RELATIONSHIP OF GENETIC POLYMORPHISMS WITH SMOKING BEHAVIOR IN THE MALAY POPULATION IN KELANTAN

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by

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LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptamine
BMI	Body mass index
bp	Base pair
CHRNA4	Cholinergic receptor nicotinic alpha 4
CNS	Central nervous system
COPD	Chronic obstructive pulmonary disease
DBP	Diastolic blood pressure
df	Degree of freedom
DALYs	Disability adjusted life years
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine-tetraacetic acid
EtBr	Ethidium Bromide
FTND	Fagerstrom test for nicotine dependence
GATS	Global Adult Tobacco Survey
IQR	Interquartile range
KCl	Potassium Chloride
kg	kilogram
LGIC	ligand-gated ion channels
m	meter
$MgCl_2$	Magnesium Chloride
MOH	Ministry of Health
NAc	Nucleus accumbens
nAChR	Nicotinic Acetylcholine Receptor
nm	nanometer
OD	Optical density

PCR	Polymerase Chain Reaction
RFLP	Restriction fragment length polymorphism
SBP	Systolic blood pressure
SD	Standard deviation
SNP	Single Nucleotide Polymorphism
TBE	Tris Borate EDTA
TE	Tris-EDTA
UTR	Untranslated region
UV	Ultraviolet
VTA	Vetral tegmental area
WHO	World Health Organization
YLL	Years of life lost

HUBUNGAN ANTARA POLIMORFIK GENETIK DAN TABIAT MEROKOK DI KALANGAN POPULASI MELAYU DI KELANTAN

ABSTRAK

Ketagihan merokok merupakan penyebab utama penyakit dan kematian di seluruh dunia. Bagaimanapun, disebalik risiko kesihatan yang diketahui, kelaziman merokok masih meningkat di seluruh dunia. Nikotina merupakan bahan utama yang menyebabkan ketagihan terhadap rokok. Faktor-faktor genetik memainkan peranan penting dalam setiap aspek ketagihan nikotin. Oleh itu, gen yang terlibat dalam farmakodinamik dan farmakokinetik nikotina merupakan kemungkinan penyebab terhadap ketagihan merokok. Empat gen telah dipilih di antara senarai gen yang mungkin terlibat dengan ketagihan merokok yang telah diterbitkan, iaitu reseptor asetilkolina nikotina (nAChR) rs2273502, rs2236196, pengangkut serotonin (5HTTLPR) dan reseptor serotonin (5HT2A). Hubungkait di antara gen-gen ini dengan tabiat merokok di kalangan populasi Melayu telah dikaji di dalam kajian ini.

Kajian ini melibatkan seramai 248 orang lelaki Melayu yang merokok dan 248 orang lelaki Melayu yang tidak merokok. DNA diekstrak daripada leukosit. Hasil PCR bagi nAChR rs2273502 dan rs2236196 dan 5HT2A masing-masing dicerna oleh enzim penyekatan *AfeI, Sau96I* dan *HpaII*. Manakala gen 5HTTLPR dianalisis menggunakan teknik PCR sahaja dan hasilnya diklasifikasi sebagai pendek (S) atau panjang (L).

Tiada mutasi bagi rs2273502 (genotip TT) dikesan dalam kumpulan bukan perokok manakala kekerapan bagi homozigos genotip CC dan heterozigos genotip CT dalam

kumpulan bukan perokok masing-masing ialah 75.8% dan 24.2%. Manakala bagi kumpulan perokok, kekerapannya masing-masing ialah 73.4%, 2.0% dan 24.6%. Tiada perbezaan yang signifikan dilihat bagi kekerapan genotip (χ^2=5.106, p=0.078) dan alel (χ^2=1.064, p=0.302) kedua-dua kumpulan. Taburan kekerapan bagi rs2236196 dalam kumpulan perokok ialah 80.6% untuk homozigos genotip AA manakala bagi kumpulan bukan perokok ialah 77.0%. Tiada mutasi (genotip GG) dikesan dalam kedua-dua kumpulan. Genotip AG untuk kumpulan perokok ialah 19.4% manakala bagi kumpulan bukan perokok ialah 23.0%. Tiada perbezaan yang signifikan dilihat dalam kekerapan genotip (χ2=0.979, p=0.323) dan alel (χ2=0.863, p=0.353) di antara kedua kumpulan yang dikaji. Kekerapan genotip bagi 5HT2A bagi kumpulan perokok pula ialah CC= 10.1%, TT= 46.8% dan CT=43.1% manakala bagi kumpulan bukan perokok ialah CC=8.1%, TT=46.4% dan CT=45.6%. Tiada perbezaan yang signifikan dilihat dalam kekerapan genotip (χ2=0.724, p=0.696) dan alel (χ2=0.075, p=0.784). Bagi 5HTTLPR pula, kekerapan genotip bagi SS, LL dan heterozigos SL masing-masing ialah 41.1%, 12.9% dan 46.0% dalam kumpulan perokok. Manakala bagi kumpulan bukan perokok, kekerapan bagi SS, LL dan heterozigos SL masing-masing ialah 39.1%, 11.3% dan 49.6%. Tiada perbezaan yang signifikan bagi taburan kekerapan bagi genotip 5HTTLPR (χ^2 = 0.734, p=0.693) dan alel 5HTTLPR (χ2 = 0.004, p=0.947) bagi kedua-dua kumpulan yang dikaji. Kesimpulannya, tiada perbezaan signifikan antara reseptor asetilkolina nikotina (nAChR) rs2273502 dan rs2236196, pengangkut serotonin (5HTTLPR) dan reseptor serotonin (5HT2A) polimorfik dengan tabiat merokok di kalangan populasi Melayu di Kelantan.

RELATIONSHIP OF GENETIC POLYMORPHISMS WITH SMOKING BEHAVIOR IN THE MALAY POPULATION IN KELANTAN

Smoking addiction is a leading cause of diseases and mortality worldwide. However despite the well-known associated risk to health, smoking prevalence is still increasing worldwide. Nicotine is the main addictive substance in cigarettes that is responsible for the development as well as maintenance of smoking addiction. Genetic variables appear to play a key role in every aspect of nicotine addiction. Therefore genes involved in pharmacodynamic and pharmacokinetic of nicotine are logical candidates for nicotine addiction. Among listed candidate genes published for their known association with nicotine, four of them were selected which were nicotinic acetylcholine receptor (nAChR) rs2273502 and rs2236196, serotonin transporter 5HTTLPR and serotonin receptor 5HT2A and their relationships with smoking behavior in the Malays were investigated in this thesis.

The study involved 248 Malay male smokers and 248 Malay male nonsmokers. DNA was extracted from leucocytes. The PCR product of nAChR rs2273502 and rs2236196 and 5HT2A were digested with restriction enzymes *Afe*I, *Sau96I and HpaII* respectively. The 5HTTLPR genotypes were analyzed by using PCR method and the results were classified as short (S) alleles or long (L) alleles.

No mutation of rs2273502 (TT genotype) was detected in the nonsmoker group whilst the frequencies of homozygous CC genotype and heterozygous CT in nonsmokers were 75.8% and 24.2%, respectively. While in smokers, the frequencies were 73.4%, 2.0% and 24.6%, respectively. No significant difference was observed in genotype (χ^2=5.106, p=0.078) and allele (χ^2=1.064, p=0.302) frequencies among both group. The frequency distribution for rs2236196 polymorphism in smoker

group was 80.6% for homozygous AA genotype while in nonsmoker 77.0%. No mutation (GG genotype) was detected in both groups. The AG genotype for smoker group was 19.4% while in nonsmoker group 23.0%. There was no significant difference observed in the genotype ($\chi 2$=0.979, p=0.323) and allele frequencies ($\chi 2$=0.863, p=0.353) between both groups. The genotype frequencies for 5HT2A polymorphism in smokers are CC= 10.1%, TT= 46.8% and CT=43.1%. While for nonsmokers are CC=8.1%, TT=46.4% and CT=45.6%. There was no significant difference observed in the genotype 5HT2A ($\chi 2$=0.724, p=0.696) and allele frequencies ($\chi 2$=0.075, p=0.784). On the other hand, for 5HTTLPR polymorphism, the frequencies of variant alleles S, L and heterozygous SL in nonsmokers were 39.1%, 11.3% and 49.6%, respectively. While in smokers, the frequencies of variant alleles S, L and heterozygous SL were 41.1%, 12.9% and 46.0%, respectively. No significant difference in the frequency distribution of alleles was found between smokers and nonsmokers of genotype 5HTTLPR (χ^2 = 0.734, p=0.693) and allele frequencies ($\chi 2$ = 0.004, p=0.947) for both group studied.

In conclusion, the nicotinic acetylcholine receptor (nAChR) rs2273502 and rs2236196, serotonin transporter (5HTTLPR) and serotonin receptor (5HT2A) polymorphisms were not found to be associated with the smoking behavior in Malay male subjects in Kelantan.

CHAPTER 1

LITERATURE REVIEW AND INTRODUCTION

1.1 Smoking

Tobacco consumption is well known as a leading cause of preventable morbidity and mortality worldwide, hence is a critically public health concern. It is a major contributor to many diseases such as stroke, heart disease, chronic obstructive pulmonary disease (COPD), periodontal disease, peripheral vascular disease, pneumonia, lung and oral cancer (Lung *et al.*, 2005)

The tobacco epidemic kills 5.4 million people in average per year from lung cancer, heart disease, and other illnesses. If this smoking trend continues, there will be more than 8 million deaths every year, with more than 80% of tobacco-related deaths in developing countries by 2030 (Mathers and Loncar, 2006). As a result, tobacco will kill a billion people due to smoking related disease during this century.

In Malaysia, smoking-related diseases have been the primary cause of mortality for the past three decades. It is estimated that one-fifth of disability adjusted life years (DALYs) and one-third of years of life lost (YLL) for Malaysians were due to smoking-related diseases (Yusoff *et al.*, 2005)

1.1.1 Smoking prevalence in Malaysia

In 2012, The Global Adult Tobacco Survey Malaysia (GATS Malaysia) reported 23.1% or 4.75 million Malaysian adults aged 15 years or older were current smokers of tobacco with 43.9% (4.64 million) of men and 1.0% (0.10 million) of women. On average, a daily Malaysian adult smoker smoked 14 cigarettes per day in 2011. More than half (51.8%) of those aged 20-34 years who had ever smoked on a daily basis had started smoking daily before the age of 18. Among the three main racial/ethnic groups in Malaysia, 22.3% of Malays, 17.2% of Indians, and 14.2% of Chinese were daily smokers. The proportions of nonsmokers were 84.6% of Chinese, 80.4% of Indians and 75.4% of Malays. The survey shows significantly higher proportion of Muslims (23.2%) than non-Muslims (16.2%) smoked a tobacco product daily, while the percentage of nonsmokers was significantly higher for non-Muslims (81.7%) than Muslims (74.4%) (WHO, 2012).

According to the Ministry of Health Malaysia (MOH), tobacco use in Malaysia accounts for 35% of in-hospital deaths, principally from cancer, heart disease and stroke. More than 10 000 Malaysians die from smoking-related illnesses each year (WHO, 2012). Statistics from the Ministry of Health Malaysia in 2006 revealed that diseases related to smoking remained the top causes of death in MOH hospitals, accounting for more than 15% of hospitalizations and 35% of in-hospital deaths. Heart diseases and diseases of pulmonary circulation ranked first, accounting for 15.7% of these deaths, followed by malignant neoplasms, 10.6%, and cerebrovascular diseases, 8.5% (MOH, 2006).

1.1.2 Tobacco

Tobacco is an agricultural product and an end product of the fresh tobacco leaf. It is from the genus of Nicotiana of the Solanaceae family plants. Dried leaves are used to produce tobacco products. There are several types of tobacco products such as cigarette, chewing tobacco and cigarette pipe tobacco (Giuseppe *et al.*, 2013). Tobacco for cigarettes is generally derived from two varieties of plants:

1) Virginia, a yellowish "bright" leaf contain about 3% of nicotine
2) Burley, with higher quantity of nicotine (Giuseppe *et al.*, 2013)

Tobacco use is a repetitive and compulsive behavior characteristic of drug addiction.

1.1.3 Nicotine and nicotine addiction

Several compounds of cigarettes are toxic, but they are not addictive. The addiction is caused by nicotine, which is the major tobacco alkaloid. Nicotine is an *N*-alkylpyrrolidine that consists of *N*-methylpyrrolidine bearing a pyridin-3-yl substituent at position 2 (Figure 1.1).

It is commonly used as a psychoactive drug that is orally self-administered via the chewing or combustion of tobacco products, the latter typically via cigarette use. According to Henningfield *et al.*, (1985), cigarettes represent "the most toxic and addictive form of nicotine delivery"

Nicotine is primarily responsible for the development and maintenance of tobacco dependence (Henningfield *et al.*, 1985). Understanding the neural substrate of

nicotine addiction is the first step to develop medication for smoking cessation therapy. According to Palca (1988), the behavioral and pharmacological characteristic for nicotine addiction are similar to cocaine and heroin addiction.

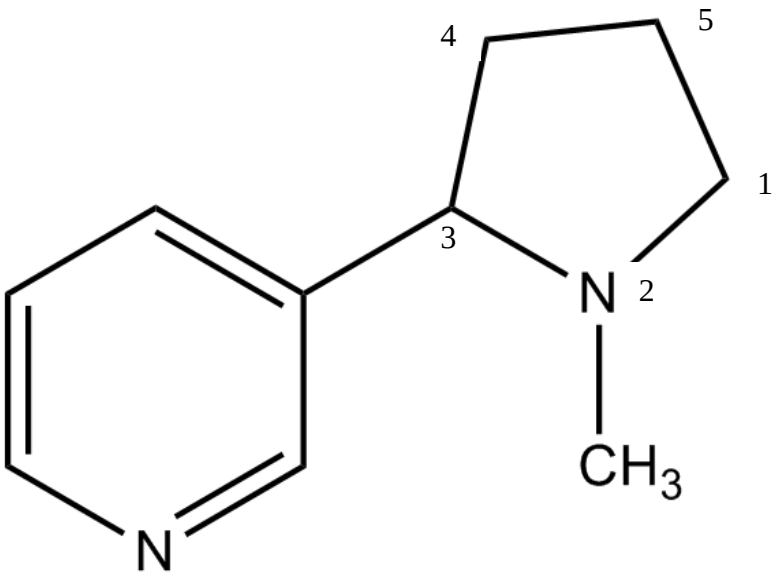

Figure 1.1 Structure of nicotine molecule

1.1.4 The biology of nicotine addiction

Nicotine acts on nicotinic cholinergic receptors, then triggering the release of neurotransmitters that produce psychoactive effects that are rewarding. After repeated exposure, tolerance will develop to the effects of nicotine, thus reducing its primary reinforcing effects and inducing physical dependence such as withdrawal symptoms in the absence of nicotine. According to Benowitz (2010), smoking behavior is influenced by pharmacologic feedback as well as environmental factors. Examples of environmental factors are smoking cues, friends who smoke, stress, and product advertising. Levels of nicotine in the body in relation to a particular level of nicotine intake from smoking are modulated by the rate of nicotine metabolism. Other factors that influence smoking behavior include age, sex, genetics, mental illness, and substance abuse (Figure 1.2)

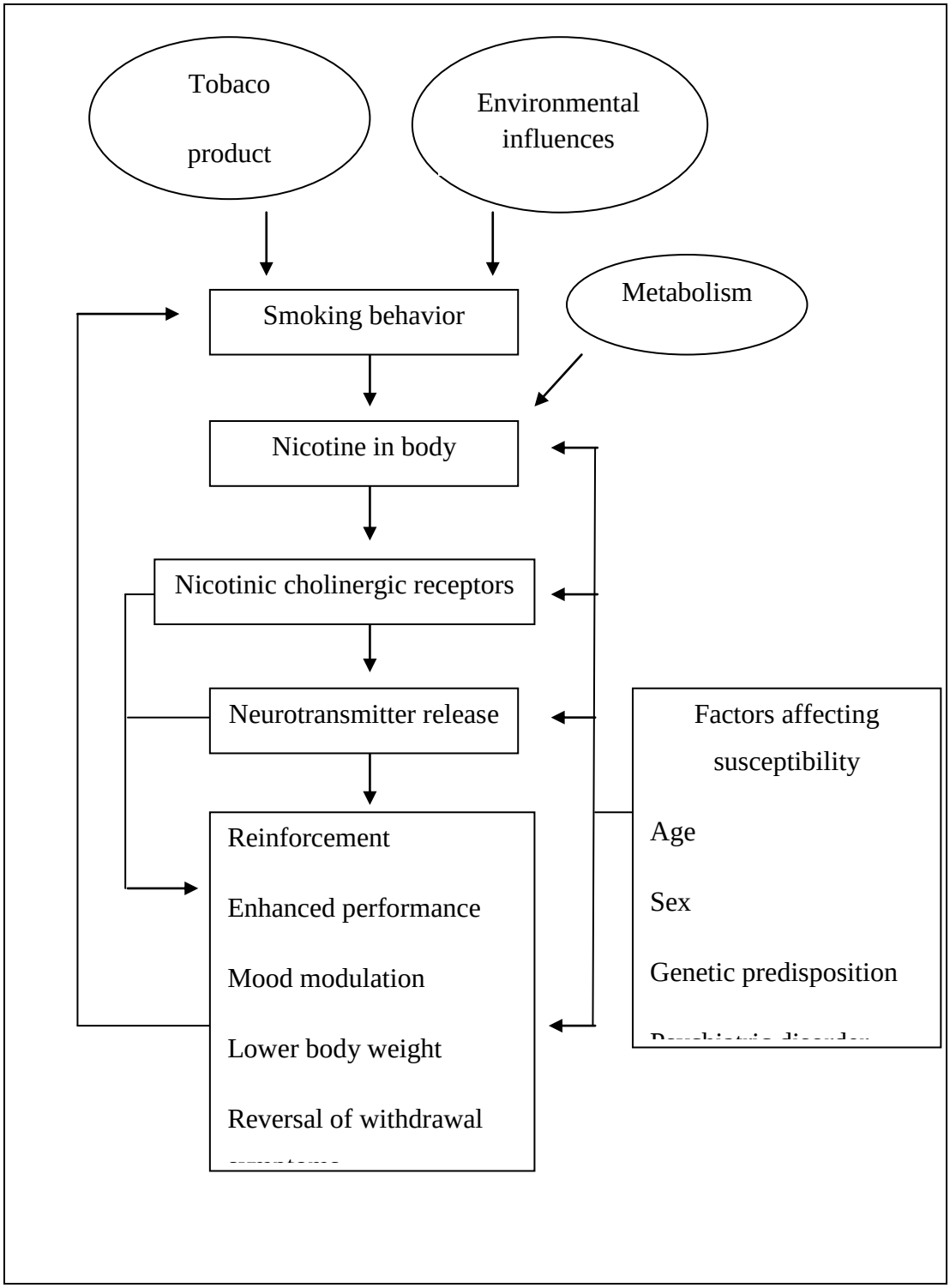

Figure 1.2 The Biology of Nicotine Addiction. (Adapted from Benowitz, 2010)

1.1.5 Metabolism of nicotine in the body

The effects of nicotine are strongly influenced by its complex metabolism. Nicotine influences mood, cognition, and body function by binding to and activating nicotinic acetylcholine receptor (nAChR). This receptor is located on neurons in the central nervous system (CNS). During activation by either nicotine or the endogenous neurotransmitter acetylcholine, the nAChR opens a channel that allows ions to pass through the neuron's membrane from the exterior to the interior of the cell. This will trigger changes that activate the cell (D'Souza and Markou, 2011). This binding is at the base of the neurobiology of nicotine addiction and is the major obstruction for smokers who try to quit (Benowitz, 2010)

When tobacco is smoked, the nicotine in the smoke enters the blood vessels in the lungs and reaches the brain within 10 sec of the first puff (Benowitz *et al.*, 2009) Consequently, nicotine binds to receptors located on the cell bodies of neurons in the vetral tegmental area (VTA) and the terminals of these neurons. Normally, the neurotransmitter acetylcholine bind to these receptors. As nicotine has a very similarly structured to acetylcholine, it is able to bind to the acetylcholine receptor. Nicotine has specific acetylcholine receptors (nAChRs) in the brain and other organs. The stimulation of presynaptic acetylcholine receptors increases the release of acetylcholine as well as the metabolism (Pidoplichko *et al.*, 2004)

Similarly to other drugs of abuse, nicotine triggers the dopamine reward system and increases the extracellular level of dopamine in nucleus accumbens (NAc). NAc has

a fundamental role for the reinforcing behavior, stimulant, and dependence properties of nicotine (Pidoplichko *et al.*, 2004).

According to Quaak *et al.,* (2009), smokers with a reduced nicotine metabolism and increased dopamine levels are less seems to have addicted to smoking, smoke less, and have a higher chance to quit. On the other hand, smokers with an increased nicotine metabolism and reduced dopamine levels, seem to be more addicted. They have a higher chance to become a smoker, start smoking at a lower age, smoke more cigarettes, and also undergo fewer and less successful smoking cessation attempts.

1.2 Genetic polymorphism and smoking behavior

Genetic polymorphism is define as mutant or variant genes that exist at a frequency of more than 1% in the normal population (Meyer, 2000). A major difference between genetic and environmental variation is that an inherited mutation or trait is present throughout life has to be tested for only once in a lifetime, whereas environmental effects are continually changing.

Genetic polymorphisms explain why a small proportion of the population may be at higher risk of drug inefficacy or toxicity. The gene polymorphism's study has given rise to the field of pharmacogenetics. Genetic polymorphisms are a source of variation of drug response in the human body. In relation to cigarette smoking, most interest has focused on the involvement of pharmacokinetic factors and, in particular, nicotine metabolism. Nowadays, there is increasing recognition that genetic variation

in nicotine targets (pharmacodynamic factors) might also influence to smoking behavior.

1.2.1 Single Nucleotide Polymorphism (SNP)

Single nucleotide polymorphisms (SNPs) are differences in single bases of DNA. SNPs occurs in about 90% of sequence variants in humans throughout the human genome. The International SNP Map Working Group has published a map of 1.42 million SNPs throughout the genome, occurring at an average density of one SNP every 1.9 kilobases (The International SNP Map Working Group, 2001)

1.2.2 Candidate genetic polymorphism and smoking behavior

Candidate gene studies focus on genes that have been selected either because of their location within an identified linkage region (positional candidate genes) or because of their hypothetical aetiological role in disease (functional candidate genes). Functional candidate gene selection process is made with a careful investigation of the earlier published studies and by evaluating known hypothesised biological pathways involved in the studied phenotype.

Several genes have been shown to be associated with tobacco smoking. To date, candidate gene association studies have focused on genes in a few candidate pathways. Variants in two broad classes of candidate genes have been suggested to contribute to smoking behavior:

1) Genes that may influence the response to nicotine (e.g. nicotine metabolism, nicotinic receptors)

2) Genes that may predispose to addictive behavior due to their effects on key neurotransmitter pathways (e.g. dopamine, serotonin, opioid) (Quaak *et al.*, 2009).

1.3 Specific candidate genes in smoking behavior

A 'reward deficiency syndrome' has been recognized as of the one reasons to be the role of diverse neurotransmitters in nicotine dependency (Blum *et al.*, 2000). Therefore, many studies have evaluated genes in opioid (Lerman *et al.*, 2004), serotonin (Lerman *et al.*, 2000; Iordanidou *et al.*, 2009; Iordanidou *et al.*, 2010), dopamine (Shields *et al.*, 1998; Blum *et al.*, 2000), drug metabolizing enzymes (Malaiyandi *et al.*, 2005; Benowitz *et al.*, 2006), nicotinic and muscarinic cholinergic receptor pathways (Li *et al.*, 2005). Some candidate genes involved in dopaminergic function are area of research interest involving a wide range of addictive and other behavioral disorders. Other genes, including some of the cytochrome P450 family as well as the nicotinic acetylcholine receptor are also considered reasonable candidates to influence smoking behavior. This is because they are involved in neural activity or metabolism of nicotine.

Polymorphisms of a variety of genes involved in serotonergic neurotransmission have been identified. The serotoninergic system may be involved in smoking behavior because nicotine increases brain serotonin secretion, nicotine withdrawal decreases serotonin levels, and a selective serotonin reuptake inhibitor antagonizes the response to nicotine (Ishikawa *et al.*, 1999)

It has been suggested that nicotine-induced release of dopamine drives tobacco usage, while receptor inactivation due to chronic nicotine use may play a role in the processes of tolerance and withdrawal (Dani and De Biasi, 2001). Genetic differences in nicotinic receptor activity or nicotine metabolism might reasonably be hypothesized to play a role in smoking addiction. Nicotinic receptors are present in the brain, autonomic ganglia, and the neuromuscular junction. Neuronal nicotinic acetylcholine receptors are believed to be most relevant to nicotine addiction.

Therefore, the polymorphism on nicotinic acetylcholine receptors genes and serotonin genes represent logical candidates for an association study with smoking behavior.

1.3.1 Nicotinic Acetylcholine Receptor (nAChR)

The behavioral and physiological effects of nicotine are mediated largely by neuronal nicotinic acetylcholine receptors (nAChRs). Nicotine functions by binding to nAChRs, which, in turn, modulate the release of dopamine in the mesolimbic system (Pidoplichko *et al.*, 1997).

1.3.1.1 Structural characteristic and function of nAChR

The nicotinic acetylcholine receptors (nAChRs) belong to the superfamily of the Cys-loop ligand-gated ion channels (LGICs), which also include the GABA, glycine, and 5-HT3 receptors. They are formed by the assembly of five transmembrane subunits selected from a pool of 17 homologous polypeptides (α1–10, β1–4, γ, d, and e). There are many nAChR subtypes, each consisting of a specific combination of subunits, which mediate diverse physiological functions. These receptors are widely

expressed in the central nervous system, and, in the periphery, they mediate synaptic transmission at neuromuscular junctions and ganglia (Lee *et al.*, 2011). Among them, α 4 and β 2 subunits are the most widely and concurrently expressed high-affinity nAChR subunits in the brain and are both upregulated under chronic nicotine exposure (Whiteaker *et al.*, 1998). The gene that encodes the human nAChR α 4 subunit (locus ID 1137) was mapped by FISH to 20q13.2-13.3 (Steinlein *et al.*, 1994). The gene is ~17 kb in size and comprises six exons (Steinlein *et al.*, 1996).

1.3.1.2 Polymorphism in CHRNA4 and association with smoking behavior

CHRNA4, the gene that encodes the nicotinic acetylcholine receptor α4 subunit, is a potential candidate gene for nicotine dependence. However, studies of the association of CHNRA4 with smoking behavior have shown inconsistent results. CHRNA4 is highly expressed in the central nervous system, and its protein product is part of high affinity receptors (α4β2) for nicotine. It plays a major role in tolerance, reward, and the modulation of mesolimbic dopamine function, all of which are critical to the development of nicotine dependence (Tapper *et al.*, 2004). Although some genetic association studies support the role of CHRNA4 in smoking-related behaviors (Feng *et al.*, 2004; Li *et al.*, 2005; Hutchison *et al.*, 2007; Breitling *et al.*, 2009), there have been conflicting results (Ehringer *et al.*, 2007; Etter *et al.*, 2009).

1.3.1.2.1 rs2273502

Mutation in rs2273502 involved C $\longrightarrow$ T transition located in exon 5 in CHRNA4. rs2273502 polymoprhism has been reported has association with attention deficit/

hyperactivity disorder ADHD (Weidong *et al.*, 2009). Emerging research shows a higher incidence of smoking in individuals with ADHD compared with the general population. Data from a number of longitudinal studies have shown clearly that individuals with ADHD are at increased risk for cigarette smoking compared to their peers who do not have ADHD (Lambert and Hartsough, 1998). ADHD symptoms have also been shown to influence the route of smoking behavior from initial use to regular use and dependence. One study found that a lifetime diagnosis of ADHD was a significant predictor of progression from initiation of smoking to daily use (Rohde *et al.*, 2004). Study done by Spruel *et al.*, (2012) shows there was association of rs2273502 with smoking abstinence.

1.3.1.2.1 rs2236196

Polymorphism in of CHRNA4 rs2236196 located in the 3' UTR region (A → G transition). Several analyses have been carried out on CHRNA4 rs2236196. According to Hutchison *et al.*, (2007), there was a significant association with abstinence at 6 months, while in another, the association was seen with nicotine dependence in both European Americans and African Americans (Li *et al.*, 2005). An association study in 5,500 Germans showed that rs2236196 was significantly associated with Fagerstrom Test Nicotine Dependence (FTND) and the ''G'' allele was associated with a higher risk of nicotine addiction and higher odds of being a smoker (Breitling *et al.*, 2009). The accumulating evidence from genetic association studies and the additional findings on clinical effects (Hutchison *et al.*, 2007) suggest that rs2236196 could be one of the most promising risk variants for smoking related behaviors.

1.3.2 Serotonin transporter

1.3.2.1 Structural characteristic and function of serotonin transporter

Serotonin transporter 5-HTT is a member of the sodium and chloride dependent neurotransmitter transporter (SLC6) family (Saier, 2000). It is a 630 amino acid protein and it has 12 transmembrane domains. Its C and N terminal resions lie in the cytoplasm (Rudnick and Clark, 1993). They are responsible for regulating the magnitude and duration of serotonergic neurotransmission.

This protein selectively transports serotonin together with Na^+ ans Cl^- into cells and in the same reaction transports K^+ out of the cell modulating serotonergic signaling and neurotransmission (Blakely *et al.*, 1994). Serotonin transporter, 5HTT, controls the duration and concentration of serotonin neurotransmission in synaptic cleft (Uhl and Johnson, 1994). It is encoded by a single gene (*SLC6A4*) located on the longer arm of the seventh chromosome (17q12) (Ramamoorthy *et al.*, 1993). The 5HTT gene has been linked to psychological traits such as anxiety-related personality traits (Lesch *et al.*, 1996) and depression (Collier *et al.*, 1996; Ogilvie *et al.*, 1996), both are related with nicotine dependence (Lerman *et al.*, 1996; Audrain *et al.*, 1998) .

1.3.2.2 Polymorphism in serotonin transporter and association with smoking

A 44 bp insertion or deletion polymorphism, 5HTTLPR, was identified within this gene with two allelic variants, the long (L) and the short (S) allele which altered the transcriptional efficiency of the 5HTT gene (Heils *et al.*, 1996). S allele was associated with reduced serotonin uptake leading to hypothesis that individual with S allele is not likely to be a smoker (Heils *et al.*, 1996). This hypothesis is supported by

two studies in Japanese and Chinese population that found individuals with homozygous S genotype were less likely to initiate smoking behavior and could easily stop smoking than others (Ishikawa *et al.*, 1999; Chu *et al.*, 2009) . However the hypothesized role of S allele in smoking behavior has yet not been confirmed as other studies that attempt to replicate these findings have obtained contradictory results (Trummer *et al.*, 2006; Sieminska *et al.*, 2008b; Iordanidou *et al.*, 2009; Rasmussen *et al.*, 2009) .

1.3.3 Serotonin receptor

1.3.3.1 Structural characteristic and function of serotonin receptor

Serotonin, 5-hydroxytryptamine (5-HT), is a neurotransmitter synthesized by tryptophan hydroxylas with the decarboxylation of the essential amino acid tryptophan. It is present in the central nervous system. About 90% of 5-HT is wrapped by the chromatophil cells of the alimentary canal, 9% exists in blood platelets and 1% exist in the brain. Central 5-HT is synthesized in the raphe nucleus cells. It is transported to the nerve endings, where it is stored in granules. When a stimulant impulse connects with the nerve endings of the raphe nucleus origin, depolarization occurs and 5-HT is emitted to the synaptic cleft, where it combines with the serotoin receptor (5-HTR) of the postsynapse. 5-HT will trigger a strong vital reacton by the receptor that combines through the signal transduction system in the cells (Terayama *et al.*, 2004).

5-HTR is related to affectivity, regulation and pharmacologic effects of antidepressant, anti-anxiety and anti-psycotic medications. It is thought to be

associated with various psychiatric disorders (including schizophrenia, eating disorders, alcoholism and anxiety disorders) (Abbas and Roth, 2008).

1.3.3.2 Polymorphism in serotonin receptor and association with smoking

The serotonin receptor is classified into seven groups (5HT1-7). Group 2 is divided into three subtypes (A, B, and C). The 5HT2A gene is located on chromosome 13q14–q21 (Peroutka, 1998). The 5HT2A gene has been associated with emotional disorders and alcohol dependence, which are related to smoking behavior (Polina *et al.*, 2009). One of the polymorphisms that has been described in this receptor is T102C, which involves the replacement of a cytosine with a thymine (Peroutka, 1998). This polymorphism has been associated with smoking maintenance but is less likely to be involved in the initiation of smoking behavior (do Prado-Lima *et al.*, 2004). 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 that suggest the occurrence of the TT genotype was associated with current smokers (White *et al.*, 2010). However, this finding was not replicated in several other studies in other populations (Terayama *et al.*, 2004; Huang *et al.*, 2005; Suriyaprom *et al.*, 2012)

1.4 Study objectives

To date, studies examining the association gene polymorphism with smoking behavior have yield contradictory result among different population. It is important to be able to test the linkage of candidate genes in several populations with different ethnicity in order to confirm and solidify the actual gene that affects smoking behavior. No investigation on the association of these gene polymorphisms and smoking has been carried out so far in Malay population. Therefore, the objectives of this study are:

1) To develop and optimize polymerase chain reaction method – restriction fragment length polymorphism (PCR-RFLP) techniques and use these methods to study nicotinic acetylcholine receptors (nAChRs) rs2273502 and rs2236196, serotonin receptor (5HT2A) and transporter (5HTTLPR) genes polymorphisms among Malay populations

2) To estimate the prevalence of nicotinic acetylcholine receptors (nAChRs) rs2273502 and rs2236196, serotonin receptor (5HT2A) and transporter (5HTTLPR) in the Malay population in Kelantan

3) To determine the association between nicotinic acetylcholine receptors (nAChRs) rs2273502 and rs2236196, serotonin receptor (5HT2A) and transporter (5HTTLPR) genes polymorphisms with smoking behavior among Malay smokers and nonsmokers.

CHAPTER 2

MATERIALS AND METHODS

2.1 Clinical Study

2.1.1 Ethical Approval

The study protocol was approved by the Research and Ethics Committee of School of Medical Sciences, Universiti Sains Malaysia in the year 2011 [USMKK/PPP/JEPeM [233.3.(05)]. The ethical approval letter was attached in the Appendix A.

2.1.2 Materials and instruments

The materials used for the clinical study are as listed in Table 2.1

Table 2.1 List of commercial kits and consumables used in the clinical studies.

Material	**Model and Supplier**
K_2EDTA tube	Vacutest, Arzergrande, Italy
Alcohol swab 70% isopropyl alcohol	JR, Selangor, Malaysia
Syringe (5 ml)	Becton Dickinson, Singapore
21G Needle	Teromo, Tokyo, Japan
Blood Pressure Monitor	Omron, Kyoto, Japan

2.1.3 Sample size calculation

Sample size for this study was calculated according to a case-control study done by Polina *et al.,* (2009). They found that the prevalence of 5HT2A variant of wild-type among European who lived in Brazil in nonsmokers group was 36% ($p1$). For smokers group, it is estimated that prevalence of this gene is 24% ($p2$) after reviewing other literatures.

By using a two proportions (Independent Observations) formula with 95% confidence interval, a minimum sample size (n) of 248 subjects was obtained.

Calculation of sample size

$$n = \frac{p1\,(1-p1) + p2(1-p2)}{(p1-p2)^2}\,(z_\alpha + z_\beta)^2$$

$$= \frac{(0.36)(1-0.36) + (0.24)(1-0.24)}{(0.36-0.24)^2}\,(1.96+0.84)^2$$

= 224.74 x 10% dropout

= 248

Where, n = sample size

z_α = 1.96 for α set at 0.05

z_β = 0.84 for β set at 0.20

(power 80%)

$p1$ = proportion 1

$p2$ = proportion 2

2.1.4 Subject recruitment

The subjects were selected from healthy Malay adult volunteers aged between 18 and 50. They were categorized as smokers group and nonsmokers group (control) based on their smoking status. The inclusion and exclusion criteria of the subjects are described in Table 2.2.

All the subjects involved in this study were given a detailed description about the study procedure; the objectives, benefits as well as risk before they agreed and signed written the informed consent (Appendix B). Information obtained from the subjects was kept as confidential and each subject was given a copy of information sheet.

The recruitment of the subjects was done at Hospital Universiti Sains Malaysia (HUSM), Institut Perguruan (IPG) Pengkalan Chepa and several places around the state of Kelantan, based on the inclusion and exclusion criteria.

Height of the subjects was taken in cm and weight measurements were taken in kg. Body mass index (BMI) was then calculated as weight (kg) divided by height squared (m^2). Blood pressure was measured on the subject's right arm after the subjects being rested in a sitting position for 5 min, using an automated blood pressure monitor. The blood pressure was taken twice at 2 min interval. Mean systolic and diastolic blood pressure was obtained by calculating the averages of systolic and diastolic blood pressure.

Table 2.2 Inclusion and exclusion criteria of the subjects

Inclusion criteria	Exclusion criteria
• Malay (3 generations) • Male • Healthy volunteers aged between 18-50 • Being a smoker during study period (Smokers group) • Never (Nonsmokers group)	• Non Malay • Female • Aged less than 18, and more than 50 • Ex-smoker • Under smoking cessation treatment • History of mental disorder

2.1.4.1 Data collections

All subjects were given data collection sheets to record the subject's information. The data collection sheets were given based on the smoking status group (Appendix C). Smoking history and family background of the subjects were obtained.

2.1.4.2 Fagerstrom Test

The Fagerstrom Test for Nicotine Dependence (FTND) is a widely used six-item questionnaire, which was used as a measure of physical dependence on nicotine. The higher the Fagerstrom score, the more intense is the individual's physical dependence on nicotine. FTND was given to subject who has been identified as smoker (Appendix D). The three yes/no items are scored 0 (no) and 1 (yes). The three multiple-choice items are scored from 0 to 3. The items are summed to yield a total score of 0-10. The score of the subject was calculated and the subjects were classified into five level categorization 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) according to the score (Table 2.3)