A PILOT STUDY ON THE A118G MU OPIOID RECEPTOR POLYMORPHISM AMONG DRUG ADDICTS IN MALAYSIA

by

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LIST OF ABBREVIATIONS

%	Percentage
µ	Mu
β	Beta
α	Alpha
~	Approximately
µg	Micro gram
µl	Micro liter
µM	Micro Molar
A	Adenine
ADK	Agensi Dadah Kebangsaan
Asn	Asparagine
Asp	Aspartic acid
Arg	Arginine
Ala	Alanine
BLAST	Basic Local Alignment Search Tool
bp	Base pair
C	Cytosine
cm	Centimeter
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphate
e.g.	*exempli gratia*/for example
EDTA	Ethylenediamine tetraacetic acid
et al.	*Et alii*
F	Forward
g	Gravity
G	Guanine
gm	Gram
hr	Hour
HCl	Hydrochloric Acid
K	Potassium
kb	Kilobase

kg	Kilogram
L	Liter
M	Mutant
mg	Milligram
$MgCl_2$	Magnesium chloride
min	Minute
ml	Milliliter
mm	Millimeter
mM	Millimolar
NA	Not applicable
NCBI	National Centre of Biotechnology Information
NaCl	Sodium Chloride
nm	nanometer
^oC	Degree Celsius
OD	Optical density
OPRM1	Opioid Receptor Mu Gene
OPRD1	Opioid Receptor Delta Gene
OPRK1	Opioid Receptor Kappa Gene
PCR	Polymerase chain reaction
pg	Pico gram
pmole	Pico mole
Prof.	Professor
RNA	Ribonucleic Acid
R	Reverse
rpm	Revolution per minute
RFLP	Restriction Fragment Length Polymorphism
SDS	Sodium dodecyl sulphate
sec	Seconds
Ser	Serine
SNP	Single Nucleotide Polymorphism
SPM	Sijil Pelajaran Malaysia
SRP	Sijil Rendah Pelajaran Malaysia
STPM	Sijil Tinggi Pelajaran Malaysia

T	Thymine
T_a	Annealing temperature
Taq	*Thermus aquaticus*
TBE	Tris Borate - EDTA Buffer
tet	Tetracycline
T_m	Melting temperature
U	Unit
UPSR	Ujian Penilaian Sijil Rendah
+U.S.A	United States of America
V	Volt
Val	Valine
vol	Volume
W	Wild

KAJIAN RINTIS KE ATAS POLIMORFISME RESEPTOR A118G MU OPIOID DIANTARA PENAGIH DADAH DI MALAYSIA

ABSTRAK

Penagihan dadah merupakan masalah sosial yang utama di kebanyakkan Negara. Faktor genetik dan persekitaran adalah penyumpang kepada kecendurangan penagihan dadah. Hipotesis "Opiodergic" mencadangkan bahawa terdapat penglibatan variasi genetik pada lokus gen reseptor mu opioid 1 (OPRM1) dengan penagihan opioid. Kajian ini bertujuan mengkaji frekuensi variasi ini dalam subjek warganegara Malaysia serta menkaji kaitan diantara penagihan dadah dan faktor persekitaran. Alel A118G dalam lokus gen OPRM1 telah dicirikan dengan menggunakan PCR spesifik alel. Frekuensi bagi alel A dan G adalah 51% dan 49% untuk penagih dadah manakala bagi sukarelawan sihat adalah 73% dan 27% masing-masing. Frekuensi alel G adalah 1.77 kali lebih tinggi di kalangan penagih dadah dengan pengiraan nisbah ganjil pada Cl 95% yang menunjukkan bahawa alel G satu faktor penyumbang kepada penagihan (X^2 =15.31 P< 0.0001; nisbah ganjil 2.51; Cl 95%(1.575 – 3.994) berbanding dengan sukarelawan sihat. Hubungan yang signifikan dapat diperhatikan diantara polimorfisme A118G pada gen reseptor μ opioid dan penagihan dadah. Analisis demografi juga menunjukan majoriti penagih dadah membesar di kawasan yang berisiko tinggi yang terlibat dengan dadah. Oleh itu, boleh disimpulkan bahawa faktor genetik dan persekitaran memainkan peranan penting dalam peningkatan penagihan dadah di Malaysia.

A PILOT STUDY ON THE A118G MU OPIOID RECEPTOR POLYMORPHISM AMONG DRUG ADDICTS IN MALAYSIA

ABSTRACT

Drug addiction is an important social problem in many countries. Genetic and environmental factors contribute to the predisposition of drug addiction. The opioidergic hypothesis suggests an association between genetic variations at the opioid receptor mu 1 (OPRM1) gene locus and opiate addiction. The present study aim to delineate the frequency of these variants in subjects of Malaysian origin and study their association with the phenotype of opioid dependence together with environmental factors. A118G allele of OPRM1 gene locus was genotyped by using allele specific PCR. The frequency of A allele and G allele was 51% and 49% respectively for addicts and about 73% and 27% respectively for healthy volunteers. The frequency of G allele was 1.77 fold higher in addicts by odds ratio calculation at Cl 95%, which indicate the G allele to be strongly associated with addiction (X^2 =15.31 P< 0.0001; odds ratio 2.51; Cl 95% (1.575 – 3.994), compared to healthy volunteers. A significant association was observed between A118G polymorphism in μ Opioid receptor gene and drug addiction. The demographic data analysis showed that the majority of the addicts grew up in the environment which makes them prone to addiction. Hence it can be deduced that the genetic and environmental factors play important roles in the development of addiction among Malaysian population.

CHAPTER 1

INTRODUCTION & LITERATURE REVIEW

1.1 Addiction

Drug addiction affects all segments of society in many countries, most importantly it destroys the world's most valuable asset, the youth. It destroys lives, communities, stability of nations and finally the dignity and hope of millions of people around the globe. In total according to The World Drug Report (2008) has mentioned that 449,700 persons have been treated for drug addiction worldwide.

Many individuals are self exposed to drug, alcohol and nicotine abuse. Many continue to use them on an occasional or even on regular basis. However, only some individuals develop specific addictions. This suggests changes in the sensitivity of brain system in individuals can cause one to be susceptible to drug addiction or in the inhibitory systems in the brain can prevent one from becoming an addict (Gerrits et al., 2003). Even after a prolonged period of abstinence, addicts can relapse into their former habit; a factor that leads to relapse is craving and a desire to experience the effect of substance abuse (Markou et al., 1993).

There are at least three different categories of factors that contribute to the vulnerability of developing a specific addiction. The first category is the environment, including prenatal and postnatal events, events which occur during childhood and later events such as peer pressure, cues, setting for drug self exposure. The second category of factors that contribute to the development of drug addiction includes drug induced factors as each of

the short acting substance of abuse, when administered, leads to variety of molecular neurobiological changes, including changes in gene expression, protein concentration and synaptogenesis which can cause altered behavior (Kreek and Laforge et al., 2007).

The third category is genetics. The heritable basis for addictions has been firmly established by many human genetic family studies, in which the influence of family environment, non- family environment, and genetic factors on addiction can be differentiated (Kreek and Laforge., 2007).

1.2 Epidemiological data of drug addiction

1.2.1 Asia

In many Asian countries, the increased availability and variety of drugs has led to a high prevalence of drug abuse among its population. The most abused drug constitute those in opiate group 63.3%, followed by amphetamine 18.4% and cannabis 11.5%. In southwest Asian countries like Pakistan, data from 2006 shows that some 640,000 persons regularly abuse opiates. This number approximates 0.7 % of the adult population and of those, 77% are believed to be under the influence of heroin. India has the highest number of drug addicts, and statistics show around about 81,802 people are being treated of which 61% are opiate users, 15.5% are cannabis users and only 1.5% are cocaine users. However in Bangladesh, the number of patients undergoing treatment for drug abuse decreased from 13,300 in 2004 to 4870 in 2007 and to 909 people in 2008 (World Drug Report, 2008).The majority of them used heroin as primary drug of abuse.

In East Asia, statistics indicate that in China there are over 1,160,000 registered drug users and that 105,151 people are being treated for drug abuse. Government reports states that 78.3% of all registered drug users are heroin addicts who are youths aged between 17 and 35 years (World Drug Report, 2008). Indonesia has 3.2 million addicts who constitute 1.5% its population as drug users. On the other hand, the number of addicts in Philippines decreased from 6.7 million in 2004 to 3.4 million in 2007 (International Narcotic Control Strategy Report, 2008).

1.2.2 Africa

In African countries, 1.4 million people, in the 15 - 64 age group are abusing opiates mostly heroin. The annual prevalence rate of opiate use is highest in Mauritius (2 %), followed by Egypt (0.7%). Recently it has been reported that the demand for treatment for heroin abuse in South Africa has also increased (International Narcotic Control Strategy Report, 2008). While Kenya been identified as one of the country that has registered an increase in the use of heroin (World Drug Report, 2008).

1.2.3 Europe

Around 60.3% of the population use opiates in Europe. For several years, the abuse of heroin and other opiates had been stagnant or on the decline in Western Europe. However it appears now that there is a resurgence of heroin abuse (World Drug Report., 2008). In France, the abuse of heroin has emerged amongst the young and more socially integrated segments of the population. According to UNDOC, the number of people in the group 14 – 64 age group in Eastern Europe who abuse opiates is estimated to be up

to 2 million, i.e about 1.4% of the population (International Narcotic Control Strategy Report, 2008).

1.2.4 America

Cocaine (47.5%) remains the most abused drug, followed by cannabis (31.3%) and opiate (1.7%) in North America (World Drug Report., 2008). Heroin abuse is stable at a relatively low level (0.6% life time prevalence) in USA, despite the fact that heroin is widely available in most large urban areas, in some suburbs and in the rural areas (International Narcotic Control Strategy Report, 2008). In Canada, 61.4% of those in the age group 15 -24 age group have used cannabis at least once in their lives.

1.2.5 Drug addiction in Malaysia

Drug abuse in Malaysia is not a recent phenomenon. It is closely associated with the early development of the country. Drug addiction is considered to be the major social problem in Malaysia.

Studies have shown that between 1988 and 2006, there were 300,241 registered drug addicts in Malaysia. In 2007, 14,489 addicts were reported as compared to 22,811 in 2006. In the January – September 2008 period, there were about 9989 drug addicts, a decrease by 16.90% to that of the previous year. Table 1.1 reveals that in 2008, new addicts comprised 46.81% of the total while 53.19% were relapse addicts. Statistics also indicate that 76.45% of the addicts are Malays, 11.96% Chinese, 9.22% Indian and 2.36% others. Most of them fall in the 19 – 39 age group, about 69.22%, followed by the 40 - >50 age group (28.66%) and only about 2.12% were from the 13 – 18 age group.

Majority of drug abusers were labourers (19.96%) with only 0.26% from the entertainment sector. Drug addiction also correlates with education background. The data shows that 77.61% of addicts were either not educated or having only primary level school education or were SRP drop outs. 54.76% of drug addicts were influenced by friends, 22.08% tried it for pleasure while 14.31% tried it for fun. In terms of type of drug being abused in the country, heroin continues to be the main drug being abused by Malaysian; constituting 39.47%, of the types of drug abused. The highest heroin abuse was observed in Wilayah Persekutuan (1322), Johor (621), Perak (432) and Selangor (423). Amongst Malay addicts, 74.13% preferred to take heroin (ADK 2007 and 2008).

Table 1.1: Number of addicts reported in 2007 and 2008
Adapted from: ADK report 2008

Status	**Jan – Sept 2008**	**Jan – Sept 2007**	**Difference 2007/2008**
New addicts	4676	5476	- 14.61%
Relapse	5313	6545	- 18.82%
Total addicts	9989	12021	-16.90%

1.3 Genetic Basis of Addiction

The difficulty in finding the genes that contribute to risk of addiction parallels the difficulty in finding genes for other psychiatric disorders. This is because addiction is a complex trait and thus a single gene defect might produce a relatively small effect which would be difficult to be detected experimentally (Neslter et al., 2000).

Although a hereditary basis for addiction has been established, the specific gene involved in the etiology of these disorders has not been well defined. Researchers have hypothesized that specific combinations of alleles of specific genes may result in innate differences in phenotypic expression of cellular or physiological systems known to be important in mediating the responses of drugs abuse (Kreek et al., 2000). Other studies have shown that opiates, cocaine, other drugs, alcohol and nicotine, profoundly alter physiological and cellular systems. These changes are specific to the route and pattern of administration and length of time exposure (La Forge et al., 2000).

Some of the induced alterations may be long lasting or even permanent. Therefore, cellular or physiological systems which show alterations in response to substances of abuse might respond differently in individuals due to innate genetic differences that result from polymorphisms (La Forge et al., 2000). These variances in gene which code for protein, especially when their expression results in altered protein amounts when they code for aberrant forms of proteins, may also underlie the development of these disorders (Kreek et al., 2005)

A growing number of genes are significantly associated with addiction. In fact, research shows that few selected genes from various populations are likely to be involved in contributing to vulnerability to drugs, alcohol and nicotine (Kreek et al., 2002).

1.3.1 Gene and Alcoholism

Alcoholism has an estimated heritability of 50 % -70% depending on diagnostic criteria, population and gender (Tyndale et al., 2003). A large number of genes interact with each other with in specific environments and some of these genes influence vulnerability to alcohol. For example ADH 1B * 2 allele which is common in Asians (Japanese, Chinese and Korean) protects these ethnicities from alcoholism (Chen et al., 1996 and Shen et al., 1997) but African American youths are associated with greater alcohol dependence due to the presence of ADH1B * 3. Another genetic variation DAT1 (DA transporter gene) is found to influence drinking behavior in Finland (Linda et al., 2009) while OPRL 1 (nociceptin receptor gene) is shown to be involved in alcohol dependence in the Scandinavian population (Huang et al., 2008). Apart from that, studies have been conducted to investigate the effect of A118G polymorphism of the μ opioid gene, in alcohol addiction. A stronger urge to drink alcohol was detected in an individual with a copy of G allele in Netherlands (Van et al., 2007). In central Sweden, Japan, Korea and China the functional variant A118G allele was associated with increased risk for alcohol dependence (Bart et al., 2005, Nizhizawe et al., 2006, Franke et al., 2000 and Szeto et al., 2001).

1.3.2 Gene and Smoking

Globally, smoking is responsible for the death of five million people each year and increased susceptibility to many forms of cancers. In recent years, it is known that environmental factor together with genes play an important role in nicotine dependence (Davies et al., 2009). One of the genes which contribute to nicotine dependence is nAchRs (Nicotinic acetylcholine receptors) gene polymorphism which has a strong linkage disequilibrium and is associated with increased risk of heavy smoking (Steven et al., 2008). Other genes are (CNR 1) Cannabinoid Receptor 1 (Chen et al., 2008), SNP of Neuronal Nicotine Acetylcholine (CHRNB3 and CHRNA6) (Zeiger et al., 2008). The A118G in μ opioid gene has also been shown to be associated with craving amongst women in USA (Ray et al., 2006).

1.3.3 Gene and Drug addiction

Drug addiction is a complex disorder and vulnerability to addiction has been shown to have a robust genetic component. A few selected genetic variants have been identified which have an association with drug addiction. Studies were carried out to look into the association between 4 polymorphisms of 5 – HT (2A) receptor and 5 – HT transporter gene in heroin addicts. It supports the role of the 5 – HT (2A) receptor gene in enhancing susceptibility to heroin dependence (Saiz et al., 2008). Another gene which contributes to drug addiction is DRD4 (Dopamine D4 receptor gene) which has VNTR polymorphisms up to 10 repeats, which results in an altered function of the receptor with reduced efficiency (Asghari et al., 1995). In a study done on Sephardic Jews, Israel Arabs, Han Chinese from Chengdu, Southwest China, and a cohort American

Caucasians, polymorphisms in DRD4 gene was found to be associated with heroin abuse (Kotler et al., 1997, Li et al., 1997 and Vandenbergh et al., 2000).

The μ opioid receptor gene is a member of the endogenous opioid system. A polymorphism of receptor gene has been the primary focus of the potential associations with drug addiction. In fact certain, SNP in the μ opioid receptor has been associated with drug addiction. An allelic frequency of G38T and C17T in the μ opioid receptor gene has been associated with drug addictions. But only a higher frequency of the C17T allele was observed in drug dependent groups compared to that in controls (Berrettini et al., 1997). Similarly, another study also showed a higher proportion of C17T allele in opioid dependence in combined ethnic groups (Bond et al., 1998).

Studies on the SNP of A118G of the μ opioid receptor gene among African Caucasians from America, Han Chinese from Nanjing, Malays and Chinese from Singapore showed no significant difference in allele frequencies between opioid dependence and non - opioid dependence amongst the study subjects (Bond et al., 1998, Shi et al., 2002 and Tan et al., 2003). In contrast, an association of the A118G polymorphism with opioid dependence was detected in Indians from Singapore, Swedish from Central Sweden and Han Chinese from Hong Kong, it occurs more often in dependents compared to controls (Szeto et al., 2001 and Tan et al., 2003). However, within the Hispanic study subject group, the A118G variant allele was present significantly in higher proportions in non-opioid dependent subjects than in opioid dependence subjects (Bond et al.,1998). The authors suggest that the A118G polymorphism might confer some level of protection against opiate addiction. The susceptibility to heroin addiction of the human opioid

receptor gene involving SNP of δ opioid receptor T921C also has been studied. Among the German Caucasian population group, T921C substitution was found in a significantly higher allelic proportion of heroin dependent individuals than in controls (Mayer et al., 1997). However, a study conducted by Franke et al, in 1999 did not support the role of T921C polymorphisms in heroin dependence. Another SNP of δ opioid receptor, the T82G, was also shown to be not associated heroin addicts (LaForge and Kreek., 2000).

Allelic variations in the promoter region of the preprodynorphin gene have recently been associated with differences in individual vulnerability to cocaine addiction. Since the kappa opioid gene is activated by dynorphin peptides, it is hypothesized that allelic variations in the OPKR1 gene may alter functions or expression of the receptor, which may contribute to individual differences in vulnerability to develop opioid and cocaine addiction. In this regard, Single nucleotide polymorphisms G36T were found to be significantly high between dependence and than controls among American, Caucasian and Hispanic in New York (Yuferov et al., 2004).

1.4 Genetic Polymorphisms and Opiate Addiction

1.4.1 Endogenous Opioid System

In the search for specific alleles of specific genes that may contribute to the development of the addictions, many researchers have focused on the μ opioid gene of the endogenous opioid system, which mediates a diverse array of neurological, physiological, and behavioral functions. The endogenous opioid system is also centrally important in mediating the effects of drug of abuse and alcohol (La Forge et al., 2000).

The opioid system controls pain, reward and addictive behaviors; opioids exert their pharmacological action through opioid receptors. Stereo - specific ligand binding assays were used to identify these receptors (Ingoglia and Dole, 1970; Goldstein et al., 1971), and opioid receptors were discovered independently by three groups (Pert and Synder., 1973, Simon et al., 1973 and Terenius., 1973).

Endogenous opioid peptides are small molecules that are naturally produced in the central nervous system (CNS) and in various glands throughout the body, such as the pituitary and adrenal glands. The first endogenous ligands for opioid receptor were identified by Hughes and colleagues (1975), who isolated Leu and Met – enkephalin, followed by two other endogenous opioid peptides namely β– endorphin and dynorphin (Bradbury et al., 1976, Cox et al., 1976, Li and Chung., 1976 and Goldstein et al., 1979). Opioid receptors can also be activated exogenously by alkaloid opiates such as morphine and heroin which produce the same effect as peptides. By acting at the opioid receptors, opiates such as morphine and heroin are not only extremely potent pain killers, but also addictive drugs.

Endogenous opioid peptides can serve as hormones as well as neuromodulators. Peptides that serve as hormones are secreted and delivered to target tissues where they induce physiological response, whereas peptides that serve as neuromodulators are produced and secreted by nerve cells and react together with the brain, spinal cord and the neurotransmitters.

Binding assays with selective ligands allow classification of opioid receptors and they must bind to specific molecules or receptors to affect the function of their target cells. There are several receptors on the surfaces of cells but three major categories of opioid receptors are: (i) OPRM 1 which has endogenous ligand enkephalins and β endorphin, (ii) OPRD 1 which binds with enkephalins and (iii) the OPRK 1 which binds with dynorphins. These receptors belong to the family of seven transmembrane G protein coupled receptors (Knapp et al., 1994). These receptors differ both in their functions and in their binding characteristics. A series of biochemical events including analgesia and euphoria are initiated by the binding of opioid peptides to these receptors. Once released from the neurons, opioid peptides act through opioid receptors to transmit messages that primarily inhibit secondary systems, such as pain perception, and induce euphoria. (Kreek et al., 2005)

1.4.2 Opioid Receptor Gene OPRM1

1.4.2.1 General

OPRM1 was selected for human genetic studies for many reasons briefly because it is the molecular target of the active biotransformation product of heroin 6 – monoacetylmorphine and morphine as well as most opiates.

Earlier research on this gene led to the development of methadone maintenance treatment for heroin addiction in the 1960s wherein μ selective agonists with long acting pharmacokinetics like methadone and levo - α – acetylmethadol (LAAM) were used to treat this disorder effectively (Dole et al., 1966; Kreek et al., 2002). In studies of the quantitative trait loci in mice, the chromosomal region containing the opioid receptor

gene was identified as contributing significantly to the variance in analgesic and reward responses to morphine (Belknap and Crabbe., 1992, Kozak et al., 1994; Belknap et al., 1995 and Crabbe et al., 1999).

Studies of mice with targeted deletion of the μ opioid receptor gene definitively established this receptor as essential for morphine analgesia, physical dependence, and reward as measured by antinociception, withdrawal, conditioned place preference, and self-administration studies (Matthes et al., 1996, Sora et al., 1997, Kitanaka et al., 1998 and Becker et al., 2000).

1.4.2.2 Polymorphisms in the human μ - opioid receptor gene

Due to its role in mediating the analgesic and rewarding effects of opiate drugs, researchers have focused attention on the μ opioid receptor gene as a candidate in studies of polymorphism. Several recent studies have identified polymorphisms in this gene, including single nucleotide polymorphism (Befort et al., 2001, Beretteni et al., 1997, Bond et al., 1998, Hoehe et al., 2000, Szeto et al., 2001 and Wendel and Hoehe., 1998.) Single nucleotide polymorphisms of this gene are summarized in Table 1.2 and those that are found at positions of coding regions of the genes are identified in Fig 1.1.

Table 1.2: Single nucleotide polymorphisms in the human μ opioid receptor gene. Adapted from : Laforge et al, 2000

Nucleotide	Location gene	Amino acid substitution	Functional domain	Detection method
G – 54T	Exon I	NA	5' untranslated region	A
G – 38T	Exon I	NA	5' untranslated region	A, B
C12G	Exon 1	Ser4Arg (S4R)	N- termina	A
C17T	Exon 1	Ala6Val (A6V)	N – terminal	A
G24A	Exon 1	Synonymous (Thr8)	N- terminal	A
A118G	Exon I	Asn40Asp (N40D)	N- terminal	A, C, D
C440G	Exon II	Ser147Cys (S147C)	Transmembrane domain 3	A
A454G	Exon II	Asn152Asp (N152D)	Transmembrane domain 3	A
IVS2 G31A	Intron 2	NA	NA	A
IVS2 C691G	Intron 2	NA	NA	A,C

Abbreviation: NA- Not applicable

(A) sequencing of PCR amplified DNA
(B) Single strand conformation polymorphisms analysis
(C) PCR – restriction fragment polymorphisms analysis
(D) Allele Specific PCR

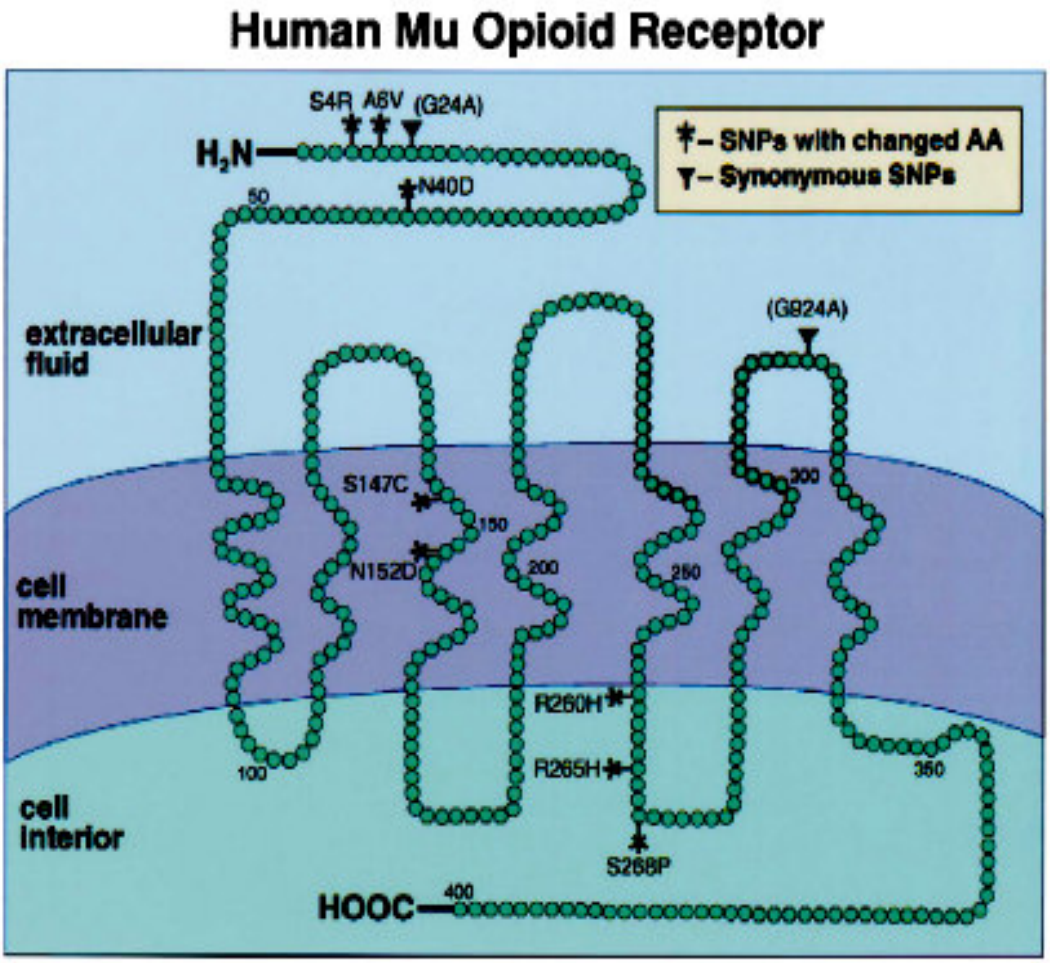

Figure 1.1: The human μ - opioid receptor gene and positions of identified coding regions of the single nucleotide polymorphisms. Adapted from: (Laforge et al, 2000)

1.4.2.3 A118G polymorphisms and drug addiction

The μ opioid receptor gene (OPRM1) has been the primary focus in studies of the potential association of endogenous opioid system genes with alcohol and drug abuse or with dependency. With one exception (Bergen et al,. 1999), all studies reported to date have used a classical case - control association study design. In the body and brain, heroin is rapidly biotransformed by stepwise deacetylation to monoacetyl morphine and then to morphine, both of which are potent μ opioid receptor agonists. A genetic polymorphism in the μ opioid receptor gene that causes differences in physiological response to long acting peptides such as β endorphin may therefore have relevance for this disorder. Studies of μ opioid knockout mice show that such mice have reduced prevalence and decreased self - administration of morphine and other μ opioid receptor agonists (Becker et al., 2000 and Matthes et al., 2000).

Several of these SNP are located at the coding region of the gene and alter the structure and function of the predicted amino acid receptors. Among all OPRM 1 polymorphisms, the A118G variant in exon 1 which alters amino acid sequence was one of the first discovered single nucleotide polymorphisms in the OPRM1 gene and has been evaluated in a number of genetic studies pertaining to opiate, alcohol and drug addiction (Bergen et al., 1997 and Bond et al., 1998).

This polymorphism substitutes amino acid asparagines to aspartic acid, which results in a charge difference and removal of a putative N – linked glycosylation site in the N terminal domain of the receptor (Bergen et al., 1997 and Bond et al., 1998). The altered function of A118G variant includes in vitro evidence for increased binding affinity of the

endogenous μ opioid ligand β – endorphin and increased activation of G protein. (Bond et al., 1998).

The differences in binding of β -endorphin and activation of the A118G variant receptors following β -endorphin binding in these in vitro cellular studies led us to predict that persons carrying the gene expressing the variant receptor might show altered function of physiological systems under the control of the μ opioid receptor, including, pain perception, reproductive function, and responses to stress mediated by the HPA stress-responsive axis, which is under tonic inhibitory control of this receptor (Bond et al., 1998; Kreek 2000 and LaForge et al., 2000a).

Two studies have identified such a difference in the HPA stress-responsive axis of persons with the 118A/118G or 118G/118G genotype (Wand et al., 2002 and Hernandez-Avila et al., 2003). In these studies, healthy control individuals were administered naloxone; subjects who carried one or more 118G alleles showed a greater activation of the axis, as measured by plasma cortisol, demonstrating a physiogenetic role for the A118G variant.

The A118G variant has been studied in a number of genetic studies, primarily case-control studies of opiate and other addictions. In some studies, evidence of an association of specific alleles in specific populations has been found while other studies have not obtained such evidence (Kreek et al., 2005).

Single nucleotide polymorphism A118G also shows no association or linkage to drug dependence among African Americans and Caucasians. However, within the Hispanic study - subject group, it was found that 118G allele was present in a significantly higher proportion of non - opioid dependence subjects compared to that in opioid dependence subjects. The author suggests that the A118G polymorphisms might confer some level of protection against opiate addiction, which is of particular interest given the differences in receptor activity (Bond et al., 1998).

A new PCR-RFLP method was developed to detect the polymorphism of A118G and studied in control and substance-dependent populations of African American (AA), European American (EA) and Hispanic origins, and in a series of populations differing in geographic origin (Japanese, Ethiopians, Bedouins, and Ashkenazi Jews) 891 subjects overall. The results shows allele frequencies for 118G were significantly different between AA and EA subjects, and there was significant heterogeneity among the more extensive set of populations. Furthermore, there were no significant differences in allele frequency by diagnosis indicating that polymorphism appears not to be a direct risk factor for substance dependence (Gelernter et al., 1999).

Polymorphism A118G was also studied in 282 Chinese heroin addicts from Sichuan Province, Southwest China, and their allele and genotype frequencies were compared to those in 258 normal controls from the same geographic region. The Asn40Asp did not differ significantly for allele ($p = 0.16$; $p = 0.21$) in heroin-addicted cases and in normal controls. This indicates that the μ opioid receptor is not likely to be a major genetic risk factor for heroin abuse in this population (Li et al., 2000).

The hypothesis of A118G (Asn40Asp) polymorphism of the μ opioid receptor gene (OPRM1) as a particular vulnerability factor for heroin and alcohol dependence was tested in two independent large samples by two different methods: a case-control sample (comprising n = 287 heroins versus n = 365 nondependent controls) in Germany. The studies were unable to corroborate the hypothesis of OPRM1 A118G polymorphism as a particular risk factor for any kind of substance dependence, including opioid addiction (Franke et al., 2001).

Polymorphism A118G was also studied in Nanjing, China among a Han Chinese cohort. The result shows that addicts having one G, which is heterozygous showed higher heroin intake but there, no correlation to opioid dependence when they were compared to controls (Shi et al., 2002).

Crowley and colleagues in 2003 studied 225 opioid-dependent and 200 screened controls matched for ethnicity (European American and African American) and recruited from the Philadelphia area. No differences between cases and controls in genotype or allele frequencies for the A118G SNP were found within either ethnic group (Crowley et al., 2003).

A significant difference in allele and genotype frequencies in 118G was found in Singapore where highly significant association with heroin dependence was found among Indians for both genotype distribution ($p = 0.024$) and allele frequency ($p = 0.009$) (Tan et al., 2004).

Heroin addiction was studied in Sweden, a geographic region known to have minimal admixture (139 cases and 170 controls). There is an association of the 118G allele in exon 1 OPRM1 gene contributing to increased OR for heroin addicts; the results show up to 21% of the attributable risk of heroin addiction mediated by the 118G allele. The subgroup of analysis was performed on subjects identifying themselves and both parents as Swedish (Bart et al., 2004).

A study was also done to delineate the frequency of these variants in the subjects of Indian origin and study their association with the phenotype of opioid dependence. The A118G was genotyped using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. The control subjects (n = 156) showed a frequency of 0.12 while the opioid dependents (n = 126) had an approximately 2.5-fold higher frequency of 0.31 (Odds Ratio 3.501; CI (95%) 2.212-5.555; $p < 0.0001$). A significant association was observed between the A118G and opioid dependence (Kapur et al., 2007).

About 336 Chinese Han heroin addicts and 245 healthy volunteers were recruited in Shanghai. Association analyses with the genotypes and alleles in nine tagging single nucleotide polymorphisms (tSNPs) including A118G in OPRM1 with subjective responses were performed. Similar analysis with haplotypes of these tSNPs was also performed. It was found that allele frequencies of three tSNPs including two of each A118G were significantly different between the positive and negative groups. Moreover, subjects with heroin-induced positive responses on first use consumed more drugs than

the negative group. The findings suggest that heroin-induced positive responses are likely to be associated with more heroin consumption (Zhang et al., 2007).

Some studies have provided support for the hypothesis that specific alleles of the μ opioid receptor gene were associated with opiate or other drug dependence but other studies have not. The majority of genetic association studies reported to date have evaluated the common single nucleotide polymorphism in the N-terminal domain of the receptor, which leads to potentially functional amino acid changes in the predicted primary structure of the receptor. Existing evidence with respect to specific addictive diseases supports either no effect or a protective effect from the 118G allele (La Forge et al., 2000). For a better understanding, characterized populations are necessary with interplay of genes, the environment and the specific drug (Kreek et al., 2003). Though μ opioid receptor polymorphism has been studied in USA, Europe and in few Asian countries, to the best of our knowledge it was not studied among Malaysian drug addicts.

1.5 Rationale of the study

a) This study will provide first - hand information on the genetic polymorphisms of μ opioid receptor gene in Malaysian drug addicts.

b) This research will help us to understand the interplay of genetic and environmental factors on the development of drug addiction.

1.6 Objectives

a) To investigate the prevalence of genetic polymorphism of μ opioid receptor gene among drug addicts and normal populations in Malaysia.

b) To correlate the genetic polymorphisms of the A118G allelic variant in μ opioid receptor gene with socioeconomic factors.

CHAPTER 2

METHODOLOGY

2.1 Demographic studies

2.1.1 Study Design

This study involved two parallel designs; namely with healthy volunteers who had no history of drugs or alcohol, and those who were long - term former heroin addicts.

2.1.2 Location of Study

The study was conducted at the Drug Rehabilitation Centre, Bukit Mertajam, Penang, Malaysia.

2.1.3 Number of subjects

About 80 drug addicts (male) who were long - term former heroin addicts currently in treatment programmes at the rehabilitation centre and 80 healthy volunteers (male) subjects were selected. Healthy control subjects were recruited primarily through notices and newspaper advertisements. The demographic profiles as well as the drug abuse patterns of the addicts were gathered through a questionnaire (Appendix A). All study subjects were interviewed extensively with respect to drug abuse (whether other substances were used or abused, e.g. cannabis, cocaine), the addictive diseases, and their psychological, psychiatric and medical profile. Individuals with psychiatric disorders were excluded. Information about family backgrounds regarding family origin, ethnic back ground, including state of birth or geographic area of birth, were also collected.

2.1.4 Inclusion Criteria

Volunteers were recruited from a list of registered volunteers. Volunteers met all the following criteria:

- Age 18 – 50 years
- No other drugs or medications, including over the counter preparations, ingested in the preceding week.
- Adequate venous access
- Written consent given after reading the information leaflet. Participation must be voluntary and volunteers were fully informed of the possible side effects.

Addicts met all the following criteria:

- Age 18 – 50 years
- Heroin Addicts
- Adequate venous access
- Written consent given after reading the information leaflet. Participation must be voluntary and volunteers were fully informed of the possible side effects