibit no association with smoking behavior among Malay Males in Kelantan, Malaysia

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Abstract Background: Smoking behavior is influenced by both genetic and environmental factors. Nicotine is the major addictive substance in cigarettes. Nicotinic acetylcholine receptors (nAChRs) are thought to play an important role in nicotine addiction of smokers. One of the genes, α-4 subunit of nicotinic acetylcholine receptor (CHRNA4) gene was reported to be associated with smoking behavior in many populations.

Aim: The aim of this study is to determine association between α-4 subunit of nicotinic acetylcholine receptor single nucleotide polymorphism (rs2236196 and rs2273502 loci) and smoking behavior among Malay Males.

Methods: The study was conducted in Malay smokers (n = 248) and non-smoking controls (n = 248). DNA was extracted from leucocytes and the two SNPs were determined by a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. The PCR product was digested with restriction enzymes AfeI and Sau96I, respectively.

Results and conclusion: We found that the AA genotype frequency for CHRNA4 rs2236196 polymorphism in the smoker group was 80.6% while in nonsmoker 77.0%. No mutation (GG genotype) was detected in both groups. The AG genotype for the smoker group was 19.4% while in the nonsmoker group 23.0%. There was no significant difference observed in the genotype (χ² = 5.106, p = 0.078) and allele frequencies between both study groups. On the other hand, no mutation of CHRNA4 rs2273502 (TT genotype) was detected in the non-smoker group while the frequencies of genotype CC and heterozygous CT in non-smokers were 75.8% and 24.2%, respectively. In the smoker group, the frequencies were 73.4%, 2.0% and 24.6%, for TT, CC and CT, respectively. There was no significant difference observed in rs2273502 (χ² = 5.16, p = 0.078) and smoking behavior.
1. Introduction

Smoking behavior is influenced by both genetic and environmental factors. Previous studies have shown that genetic factors strongly influence smoking behavior and nicotine addiction [1].

According to Arinami et al. [2] the genetic predisposition to smoking is determined by multiple genes. A variety of genes have been investigated for their association with smoking behavior, including loci involving dopamine receptors and transporters [3–6], serotonin receptors and transporters [7–10], nicotinic acetylcholine receptors (nAChRs) [11–13], tyrosine hydroxylase [14], tryptophan hydroxylase [15], monoamine oxidase [16], catechol-O-methyltransferase [17], adrenergic receptors [18], μ opioid receptors [19] and cannabinoid receptors [20]. Among all, nicotinic acetylcholine receptor (nAChRs) genes were selected as our main interest of investigation.

Nicotine is the major addictive substance in cigarettes. Nicotine exerts its pharmacological and physiological effects by binding to nAChRs [11]. Molecular analyses have identified the expression of nine α-subunits (α2 – α10) of nAChRs in the central nervous system [21]. According to Tapper et al. [22] α-4 subunit of nicotinic acetylcholine receptor (CHRNA4) is the most important for nicotine-induced reward, tolerance, as well as sensitization. The CHRNA4 gene, which was mapped to chromosome 20q13.2–13.3 is 17 kb long and contains six exons [23]. Therefore, the gene encoding this subunit can be considered as a good candidate gene for smoking behavior.

The rs2273502 locus is sited in the 3′-downstream region of intron 2 of the CHRNA4 gene, close to the transcription initiation site of exon 3. Li and colleague [12] reported association of CHRNA4 rs2273502 with nicotine dependence. This can be an important functional regulatory element for the expression of exon 3 of the CHRNA4 gene. The CHRNA4 rs2236196 gene was also reported to be associated with nicotine dependence or smoking behavior in many populations [12,13]. The gene was characterized by an A → G transition located in the 3′-untranslated region of CHRNA4 [11].

However, there has been no prior study reported for these two genes in Malay Males, so far. Considering the evidence for ethnic differences in nicotine metabolism in response to smoking and in the genetic influences on nicotine dependence [24–27], we were interested to investigate the association of CHRNA4 (rs2236196 and rs2273502) genes with smoking behavior among Malay male smokers in Kelantan, Malaysia. Therefore, the objective of this study was to determine the frequency of single nucleotide polymorphisms in the CHRNA4 (rs2236196 and rs2273502) genes among Malay male smokers in Kelantan, Malaysia.

2. Methods and materials

2.1. Subjects and methods

The study protocol was approved by the Ethics Committee of School of Medical Sciences, Universiti Sains Malaysia, Malaysia. The work has been carried out in accordance with The Code of Ethics of The World Medical Association (Declaration of Helsinki) for experiments in human. Most of the subjects were recruited from two institutions of higher education which were IPG Pengkalan Chepa, Kelantan, Malaysia (College for teacher education) and Universiti Sains Malaysia, Health Campus, Kelantan, Malaysia and some from various locations in Kota Bharu, Kelantan, Malaysia. Subjects were informed about the experimental procedures and study aim before written informed consents were obtained. Blood samples were obtained from 496 Malay Males, aged 18–50 years old who agreed to participate in the study. The subjects were categorized as smokers (n = 248) and non-smokers (n = 248). Ex-smokers or those who had stopped smoking prior to the study were excluded. Other exclusion criteria included a history of cancer, coronary heart disease, liver disease and undergoing treatment for drug addiction.

To be included in the study, smokers were defined as having smoked more than 100 cigarettes in a lifetime and being a current smoker at the time of the study. Non-smokers were defined as volunteers who never smoked cigarettes. Smoking behavior was assessed with a questionnaire concerning tobacco use. The questions included age of smoking initiation, number of cigarettes smoked daily, factors influencing smoking and number of quit attempts. The subjects in the smoker group were then classified based on the scores that they get from the validated Malay version of Fagerstrom Test for Nicotine Dependence (FTND-M) [28]. The six-questions of FTND-M are the valid self-reporting measure of nicotine dependence and can assist physicians in determining appropriate cessation treatment. All subjects were scored into five levels of categorizations which were very low nicotine dependence (0–2), low nicotine dependence (3–4), moderate nicotine dependence (5), high nicotine dependence (6–7) and very high nicotine dependence (8–10).

2.2. DNA extractions

Three milliliters of venous blood was drawn into a sterile tube containing EDTA and stored at −20 °C until the isolation of genomic DNA. Genomic DNA was isolated from 300 μl of blood using QIAamp DNA Blood Mini Kit (QIAGEN, Hilden). The purity of the DNA and their concentrations were measured by spectrophotometry, Biophotometer Uvette (Eppendorf, Germany) at 280 nm absorbance.
2.3. Genotyping of CHRNA4 rs2236196 gene using PCR-RFLP

For the analysis of CHRNA4 gene rs2236196 polymorphism, genomic DNA was PCR-amplified in a final volume of 25 μl containing 2.5 μl of 10× PCR buffer with KCl (Vivantis, Malaysia), 0.2 μM/L of each primer as listed in Table 1, 0.2 μmol/L of dNTP (Vivantis, Malaysia), 1.5 mmol/L MgCl2 and 2.5U Taq DNA polymerase (Vivantis, Malaysia). The following PCR conditions were used: an initial denaturation step for 3 min at 95 °C, followed by 25 cycles of 1 min at 95 °C, 30 s at 55 °C and 1 min at 72 °C with a 10 min final extension at 72 °C. Fig. 1 shows schematic representations of RFLP analysis of CHRNA4 rs2236196. A 326 bp fragment was yielded by running on a 1% agarose gel (Fig. 2a) in the presence of ethidium bromide. The PCR product was digested with 1U restriction enzyme AfeI (Vivantis, Malaysia) for 2 h at 37 °C. Digestion of the PCR product with AfeI resulted in 326 bp fragments for homozygous wild type (wt) sequence and 3 fragments of 326, 200 and 124 bp for heterozygous wild type – mutant (wt/mt) allele (Fig. 2b).

2.4. Genotyping of CHRNA4 rs2273502 gene using PCR-RFLP

For the analysis of CHRNA4 gene rs2273502 polymorphism, genomic DNA was PCR-amplified in a final volume of 25 μl containing 2.5 μl of 10× PCR buffer with KCl (Vivantis, Malaysia), 0.2 μM/L of each primer as listed in Table 1, 0.2 μmol/L of dNTP (Vivantis, Malaysia), 1.5 mmol/L MgCl2 and 0.5U Taq DNA polymerase. The PCR reaction was carried out in a total volume of 25 μl consisting of 2.5 μl of 10× PCR Buffer with KCl (Fermentas), 0.4 μM of each primer (Table 1), 500 μmol/L dNTP mixture, 3.75 mM MgCl2 and 0.5U Taq DNA polymerase. PCR amplification was performed with an initial denaturation at 94 °C for 5 min and then 35 cycles of 94 °C for 30 s, 59 °C for 30 s and 72 °C for 1 min, and then held at 72 °C for

### Table 1

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
<th>Size of PCR product (bp)</th>
<th>Endo-nuclease</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs2236196</td>
<td>Forward 5'-AGCTCCACAAAACTCTGTTC-3'</td>
<td>326</td>
<td>AfeI</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-AGTCTTCAGCAGATCTGTG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs2273502</td>
<td>Forward 5'-TCACCTGGTTACCCATAGC-3'</td>
<td>237</td>
<td>Sau96I</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-TCCCTCAGATCCCCATCTCC-3'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 1  Schematic representation of RFLP analysis of CHRNA4 rs2236196 gene. In the mutant type, AfeI cleaves a 326 bp fragment into 126 and 200 bp whereas the wild type is not cleaved.

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10 min. The amplified PCR products sized 237 bp were observed (Fig. 3a). One unit of restriction enzyme, Sau96I (New England Biolabs, MA, USA) was used to digest the 5 µl PCR product for 1 h at 37 °C. The digestion products were then resolved on a 1.4% agarose gel containing EtBr and were visualized under UV light. Samples with homozygous wild-type sequences show two bands of 72 and 136 bp. The homozygous mutant sequence was digested into 72 and 165 bp while heterozygous wild type mutant gave 72, 136 and 165 bp (Fig. 3b).

2.5. Direct DNA sequencing

Upon successful PCR, three samples from each different genotype were chosen at random and sent for sequencing. PCR products were purified using MEGAquick PCR purification kit (Intron, Kyeonggi-do, Korea) and were sent for DNA sequencing. The sequencing processes were performed by using Applied Biosystem 3730 XL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The sequencing results were verified against the published sequence for CHRNA4 (rs2236196 and rs2273502). (GenBank accession number: NT_011333).

2.6. Statistical analysis

Sample size was calculated using Power and Sample Size Calculation Software (PS Software ver.2.1.31), where alpha (α) was set at 0.05 with 80% power. All statistics in this study were performed using the SPSS package (ver. 20, SPSS, Chicago, IL). All values for demographic parameters were expressed as mean ± SD. The ages of the subjects were compared using Mann–Whitney tests. Independent-samples T-test was used to compare the differences in height, weight and BMI of the subjects.

Data were compiled according to the genotypes and allele frequencies. The 95% confidence intervals were calculated for all observed allele frequencies. The allele and genotype frequencies were calculated to equilibrium using a Hardy–Weinberg equation (p² + 2pq + q² = 1). We used non-parametric chi-square test to assess the significance of allele frequency and genotype distribution with smoking behavior among the subjects. A p < 0.05 was considered statistically significant.

3. Results and discussion

In all reactions, correct lengths of expected PCR products were obtained. The restriction enzyme used in the present study was found have worked successfully as depicted in Figs. 2b and 3b. The presence of mutations in CHRNA4 (rs2236196 and rs2273502) gene polymorphisms was confirmed by DNA sequencing. The electropherogram sequence of wild-type and polymorphic mutant of CHRNA4 rs2273502 is shown in Fig. 4. A homozygous C to T transition was observed in the sense strand of the polymorphic variant.

From our findings, there were significant differences in mean age between smokers and non-smokers (Table 2). No significant differences were found in height, weight and BMI between smoker and non-smoker groups.

The genotypes and allelic frequencies of the polymorphism CHRNA4 rs2236196 in nonsmoker and smoker groups are shown in Table 3. The allele frequencies and genotype distribution of this gene in each group satisfied the Hardy–Weinberg equilibrium law. From the results, the AA genotype frequency
in the smoker group was 80.6% while in nonsmokers 77.0%. No mutation (GG genotype) was detected in both groups. The AG genotype for the smoker group was 19.4% while in the nonsmoker group was 23.0%. No significant differences were observed in the genotype ($\chi^2 = 5.106, p = 0.078$) and allele frequencies between both study groups.

The genotypes and allelic frequencies of the polymorphism CHRNA4 rs2273502 in nonsmoker and smoker groups are shown in Table 4. The allele frequencies and genotype distribution of this gene in each group satisfied the Hardy–Weinberg equilibrium law. No significant differences were observed in the genotype ($\chi^2 = 5.16, p = 0.078$) and smoking status of the subjects.

No association was observed between the FTND-M score and the genotypes of the CHRNA4 rs2236196 (Table 5) and CHRNA4 rs2273502 (Table 6).

The SNPs of rs2273502 and rs2236196 were chosen in this study due to the significant association with smoking behavior that has been reported by other studies. However, the results indicated that the genotypes and allelic frequencies of CHRNA4 (rs2236196 and rs2273502) gene polymorphisms were not significantly associated with smoking behavior.

Table 2: Demographic characterization of subjects.

<table>
<thead>
<tr>
<th></th>
<th>Non-smokers ($n = 248$)</th>
<th>Smokers ($n = 248$)</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)a</td>
<td>24.0 (16.0)c</td>
<td>33.5 (19.0)c</td>
<td>0.000</td>
</tr>
<tr>
<td>Height (m)b</td>
<td>168.6 (0.06)</td>
<td>168.0 (0.06)</td>
<td>0.307</td>
</tr>
<tr>
<td>Weight (kg)b</td>
<td>69.58 (12.32)</td>
<td>69.24 (12.45)</td>
<td>0.760</td>
</tr>
<tr>
<td>Body mass index (kg/m²)b</td>
<td>24.47 (4.16)</td>
<td>24.50 (4.05)</td>
<td>0.945</td>
</tr>
</tbody>
</table>

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

Table 3: Genotypic and allelic distribution and statistical analyses of nAchR rs 2236196 polymorphisms in smoker and nonsmoker groups.

<table>
<thead>
<tr>
<th></th>
<th>Nonsmoker n (%)</th>
<th>Smoker n (%)</th>
<th>$\chi^2$ (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>191 (77.7)</td>
<td>200 (80.6)</td>
<td>0.979 (0.323)</td>
</tr>
<tr>
<td>GG</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td></td>
</tr>
<tr>
<td>AG</td>
<td>57 (23.0)</td>
<td>48 (19.4)</td>
<td></td>
</tr>
<tr>
<td>Allele</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>439 (88.5)</td>
<td>448 (90.32)</td>
<td>0.863 (0.353)</td>
</tr>
<tr>
<td>G</td>
<td>57 (11.5)</td>
<td>48 (9.7)</td>
<td></td>
</tr>
</tbody>
</table>

Table 4: Genotypic and allelic distribution and statistical analyses of nAchR rs2273502 polymorphisms in smoker and nonsmoker groups.

<table>
<thead>
<tr>
<th></th>
<th>Nonsmoker n (%)</th>
<th>Smoker n (%)</th>
<th>$\chi^2$ (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>182 (73.4)</td>
<td>188 (75.8)</td>
<td>5.106 (0.078)</td>
</tr>
<tr>
<td>TT</td>
<td>5 (2.0)</td>
<td>0 (0.0)</td>
<td></td>
</tr>
<tr>
<td>CT</td>
<td>61 (24.6)</td>
<td>60 (24.2)</td>
<td></td>
</tr>
<tr>
<td>Allele</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>425 (85.7)</td>
<td>436 (87.9)</td>
<td>1.064 (0.302)</td>
</tr>
<tr>
<td>T</td>
<td>71 (14.3)</td>
<td>60 (12.1)</td>
<td></td>
</tr>
</tbody>
</table>
Our results contrast with a number of other studies that have successfully shown relationships between CHRNA4 variants and smoking-related variables. Feng et al., [11] were the first to report significant associations of single CHRNA4 SNPs and also of a multilocus CHRNA4 haplotype, with susceptibility to nicotine addiction in Chinese men. They studied six single-nucleotide polymorphisms (SNPs) residing in the CHRNA4 gene (rs1044396, rs1044397, rs2273504, rs2273502, rs827020 and rs2236196) with respect to nicotine dependence. They recruited 621 male subjects with multiple nicotine-addicted siblings. They used FTND [29] to identify families affected by nicotine addiction. Their analyses for CHRNA4 demonstrated a significant association of the haplotypes formed with both nicotine addiction ($\chi^2 = 19.19$, $p < 0.001$) and the age-adjusted FTND score ($\chi^2 = 19.19$, $p < 0.001$).

Another association of single nucleotide polymorphisms (SNPs) in CHRNA4 gene is observed in European-American or African-American population [12]. The study tested six SNPs in the CHRNA4 gene for association with nicotine dependence. Nicotine dependence was determined using smoking quantity, the heaviness of smoking index and the Fagerstrom test. They recruited 2037 subjects from 602 families of either European-American or African-American ancestry in the USA. Analysis of the six SNPs within CHRNA4 of European-American sample demonstrated that the SNPs rs2273504 and rs1044396 are significantly associated with the adjusted smoking quantity and FTND score, respectively. In the African-American samples, SNPs rs3787137 and rs2236196 are each significantly associated with at least two adjusted nicotine dependence measures. Association of rs2236196 with the adjusted heaviness of the smoking index and FTND scores in the African-American samples remained significant after correction for multiple testing. SNP rs2236196 showed a significant association in African American samples but showed no association in the European-American sample group. Their findings provide convincing evidence for the involvement of the CHRNA4 subunit in nicotine addiction.

Several analyses have been carried out on CHRNA4 rs2236196. This SNP has been reported to have an association with nicotine dependence and smoking behavior in African American women [30]. They have also identified that mutation in this gene was associated with subjective responses to smoking. In their study, participants were asked to smoke a cigarette after an 8 h abstinence period and they were rated on the effects of smoking phenotypes, including the physical, cognitive, and rewarding properties of smoking. They found participants with a heterozygous genotype at SNP rs2236196 exhibited an increased sensitivity to all 4 subjective measures of smoking. A study in 5500 German subjects has also observed a significant association between rs2236196 and smoking-related phenotypes [13].

The general lack of an association between the SNPs tested in our study and smoking related behavior is in agreement with many other studies [31–33]. No association between rs2236196 and smoking status was found by Spruell et al. [33]. However, they found a weak association of rs2273502 with smoking abstinence. A study done by Etter et al. [32] also found no association of SNP at rs2236196 in CHRNA4 with smoking status or other smoking related variables among smokers in Switzerland.

### 4. Conclusion

In conclusion, the present study is the first to provide evidence for the association of allelic variants of CHRNA4 rs2273502 and rs2236196 with smoking behavior in the Malay Male population. However, the results revealed that CHRNA4 rs2273502 and rs2236196 gene polymorphisms are not significantly associated with smoking behavior in our population. 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.

### Acknowledgements

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### References


[3] Johnstone EC, Yudkin P, Griffiths SE, Fuller A, Murphy M, Walton R. The dopamine D2 receptor C2806T polymorphism (DRD2 Taq1A RFLP) exhibits no association with smoking


