ASSOCIATION ANALYSIS OF SINGLE NUCLEOTIDE POLYMORPHISM OF OPIOID DEPENDENCE GENES AMONG MALAY MALES IN MALAYSIA

by

DEVAKI NAGAYA

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LIST OF ABBREVIATIONS

5-HTT	The Serotonin Transporter
A	Adenine
AADK	Agensi Anti Dadah Kebangsaan
ABCB1	P glycoprotein Gene
ADH	Anti-Diuretic Hormone
Ala	Alanine
AD	Allelic Discrimination
AIDS	Acquired Immune Deficiency Syndrome
ALDH	Aldehyde dehydrogenase
ANKK1	Ankyrin repeat and kinase domain
Arg	Arginine
ASW	African ancestry in South West USA
Asn	Asparagine
Asp	Aspartic acid
ATS	Amphetamine type stimulant
Bp	Base pair
C	Cytosine
CEU	European
CHB	Han Chinese Beijing
CHD	Chinese in Metropoliton Denver
CHRNA	Neuronal Acetycholine receptor subunit

Cm	Centimeter
COMT	Catechol O Methyltranferase
CYP	Cytochromes P450
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphate
DOR	Delta Opioid Recptor
DRD2	D2 subtype dopamine receptor
DRD4	D2 subtype dopamine receptor
DSP	Dual Specific Phosphates
EDTA	Ethylenediamine tetraacetic acid
EU	European Unions
F	Forward
G	Guanine
GABRA	Gamma aminobutyric acid receptor subunit
GCP	Good Clinical Practice
GIH	Gujarati in Houston
GWAS	Genome Wide Association Study
HCB	Han Chinese Beijing
HCl	Hydrochloric Acid
HIV	Human Immunodeficiency Virus
HTR3A	5 Hydroxytryptamine Receptor 3A
HTR3B	5 Hydroxytryptamine Receptor 3B

HW	Hardy Weinberg
IDU	Injection Drug Users
JPT	Japanese in Tokyo
KOR	Kappa Opioid Receptor
LD	Linkage Disequilibrium
LWK	Luhya in Webuye, Kenya
MAOA	Monoamine Oxidase A Gene
Mex	Mexican in Los Angeles
MOR	Mu Opioid Receptor
NA	Not applicable
NCBI	National Centre of Biotechnology
NCAM1	Neural Cell Adhesion Molecule 1
MDMA	3,4-Methylenedioxymethamphetamine
OD	Optical density
PCR	Polymerase chain reaction
OPRM1	Opioid Receptor Mu Gene
OPRD1	Opioid Receptor Delta Gene
OPRK1	Opioid Receptor Kappa Gene
RNA	Ribonucleic Acid
SNP	Single Nucleotide Polymorphism
SPM	Sijil Pelajaran Malaysia
SRP	Sijil Rendah Pelajaran Malaysia
STPM	Sijil Tinggi Pelajaran Malaysia

SUD	Substance Use Disorder
T	Thymine
Taq	*Thermus aquaticus*
TBE	Tris Borate - EDTA Buffer
Tet	Tetracycline
U	Unit
UPSR	Ujian Pencapaian Sekolah Rendah
UNODC	United Office on Drug & Crime
U.S.A	United States of America
Val	Valine

LIST OF SYMBOLS

μ	Mu
β	Beta
α	Alpha
~	Approximately
μγ	Micro gram
μλ	Micro liter
μM	Micro Molar
e.g.	for example
Hr	Hour
Kb	Kilobase
Kg	Kilogram
L	Liter
Mg	Milligram
Min	Minute
Ml	Milliliter
Mm	Millimeter
mM	Millimolar
Nm	Nanometer
MT	Metric Tonne
^{o}C	Degree Celsius
Pg	Pico gram

Pmole	Pico mole
R	Reverse
Sec	Seconds
T_a	Annealing temperature
U	Unit
V	Volt
Vol	Volume

ANALISIS HUBUNG KAIT POLIMORFISME NUKLEOTIDA TUNGGAL PADA GEN-GEN KEBERGANTUNGAN OPIOID DALAM KALANGAN LELAKI MELAYU DI MALAYSIA

ABSTRAK

Ketagihan dadah ialah gangguan kronik dan berulang yang dikaitkan dengan genetik. Banyak kajian telah melaporkan peranan polimorfisme nukleotida tunggal (SNP) terhadap pergantungan pada dadah. Antara kesemua gen tersebut, sejumlah dua belas (12) gen kandidat yang mempunyai kaitan dengan pergantungan pada dadah akan diselidiki dalam kajian ini. Gen kandidat ini ialah *OPRD1* (Delta Opioid Receptor), *OPRK1* (Kappa Opioid Receptor), *COMT* (Catechol – O-Methyltransferase gene), *PDYN* (Prodynorphin), *DRD4* (Dopamine receptor D4), *ABCB1*(P glycoprotein), *DUSP* (Dual Specificity Phosphatase 27) dan rs10494334.Matlamat kajian ini adalah untuk menentukan kekerapan SNP rs1042114, rs702764, rs199774, rs1022563, rs910080, rs737866, rs10494334, rs1800955, rs1128503, rs1045642 dan rs2032582, selain mengkaji hubungannya dengan pergantungan pada opioid dalam kalangan lelaki Melayu di Malaysia. Pangkalan data genetik ini akan dibangunkan untuk rawatan berasaskan farmakogenetik. Peserta kajian terdiri daripada 459 orang lelaki Melayu dengan pergantungan pada opioid dan 543 orang lelaki Melayu yang sihat sebagai kumpulan kawalan. SNP digenotipkan dengan menggunakan cerakin penjenisan gen SNPTaqMan. Analisis statistik dilakukan dengan menggunakan perisian Golden Helix SVS untuk mengenal pasti pengagihan frekuensi alel dan genotip serta interaksi SNP-SNP. Pangkalan data dibangunkan menggunakan MySQL - iaitu pangkalan data piawai de-

facto yang dijalankan pada pelayan yang dilindungi. SNP rs1042114 OPRD1, rs910080 PDYN, r1800955 DRD4, rs1128503, rs1045642, dan rs2032582 ABCB1 didapati mempunyai perkaitan kuat dengan ketagihan opioid pada tahap alel dengan $p < 0.05$. Interaksi signifikan secara statistik juga dikenal pasti antara kebanyakan alel risiko bagi kesemua SNP yang dikaitkan dengan ketagihan dadah dengan polimorfisme lain yang diselidiki dalam kajian ini. Pangkalan data yang dihasilkan daripada penyelidikan ini mengenai SNP ketagihan dadah boleh digunakan oleh pegawai perubatan bagi memahami kesan SNP dan interaksi SNP-SNP terhadap pergantungan pada opioid untuk merawat pesakit ketagihan dadah dengan berkesan tanpa tindak balas ubat-ubatan yang buruk.

ASSOCIATION ANALYSIS OF SINGLE NUCLEOTIDE POLYMORPHISMS OF OPIOID DEPENDENCE GENES AMONG MALAY MALES IN MALAYSIA

ABSTRACT

Drug addiction is a chronic and relapsing disorder is associated with genetics. There are many studies have been reported the roles of single nucleotide polymorphisms (SNPs) with drug dependence and among those, twelve (12) candidate genes that were associated with drug dependence will be investigated in this study. The candidate genes are *OPRD1* (Delta Opioid Receptor), *OPRK1* (Kappa Opioid Receptor), *COMT* (Catechol – O-Methyltransferase gene), *PDYN* (Prodynorphin), *DRD4* (Dopamine receptor D4), *ABCB1*(P glycoprotein), *DUSP* (Dual Specificity Phosphatase 27) and rs10494334. The goal of this study was to determine the frequencies of these SNPs rs1042114, rs702764, rs199774, rs1022563, rs910080, rs737866, rs10494334, rs1800955, rs1128503, rs1045642, and rs2032582 and to study their association with opioid dependence in Malay males in Malaysian population. This genetic database will be established for pharmacogenetics-based treatment. Research participants were 459 Malay males with opioid dependence and 543 healthy Malay males as controls. SNPs were genotyped using TaqMan SNP genotyping assay. Statistical analysis was performed using Golden Helix SVS software suite to identify the distribution of allele and genotype frequencies and SNP–SNP interactions. Database was developed using MySQL - the de-facto standard database that runs on a protected server. SNPs rs1042114 of OPRD1, rs910080 of PDYN, r1800955 of DRD4, rs1128503, rs1045642,

and rs2032582 of ABCB1 were strongly associated with opioid addiction at allelic level with $p<0.05$. A statistically significant interaction was also identified between most of the risk alleles of all the SNPs associated with drug addictions with other polymorphism studied in this research. The database established on drug addiction SNPs from this research will be useful for clinicians in the understanding of the effects of SNPs and the SNP–SNP interaction on opioid dependence to treat the drug dependence patients effectively without adverse drug responses.

CHAPTER 1: INTRODUCTION

1.1 Drug Addiction

Drug addiction is a chronic, relapsing brain disease, characterized by compulsive drug seeking behavior with strong genetic, sociocultural and neurodevelopment component. Despite the harmful consequences (Kreek et al., 2005b) and a major contributor to the global burden of disease worldwide (Degenhardt et al., 2013) an estimated 17 million people are afflicted with drug use disorder. Brain images from drug addicts have shown physical changes especially in the areas of decision making, judgment, learning, memory and behavioral control, which explains the compulsive drug seeking behavior among addicts (Fowler et al., 2007). Each drug binds to its protein target in the brain and elicits a combination of behavioral and physiological effects once it is administrated. Current evidence shows drug abuse exerts their reinforcing effects by activating, reward circuits that promote repeated drug use which eventually leads to addiction (Nestler, 2005). 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.

According to The World Drug Report (2017), published by United Nations Office on Drug and Crime (UNODC), in 2015 the global estimate of annual illicit drug use prevalence was at 5% of the global adult population. Amongst them, almost 12 million people inject drugs and out of this number, over 13% of people (or 1.6 million) who inject drugs are living with AIDS and 50% (6.1 million) are with hepatitis C.

Higher percentage of death were reported from Hepatitis C than HIV. According to the report, opioid was the top most common drug associated with fatal and non-fatal over dose (World Drug Report., 2017).

Many individuals are self-exposed to drug and many continue to use them on an occasional or even on regular basis. However, only some individuals develop specific addictions and vulnerability to addiction differs from person to person (Gerrits et al., 2003). Even after a prolonged period of abstinence, an individual can return to use drugs due to cravings and a desire to experience the enhancing effects of substance abuse (Markou et al., 1993, McLellan et al., 2000).

No single factor determines an individual tendency to be addicted to drugs, however, it is postulated that at least three different categories of factors may contribute to the vulnerability of developing a specific addiction. The first category is the environmental factors which include events during childhood and adolescence, peer pressure and self-exposure to the drug. The second category is drug induced factors, leading to a variety of molecular neurobiological changes, which cause altered behaviour (Kreek et al., 2005a, Kreek & LaForge, 2007).

The third category is genetics; several studies have suggested that illicit drug abuse and dependence are also under a significant genetic influence. Heritability studies put the estimated range at 45% – 79% among drug dependence (Tsuang et al., 1998, Kendler et al., 2003, Agrawal et al., 2006).

1.2 Global Epidemiological Data of Drug Addiction

1.2.1 America

It is estimated around 47.7 million people in the US, aged 12 years or older used illicit drugs or misused prescription drugs, which includes the use of marijuana, cocaine, heroin, hallucinogens, inhalants, or methamphetamine, and the misuse of prescription drugs. Estimated rates of use of illicit drugs in the past year by drug type were: 0.3 per 100 persons for heroin, 1.8 for cocaine, and 0.6 for methamphetamine (International Narcotic Control Strategy., 2017). Estimated rates for prescription drug misuse by drug type were: 4.7 per 100 persons for prescription pain relievers, 2.3 for tranquilizers, 2.0 for stimulants, and 0.6 for sedatives (Matsson et al., 2017, CL et al., 2017).

Marijuana is the most commonly used illicit drug, however 11.8 million people misuse opioid that is about 4.4% of the total population. It was reported that the number of opioid use increased 2.35 fold from 2002 – 2016, similarly number of deaths due to higher dosage opioid increased 6.33 fold (533%) from 2001 – 2016 (SAMHSA., 2017). Most of the opioid addicts were aged 45- 54 years (2016). Another study also reported that usage of heroin steadily is increasing since 2007 (Merikangas & McClair, 2012) in the US, this is due to the increase in misuse of heroin as pain reliever which is easily available and cheaper (Cicero et al., 2012).

1.2.2 Europe

More than 4% of the population is affected by alcohol or drug dependence (Wittchen et al., 2011). European Drug Report issued by European Monitoring Centre

for Drugs and Drug Addiction indicated that one quarter of European Unions (EU) populations had used illegal drugs. About 23.5 million people in the age group of 15-64 years old were involved in cannabis abuse, cocaine 3.5 million, MDMA 2.7 million and Amphetamine 1.8 million. The opioid abuse is only 1.3 million and 79% of deaths due to the drug over dose are caused by opioid abuse, with the mean age of 38 years. Heroin is the most common opioid in the European Drug Market, originating from Iran or Pakistan (European Drug Report., 2017)

1.2.3 Africa

Drug abuse is a major problem in South Africa, and shows an increase in 2016, where South Africa is the largest market for illicit drugs within sub-Saharan Africa and a trans-shipment point for cocaine and heroin (International Narcotic Control Strategy., 2017).

Large quantities of heroin started arriving on the eastern coast of Africa in the late 1990s when smugglers switched from their traditional overland routes from Asia to the sea route across the Indian Ocean (2013).

Cannabis is the most commonly used illicit drug in South Africa, it is also a large source of herbal cannabis for the United Kingdom and continental Europe. Recently, South African police seized approximately $4.8 million worth of heroin from a Pakistani national believed to be part of an international syndicate (World Drug Report., 2017). Drug abuse is currently costing around 20 billion a year for the South African government and in future, it can be the biggest threat to the government. It is also

reported that 60% of the crime is related to drug abuse (World Drug Report., 2017). In 2014, South Africa initiated a long-term project with U.S. support to further professionalize all substance use treatment staff in the country through the dissemination of U.S.-developed curriculum and international credentialing through the Colombo Plan's International Centre for Certification and Education of Addiction Professionals (International Narcotic Control Strategy., 2017).

1.2.4 Asia

In many Asian countries, the increased availability and variety of drugs such as heroin, cannabis, cocaine and amphetamines has led to a high prevalence of drug abuse. It is noted that India's geographic location makes it an attractive transhipment area for narcotics and there is evidence that in the northern part of India opium poppy is grown illicitly. The National Household Survey reported alcohol (21.4%) as the primary substance used followed by cannabis (3.0%) and opioids (0.7%) (Dhawan et al., 2017b). However, it is reported that India is authorized by the international community and the United Nations to produce licit opium for pharmaceutical uses, and these pharmaceutical items and precursor chemicals are vulnerable to diversion for illicit use. There is a high demand for methamphetamine, the increased profitability from the manufacturing and distribution of methamphetamine has transformed India into a significant precursor chemical source and supply warehouse (World Drug Report., 2017). According to the Indian Ministry of Home Affairs Annual Report 2013-2014, the Government of India seized 1,412 kilograms (kg) of heroin; 2,372 kg of opium; 47 kg of cocaine; 3,205 kg of Methaqualone; 68 kg of Amphetamines; 37,466,812 tablets of Psychotropic Substances; 1,356 kg of Ketamine; and 6,935 kg of Ephedrine and Pseudo-Ephedrine(World Drug

Report., 2017). India has one of the highest proportions of children and adolescents involved in substance abuse aged <18 years which is about 45% of the population and 5–19 years 35.3% of the population (Dhawan et al., 2017a). It is reported that the estimated number of 177,000 adults are injection drug users (IDUs) (Dhawan et al., 2016). It is also reported that abuse onset of IDU typically occurs in adulthood after 20 years of age, with a gradual progression from licit, gateway drugs in early adolescence to illicit substances (Solomon et al., 2010). India observed the United Nations sponsored International Day Against Drug Abuse and Illicit Trafficking on June 26, 2014, with programs focusing on raising awareness of the harmful effects of drug abuse. India had enhanced its law enforcement capacity through training to enforcement officers (International Narcotic Control Strategy., 2017).

Indonesia is also a destination country for illegal drugs especially for cannabis, methamphetamine, and heroin. It is reported that trafficking of methamphetamine and other synthetic drugs into Indonesia had increased in 2014 and heroin trafficking remained the same throughout the years. Cannabis is the most widely used drug in Indonesia and second highest is methamphetamine which is smuggled in through Iran, whereas heroin smuggled from Southwest Asia. There are estimated 4.7 million of drug users in Indonesia, the statistical analysis showed that 22% of the users are students and the most widely used narcotics are cannabis, methamphetamine and ecstasy. There is an increase in the drug use in the year 2014 although National Narcotics Board had a lot of outreach programs for the community (International Narcotic Control Strategy., 2017). The current government response to the ‘national drug emergency’ is dominated by

criminalizing substance use disorders (SUDs) which is ineffective. Interventions are now focusing more evidenced-based approaches to SUDs in Indonesia (Ayu et al., 2016).

It is well known that Pakistan is one of the world's top transit corridors for opiates and cannabis, which is trafficked through the countries from borders with Afghanistan. It is reported that around 40% of drugs like heroin and marijuana from Afghanistan are routed through Pakistan to China, Africa and Europe. Poppy cultivation had also increased in Pakistan in 2014. Pakistan also a major transit country for precursor chemicals used to produce heroin and methamphetamine. In 2013, UNODC released the results that Pakistan is a home to 6.5 million drug users who consume 59 metric ton of heroin and cannabis annually. In 2014 it was estimated that there are actually 6.7 million drug users, more than 3% of the country's population and most of the drug users are aged 15 to 64 (World Drug Report., 2017). A recent study conducted in Pakistan showed that out of 119 participants, around 71.4% were 15-35 years, 68.1% below secondary education and single (51.3%) and unemployed (44.5%) participants were at the greatest risk of using drugs. The data showed that majority of the drug dependence started as recreation (37%), curiosity (34.5%), and due to life changing events (14.3%) (Batool et al., 2017). It is reported that Pakistan lacks the capacity to treat drug addiction and educate the community. In 2014, Pakistan intensified efforts to raise public awareness about drug abuse (International Narcotic Control Strategy., 2017).

Methamphetamine or locally known as "shabu," in Philippines has been the primary drug consumed by the locals. Philippine authorities conducted several drug seizures and seized 660 kilograms (kg) of methamphetamine. It is reported that Chinese

drug trafficking organizations dominate the methamphetamine trade in the Philippines. The Philippine government conducting a lot of education programs aimed at promoting self-awareness (International Narcotic Control Strategy., 2017).

The national household survey showed that 3.5 million people had experienced at least one kind of illicit drug use in their lifetime and most of them aged between 12 and 65 years (National Survey., 2012). The country is mainly used as a trans-shipment point for trafficking to the international market. In 2014, Thai authorities seized large quantities of heroin, cocaine, MDMA (ecstasy), crystal methamphetamine, and methamphetamine tablets ("yaa-baa"). It is noted that in Thailand, there are no significant quantities of opiates, synthetic or other drugs cultivated or produced in 2014. However, in 2014 the authorities seized 210.22 kilograms (kg) of heroin, but there was a decline from 2013 (784.6 kg of heroin for the year) but an increase in comparison to 2012 (127.5 kg for the year). Thailand conducts demand reduction programs, which includes drug addiction prevention programs with treatment for addicts. It is also noted various therapeutic camps have been provided throughout the country (National Survey., 2012). It is reported that early adolescent sexual debut is linked to substance abuse, suggesting that associated factors need to be targeted to prevent early sexual initiation. These behaviours, along with drug use might persist into young adulthood, bringing the additional risk of contracting and transmitting HIV. However, further research is warranted which examines these factors to provide in-depth understanding (Thepthien et al., 2016). In Thailand, the drug treatment programs have gained over 700,000 drug addicts since the government announced its counter-narcotics priorities in September

2011 (International Narcotic Control Strategy., 2017). Therefore, they are in urgent need of more intensive and targeted interventions.

China is a major producer of synthetic drugs and drug precursor chemicals; it is considered a significant destination and transit country for illicit drugs. According to China's National Narcotics Control Commission 2014 Annual Report on Drug Control in China, heroin is the most abused drug in China followed by synthetic drugs such as ketamine, methamphetamine, and other amphetamine-type stimulants (ATS). Heroin is smuggled into China from Burma, Laos, Vietnam, Afghanistan, Tajikistan, and Pakistan. Methamphetamine and other ATS drugs manufactured in Burma also enter China from the "Golden Triangle" region (Burma, Laos, Thailand), and Vietnam(World Drug Report., 2017).The government has a lot of outreach program to raise awareness of the negative health effects of drug abuse and reduce the demand for drugs (International Narcotic Control Strategy., 2017).

1.2.5 Drug addiction in Malaysia

Drug addiction has been prevalent in Malaysia since the 19th century, and in the early 20th century, the main drug of abuse was opium, which was abused by Chinese and Indian immigrant laborers who were introduced by British colonialists to work in Malaya (Noorzurani et al., 2008). In 1970s heroin became the most abused substance of among Malays who is the main ethnic group in Malaysia compared to other ethnic groups and by 1980s heroin abuse among Malaysian youth was a national crisis (Rusdi A, 2008, Noorzurani et al., 2008). Illicit drugs are smuggled into Malaysia from the golden triangle area (borders of Thailand, Laos and Myanmar) as well as Iran, Nigeria

and India. Most of these countries use Malaysia as a drug trafficking hub. There is no notable cultivation of illicit drug crops in Malaysia (International Narcotic Control Strategy., 2017).

It was reported that the cumulative number of registered people who experienced problems with substance use in Malaysia between 1988 and 2006 was 300%, 241%, and 60.7% of these were opioid misusers and an increase in HIV cases due to needle sharing (MyHealth., 2016). Studies have shown that between 2010 and 2015, there were 127606 registered drug addicts in Malaysia. In 2015, 26668 drug addicts were reported and there were only 21777 in 2014 (Table 1.1). In 2015, new addicts comprised 77% of the total addicts registered while 23% were relapse cases. Majority of the registered addicts were male (98%). Statistics also indicated that 80% of the addicts are Malays, 8% Chinese, 8% Indian and 4% were others. Most of them fall in the 20 – 39 age group, about 69.09%, majority of drug abusers were labourers (21%) and part time worker (27%) and followed by jobless 14%.

Drug addiction also correlates with the education background. The data showed that 20% of addicts were either not educated or having only primary level school education or were SRP/PMR dropouts. Around 84.1% of them were SRP/PMR or SPM leavers and only 4% have got tertiary education. It is reported that 61% of drug addicts were influenced by friends and in terms of type of drug being abused in the country. Heroin continues to be the main drug being abused by Malaysian; constituting 61%, followed by Methamphetamine 30%, Ganja 5% and Ecstasy and Amphetamine 5%. The usage of heroin had increased from 49% in 2010 to 75% in 2013, 68% in 2014 and drop

to 60% in 2015. It is also noted that in 2014, RM 26.405 billion heroin was seized by the Malaysian authority, it shows that there is a drastic change in the demand of heroin among Malaysia addicts (World Drug Report., 2017).

Since its implementation, methadone treatment has successfully reduced the prevalence of drug dependence and HIV infection among drug users in Malaysia from 74.7% in 2000 to 19.3% by December 2014 (Annual Report., 2014). Latest report demonstrated the effectiveness of methadone treatment in reducing heroin dependence and HIV infections as well as in improving social dependence (Ali et al., 2017)

Table 1.1: Number of drug addicts reported in 2015 and 2016

Status	2014	2015	Difference 2014/2015
New addicts	13605	20289	+32.9%
Relapse	8172	6379	+21.9%
Total addicts	21777	26668	+18.3%

Adapted from: Agensi Anti Dadah Kebangsaan (AADK) report 2017

1.3 Molecular Basis of Addiction

Heredity is a major risk of addiction; research had shown that 40-60% of addiction risk is attributable to the genetic factor, addiction genes and biological differences that make someone more or less vulnerable to addiction (Nestler, 2000). Several methods were used to study the influence of genetic on addiction, such as family, twin and animal studies. Twin studies revealed that familial aggregation of addiction was influenced by genetic factors (Agrawal & Lynskey, 2008), due to the

segregating genes that are shared by the twins. It is also influenced by age and other exposure (Agrawal et al., 2012). Substance related addiction is ranked very low at a young age and increases during adolescence and adulthood (Kendler et al., 2008). The latest report by corroborating previous findings found substance use is moderately high from early adolescences into young adulthood (Waaktaar et al., 2017) and it is supported by genetic studies (Palmer et al., 2015). Animal study is crucial in understanding the biology and pathophysiology of addiction. Knockout mice targeting each gene of a system have been created two decades ago, mutant mice represent a unique tool to test specific role of each addiction gene. In contrast to clinical studies, the subjects can be controlled according to the study need (Becker et al., 2002, Charbogne et al., 2014). Behavioural testing in mice is limited, however new methods or models are being improvised to characterize the addiction studies better. 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 (Nestler, 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, 2000). Other studies have shown that opiates, cocaine, cannabis, amphetamine, 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 (LaForge et al., 2000, Agrawal & Lynskey, 2008, Kendler & Myers, 2015).

Some of the induced alterations in the gene may be long lasting or even permanent. Therefore, cellular or physiological systems that show alterations in response to substances of abuse might show higher or lower expression of the receptor which is postulated by genetic differences from polymorphisms (LaForge et al., 2000, Sagheddu & Melis, 2015). These may also underlie the development of substance abuse susceptibility (Kreek et al., 2005a, Sweatt, 2016).

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 addiction (Kreek et al., 2002, Volkow et al., 2012, Pandey et al., 2017).

1.3.1 Molecular basis of alcoholism

Alcohol is unique among substance abuse drugs, as it is a natural by-product of fermentation (Marugame et al., 2007), it has an estimated heritability of 50 % -70% varies with diagnostic criteria, population and gender (Tyndale, 2003, Ducci & Goldman, 2012). Researchers have investigated the genetic components by studying population of a family and inheritance of alcoholism among twins. Family and twin studies have supported the conclusion that the proportions of risk for this disorder are explained by genes which are the heritability. Twin studies are expected to have a similar history for developing alcoholism between the twins due to the similar genetic expression. Thus, it is concluded that genetic makeup of each individual can accelerate to addiction (Heath et al., 1997, Prescott & Kendler, 1999, Kendler et al., 2003).

Alcohol metabolism and the rate of its degradation products are important to determine its physiological effects. The primary pathway involves the conversion of ethanol to acetaldehyde plays a major role in mediating aversive and rewarding effects of ethanol. Acetaldehyde is oxidized further to acetate by aldehyde dehydrogenase. The key enzymes involved in alcohol metabolism are *ADH* and *ALDH* (Thomasson et al., 1995, Chen et al., 1999).

It is reported that alcoholism is less common in East Asian and Polynesian populations than in European populations, due to protective *ADH* and *ALDH* alleles (Chambers et al., 2002, Gelernter et al., 2014, Galinsky et al., 2016). *ADH1B*3* allele reveals a higher activity of ethanol oxidation and also reported to have a protective effect against the risk of alcohol dependence (Edenberg & Foroud, 2006). Similarly, *ADH1B*2* is a proactive allele, it is reported to be lower among alcohol dependence (Whitfield, 2002, Konishi et al., 2004, Higuchi et al., 2004, Bierut et al., 2012), this is a result of faster aldehyde production which might lead to unpleasant alcohol reaction. It is also recognized that *ADH1B* locus is under strong selection in East Asians and a study showed an independent evolution of the same locus also in Europeans (Galinsky et al., 2016). This functional variant is negatively associated with drinking behaviours, mainly in European populations (Gelernter et al., 2014).

Allele *ADH1C*2* of *ADH1C* gene was reported to have a higher risk of alcohol dependence (Mulligan et al., 2003, Konishi et al., 2004, Zintzaras et al., 2006, Li et al., 2012a). Allele *ALDH2*2* from *ALDH* gene also lacks of activity to catalyse acetaldehyde, it is found in East Asian population it causes high concentration of

acetaldehyde after drinking alcohol and serious adverse effect reaction facial flushing, hypotension, headaches and nausea (Yoshida, 1992, Chen et al., 1999) An alcoholic with inactive *ALDH2*2* have higher novelty seeking (Marugame et al., 2007).

Polymorphisms in two other enzymes, *ALDH1A1* (Lind et al., 2008, Sherva et al., 2009) and *ALDH1B1* (Linneberg et al., 2010,), have also been associated with alcohol consumption in Finnish, European American, European, Indo Trinidadian and Danish populations, respectively. Minor alleles at *ADH1B* (i.e., rs1229984 and rs2066702) are associated with lower levels of drinking (Xu *et al*, 2015). Consistent with the genetic associations, these variants increase the alcohol oxidization rate, raising acetaldehyde levels and its related aversive symptoms, including facial flushing, nausea, headache, and tachycardia (Edenberg, 2007).

It is also noted that *ALDH* rs672 another variant which very rare or absent in non-Asian population is negatively associated with alcohol use behaviours in Asian (Quillen et al., 2014). This variant causes a lack of acetaldehyde metabolism (Peng et al., 2014), which will produce an accumulation of acetaldehyde which will cause adverse effect reaction.

Human genetic studies have identified many other polymorphisms associated with alcohol dependence in genes that comprise various neurotransmitter-signalling pathways. This is including dopaminergic including Monoamine Oxidase A (*MAOA)* Catechol – O- Methyltranferase (*COMT)*, Dopamine Receptor D2 (*DRD2)*, Ankyrin Repeat and Kinase Domain 1(*ANKK1)* Tetratricopeptide Repeat Domain(*TTC12)* and

Neural Cell Adhesion Molecule 1 (*NCAM1)* (Köhnke et al., 2005, Tikkanen et al., 2009, Hendershot, 2011).

Serotonin Transporter Protein (*5-HTT),* Solute Carrier Family 6 Member 4 (*SLC6A4)* 5 Hydroxtryptamine Receptor 3A (*HTR3A*), 5 Hydroxtryptamine Receptor 3B *(HTR3B* and 5 Hydroxtryptamine Receptor 1B (*HTR1B*); (Cao et al., 2011, Seneviratne et al., 2013). GABAergic gene (Gamma Aminobutyric) (e.g., Gamma Aminobutyric Acid Type A Receptor Alpha 1 (*GABRA1*), Gamma Aminobutyric Acid Type A Receptor Alpha 2 (*GABRA2*) and Gamma Aminobutyric Acid Type A Receptor Gamma 1 (*GABRG1*); (Covault et al., 2004, Lappalainen et al., 2005, Agrawal & Lynskey, 2006, Dick et al., 2006b, Dick et al., 2006a, Covault et al., 2008, Enoch, 2008, Ittiwut et al., 2012). The opioid receptors Opioid Receptor Mu 1 (*OPRM1*), Opioid Receptor Delta 1(*OPRD1*) and Opioid Receptor Kappa 1 (*OPRK1*) (Ray & Hutchison, 2004, Zhang et al., 2008a, Xuei et al., 2006) and tachykinin receptor 3 (Foroud et al., 2008).

1.3.2 Molecular basis of smoking

It is estimated around 7 million smokers in the world today and most of them start smoking before the age of 18. Around 47.5% were male smokers compared to female. It is the cause of 6 million deaths around the world today(WHO report., 2017). Studies had shown that genetic factors do contribute to smoking from initiation to dependence to smoking (Bierut, 2011). Heritability estimates vary in each study, adolescent twins smoking behaviour ranges from 15% to 86%, population based twin studies provided evidence that genetic plays a larger role in smoking behaviour by late adolescence (Karp et al., 2005, Kendler et al., 2008, Do et al., 2015).

Several large Genome Wide Association Study (GWAS) of smoking quantity identified associations with genetic variants in the nicotinic acetylcholine receptor *CHRNA5-CHRNA3-CHRNB4* subunit cluster on chromosome 15q25.1 in populations of European ancestry (Thorgeirsson et al., 2010, Furberg et al., 2010). A study by Saccone and colleagues observed that the non-synonymous SNP rs16969968 in exon 5 of *CHRNA5* has consistent effects on the risk for nicotine dependence in both European and African populations, despite a large difference in allele frequency for the SNP (Saccone et al., 2009a). Some studies identified few other SNPs from this cluster, rs578776 in the 3′ untranslated region of *CHRNA3* that has low linkage disequilibrium with rs16969968 is associated with smoking dependence in European Americans but not in African Americans (Chen et al., 2012).The SNP in *CHRNA5*, rs588765, confers a protective effect for smoking dependence in populations of European descent (Saccone et al., 2009b, Wang et al., 2009). A comprehensive meta-analysis confirmed that these loci in nicotinic receptor gene affect smoking dependence (Wang et al., 2009).

The main enzyme implicated in nicotine metabolism is *CYP2A6* a polymorphic enzyme gene (Hukkanen et al., 2005). Single nucleotide polymorphism (SNP) in this gene highly polymorphic and generate isoforms. It causes variation in enzymatic activity therefore the concentration of nicotine also varies among individuals (Malaiyandi et al., 2005). A number of studies have reported the association between reduced or absent *CYP2A6* enzyme activity and lower risk of smoking, including decreased cigarette consumption, smoking intensity, and withdrawal symptoms; shorter smoking duration; and increased cessation (Malaiyandi et al., 2006, Thorgeirsson et al., 2010, Liu et al., 2011, Pan et al., 2015). However, some studies have failed to detect any association

between *CYP2A6* variation and smoking addiction, which is an active smoking (London et al., 1999, Schulz et al., 2001, Tanner et al., 2017).

Some genetic studies of smoking dependence have successfully identified risk factor for addiction using both GWAS and candidate gene approaches. However, these associated genetic factors explain only a small percentage of the variance in smoking dependence, indicating that further research to detect other genetic factors influencing smoking is warranted.

1.3.3 Molecular basis and drug addiction

Drug addiction is a chronic relapsing disease of the brain caused by drug-induced direct effects and persisting neuroadaptations at the epigenetic, mRNA, neuropeptide, neurotransmitter, or protein levels (Kreek et al., 2005a, Kreek et al., 2012). The identification of specific genes and functional loci moderating vulnerability has been challenging because it involves changes in anatomy, function, cellular and molecular neuroadaptations (Hyman & Malenka, 2001).

Many genes show significant association or display evidence of linkage with drug addiction for several decades using specific molecular genetic approaches like genetic animal model, candidate gene screening, genome wide association and genome wide linkage analysis (Li & Burmeister, 2009). Significant progress has been made to identify susceptibility genes for addiction however, only a small subset of these genes has a polymorphism for which an alteration in function has been involved in susceptibility (Tsuang et al., 1998, Kendler et al., 1999, Kendler et al., 2003, Kendler &

Myers, 2015). The implication of epistatic factors, epigenetic changes and gene–environment interactions make this task even more challenging. Translational and reverse translational research methods will be useful tools for a better understanding of the impact of gene variants on biological networks involved in addiction (Bühler et al., 2015). Other than that it is always difficult to collect larger sample size for substance abuse, it is noted that large sample will achieve the level of statistical power required and will provide needed results on genetic influence on addiction (Kalsi et al., 2016).

Identification of specific genes conveying increased risks not only for understanding the causes and potential treatments for disease, but also for increasing our understanding of how genetic and environmental risks interact to shape liability to addiction. It is noted that addiction involves a wide range of genes; there is more than one gene responsible for addiction. It is complicated mechanisms, any individual technology platform or study may be limited or biased (Goldman et al., 2005). There is a need to combine data across technology platforms and studies that may complement one another. The resultant gene list, in a database with additional functional information, definitely will be a valuable resource for the community. Systematic and statistical analysis of the genes and the underlying pathways may provide a complete picture of the molecular mechanism underlying drug addiction.

1.4 Drug Addiction Genes

1.4.1 *OPRK1*

The Kappa opioid receptor OPRK1 gene encodes KOP-r and is located on chromosome 8q11.2. It is found in the mesolimbic pathway, the binding ligand is dynorphin derived from prodynorphin were reported to inhibit dopamine neurons of the mesolimbic system, reduces dopaminergic tone in the striatum causes dysphoric and adverse effect (Shippenberg & Herz, 1986, Herz, 1998, Yuferov et al., 2010). It plays an interesting role in addiction of drug abuse and other rewarding stimuli because it is considered counter the modulatory mechanism of the brain, it directly or indirectly induced dopaminergic stimulation (Kreek et al., 2005a, Kreek & LaForge, 2007). Therefore KOR agonist may be potential therapeutic agents for the treatment of drug addiction (Shippenberg & Rea, 1997). Several SNPs in the human *OPRK1* gene have been reported, which are associated with drug addiction. In Western European, heroin dependent was genotyped for rs1051660, there was a significant association with drug dependent (Gerra et al., 2007). Similar results were reported among African American, Caucasian, Hispanic and Asian American (Yuferov et al., 2010). However, in contrary a large haplotype study by Zhang et al, 2008 showed no significant association among European American opioid dependence and control group (Zhang et al., 2008a). A study by Xu.S and colleagues proved that rs6989250 of OPRK1 gene also associated with greater subsequent cocaine relapse risk (Xu et al., 2013) it is suggesting inter population variation. There is also an association with drug withdrawal symptoms from the variants of *OPRK1* such as rs7832417, rs1698853, rs702764 and rs7817710 (Wang et al., 2014) and also with variant rs6473797 (Jones et al., 2016). There may a significant influence of

genetic and drug withdrawal biochemical mechanism which needs further investigation. The ability to predict which individual may experience greater drug withdrawal symptoms may increase the success rate of a treatment.

1.4.2 *OPRD1*

The *OPRD1* gene encodes the δ-opioid receptor (DOR) is located on chromosome 1p34, a G-protein-coupled receptor that regulates reward effects in the brain through activation of downstream MAP kinase pathways (Herz, 1998), which has enkephalins as its endogenous ligands. The Delta Opioid Receptor (DOR) functions in nociceptive responses but has also been shown to be involved in modulating the effects of Mu opioid receptor (MOR) directed compounds. Mice with targeted deletion of the *OPRD1* gene do not develop tolerance to the analgesic effects of morphine, although still becoming physically dependent on the drug (Zhu et al., 1999, Filliol et al., 2000). DOR plays an important role in the development of opioid tolerance (Daniels et al. 2005) and is involved in the rewarding and analgesic effects of opioids (Le Merrer et al., 2009). The Delta opioid receptor also becomes functional in the maintenance of opioid rewarding properties after prolonged exposure to opiates (Hack et al., 2005, Ma et al., 2006). Many SNPs in the *OPRD1* gene have been defined (Mayer et al., 1997, Gelernter & Kranzler, 2000) however only a few association studies on substance dependence were conducted between variations of *OPRD1* SNPs. A study was conducted among German Caucasians with heroin addiction, a significant association was reported for C921T (rs2234918) (Mayer et al., 1997).

Latest research provided an evidence implicating *OPRD1* SNPs (rs2236857 and rs581111) in liability for heroin dependence (Nelson et al., 2014). In another study, the opioid substance dependence among European American observed significant opioid dependence risk associated with rs1042114, a coding SNP in OPRD1, but not with other *OPRD1* SNPs. Another investigation (Levran et al., 2008a) that had a larger sample of heroin dependent cases reported a putative association with three SNPs in *OPRD1*. The haplotype association study among African American between rs1042114 and rs2234918 also showed a nominally significant association with opioid and cocaine addiction (Crist et al., 2013b). In association and family study of *OPRD1* with drug dependence, Franke and colleagues failed to find an association for rs2234918 among the German origins (Franke et al., 1999).

Similarly, a recent research conducted for SNP rs2236861 showed a significant association with drug addiction with control group compared to drug dependents (Randesi et al., 2016). Although earlier research showed significant association with drug dependence (Levran et al., 2008a, Beer et al., 2013). There was also no significant evidence for the role of variant rs1042114 among subjects from China, the G allele was totally absent from both substance dependent and control subjects (Xu et al., 2002). This report supported by another study, which showed no significant association with drug dependence among the Chinese population (Xuei et al., 2007). In this current study, we will be able to provide data on the minor allele G in Malaysian population, the absence of variant G will confirm the non-existence of the variant among the Asian population.

1.4.3 *PDYN*

The human prodynorphin gene (*PDYN*) is located at chromosome 20. It consists of four exons, exon 1 and exon 2 contain the 5′-untranslated region, exon 3 encodes a signal peptide, and exon 4 encodes dynorphin peptides, including α-neoendorphin, β-neoendorphin, dynorphin A and dynorphin B (Cox, 1982, Nikoshkov et al., 2005). Dynorphin peptides and prodynorphin mRNA are particularly abundant in the nucleus accumbens, caudate, amygdala, hippocampus, and hypothalamus (Mansour et al., 1994, Hurd, 1996, Akil et al., 1998).

PDYN precursor (Schwarzer, 2009), bind to all three opiate receptors, but it shows a preference for the kappa opioid receptor. Dynorphins are believed to mediate the aversive effects of drugs of abuse as experimental administration of KOR agonists in animals produces place aversion (Mucha & Herz, 1986, Shippenberg & Herz, 1986, Land et al., 2008, Chartoff et al., 2012, Koob, 2017) and dysphoria (Shippenberg et al., 2007). This is believed to be due, in part, to a reduction in dopaminergic neurotransmission on KOR stimulation and increased PDYN gene expression (Nestler, 2004). Exposure to cocaine upregulates dynorphin immunoreactivity in the brain regions in the caudate and ventral palladium (Hurd & Herkenham, 1995, Staley et al., 1997), it is noted that a chronic exposure to heroin increases *PDYN* mRNA in the central amygdala and nucleus accumbens shell (Solecki et al., 2009). A Naloxone-precipitated withdrawal was found to accentuate the increase in morphine-induced dynorphin expression in the striatum and accumbens in rats (Nylander et al., 1995). Such studies have led researchers to hypothesize that dynorphin may contribute to the negative emotional states

experienced during withdrawal from drugs of abuse and motivate continued drug use as a consequence of negative reinforcement (Koob & Le Moal, 2008, Wee & Koob, 2010).

Variants of the prodynorphin gene have been studied in addiction for opiates, cocaine, and alcohol, as well as in *in vitro* functional studies. Many of the variants in the *PDYN* were associated with increased risk for drug addiction (Ray et al., 2005, Nikoshkov et al., 2008). In China, it was reported that rs35286281, rs1022563, rs2235749 and rs910080 associated with heroin addicts (Wei et al., 2011) and in the United States the researchers identified an increased risk of developing opioid dependence in the female for the SNP rs1022563 (Clarke et al., 2009). Another study that was conducted by a similar group and the findings showed that the OR for rs910080, rs199774 and rs1022563 were increased in the female opioid dependent among European Americans; however there was no association for female African American population (Clarke, 2012). In another association study, rs910080 was associated with cocaine addiction among European American and African American (Yuferov et al., 2009). The results show that there is a difference in the genetic profile between male and female. Future studies should be focusing on both genders; such differences have implication on drug dependence recovery therapy.

1.4.4 *COMT*

The catechol-O-methyltransferase (*COMT*) enzyme metabolizes the catecholamines dopamine, epinephrine and norepinephrine, and is a key modulator of dopaminergic and adrenergic neurotransmission. Catechol-O-methyltransferase (*COMT*) plays a major role in brain catecholamine metabolism by catalyzing the transfer of a

methyl group from S-adenosyl-methionine (SAM) to catecholamines (Chen et al., 2004). *COMT* is in the chromosome 22q11.21–q11.23, it is frequently included in the velocardiofacial syndrome (VCFS) deletion region (Grossman et al., 1992). Numerous genetic associations have been reported for several SNPs or haplotypes at the *COMT* locus. These include VCFS-related traits (Lachman et al., 1996, Shifman et al., 2002, Bearden et al., 2004, Gothelf et al., 2005), schizophrenia (Lachman et al., 1996, Shifman et al., 2002), anxiety-related personality traits (Stein et al., 2005), pain sensitivity (Diatchenko et al., 2005, Nackley et al., 2006), psychological stress response (Jabbi et al., 2007), and nicotine dependence (Beuten et al., 2006).

Variant rs737866 SNP was associated with cocaine and heroin addiction, haplotype association between AAT of rs737866, rs4680 and rs174696 were conducted among European American and African American, this haplotype was significantly associated with cocaine in this two populations. It was concluded that the association was due to its role in the metabolism of dopamine and norepinephrine, cocaine interferes the process between dopamine and the receptor by binding to it which is the reward pathway for drug dependence (Ittiwut et al., 2011, Baik, 2013). Another study in China with heroin dependents proved that TT genotype of rs737866 has a higher association with addiction compared to CT and CC. It is noted that individuals with COMT variant gene increase the enzymatic activity with substance abuse. They may experience long lasting excitement and may increase the severity of drug dependence. However, in this study, wild type genotype TT was associated with heroin; it is noted in this report that study was not compared with control group, which might be the limitation (Li et al., 2011).