CHAPTER I
INTRODUCTION

General background

Alcohol is a legal substance, which is used worldwide and socially acceptable. Alcohol drinking seems to be a normal phenomenon among males. The benefit of alcohol in a social aspect is as a social lubricant and for solidarity. It has caused many people around the world, especially male, consume a lot of alcoholic beverage. Global alcohol consumption has been increasing in recent decades, especially in developing countries, including Thailand. Given the problems associated with alcohol use and abuse. Alcohol abuse are recognised and classified as mental disorders. Hazardous and harmful use of alcohol is associated with a higher incidence of various types of cancer, liver disorders, gastrointestinal problems, diabetes complications, cardiovascular problems, birth defects, neurologic complications and mental disorder. It also has social consequences, affecting the lives and indeed the mental health of those people who live or work with an alcohol-dependent person (WHO 2005). Alcohol may contribute 3.2% of death worldwide. This proportion is much higher in male (5.6% of death) than female (0.6% of death) the major alcohol related cause of death is by traffic accident (WHO 2002).

Alcohol consumption in Thailand is estimated to be 2.0 litres of pure alcohol per capita in the people older than 15 years of age. The 62% of traffic accident victims reveaked a positive blood alcohol concentration. Approximately 45% of deaths from traffic accidents are due to alcohol consumption. The cost of alcohol-related illness per person per admission was estimated to be over 20,000 Baht in 1992 which include costs in medical treatment, indirect costs from lost earnings, decreased productivity of the patient and family, transportation, and other non-medical equipment and food (WHO 2004).

Alcoholism

Alcoholism is the technical term for alcohol use disorder which can be distinguished by DSM-IV criteria of American Psychiatric Association as alcohol intoxication, alcohol abuse, alcohol dependence and alcohol withdrawal (Zernig et al., 2000).

Alcoholism is a disease that involves physical and psychological addiction to the alcohol consumption. It is chronic, progressive, often fatal and marked at a level that interferes with physical or mental health, society, family, or occupational responsibilities. People with alcohol dependence, the most severe alcohol disorder, usually experience tolerance (a need for markedly increased amounts of alcohol to achieve intoxication or the desired effect), and withdrawal symptoms when alcohol is discontinued or intake is decreased. They also spend a great deal of time obtaining and drinking alcohol. Alcohol abusers are "problem drinkers", i.e. they may have

legal problems, such as drinking and driving, or binge drinking (drinking six or more drinks on one occasion). People who are dependent on or abuse alcohol return to its use despite evidence of physical or psychological problems, and those with dependence have more severe problems and a greater compulsion to drink. The life-span of an alcoholic is shortened by an average of 15 years, as a result of various complications of the disease.

The essential features of alcohol addiction involve tolerance, dependence and reinforcement, which is due to neuroadaptation. Tolerance to alcohol occurs following chronic consumption when higher doses of alcohol must be ingested to achieve a given effect. Reinforcement refers to the connection between behavior and stimulus, whereby the chance of repeated behavior (e.g. alcohol-seeking) is enhanced if the action results in obtaining a reinforcing stimulus (e.g. the desired effects of drinking an alcoholic beverage)(Steward et al., 1997). Reinforcement is a key phenomenon in the development of addiction to alcohol and other drugs. Positive reinforcement is the process by which an action that becomes repetitive results in pleasure, reward. Many people find the mental effects of alcohol consumption (e.g.euphoria) rewarding; and this effect may lead to positive reinforcement and persistent alcohol-seeking behavior. The brain's adaptive changes to the continued presence of alcohol result in feelings of discomfort and craving when alcohol consumption is abruptly reduced or discontinued. These feelings reinforce alcohol-seeking behavior during abstinence. The motivation of behavior based on evidence of discomfort is called negative reinforcement. Both positive and negative reinforcement play a role in alcoholism (Valenzuela 1997). Dependence represents an adaptive state that develops as a homeostatic response to repeated drug administration. Dependence is typically unmasked when drug taking stops, leading to withdrawal symptoms. The molecular mechanism of withdrawal involves the homeostatic adaptations in response to ethanol that decreases $GABA_A$ receptor expression and increases NMDA receptor expression on some neurons. The decrease in receptors for the major inhibitory neurotransmitter and the increase in excitatory receptors would make neurons intrinsically more excitable. With removal of ethanol, a drug that facilitates $GABA_A$ receptor-mediated Cl- currents and inhibits NMDA receptors, a state of increased neural excitability would be unmasked leading to withdrawal symptoms such as agitation, tremor, hypertension, and seizures. Withdrawal symptoms may even emerge during active drug use as a result of tolerance, helping to drive increasing dosages or shorter intervals between doses (Davis et al., 2002). The association of acute alcohol ingestion and euphoria leads to more tolerance and dependence.

In the USA, the proportion of alcohol use in males over 18 years of age is 67.7% and 11.8% of these are alcohol dependent (Zernig et al., 2000). In Thailand, the prevalence of alcoholic in government hospital was 1.8%, 1.9% and 2.1% in 1999, 2000 and 2001, respectively (กรมสุขภาพจิต 2546).

The etiology of alcoholism involves many factors. Firstly, it concerns family, and an important risk factor for developing the disease is having an alcoholic parent. Although environmental and interpersonal factors are important, a genetic predisposition underlies alcoholism, particularly in its more severe forms. Hereditary alcoholism (the genetic component of interindividual variation in vulnerability) is 40 to 60 percent. In the National Comorbidity Survey of 5,877 individuals, it was found

that alcohol use disorders aggregate significantly in families with an odds ratio of 1.93 (Kendler et al., 1997). Having an alcoholic parent is a significant risk factor for the development of the disease; sons of alcoholics are 4 to 9 times more likely to develop alcohol-related problems than sons of nonalcoholics.

At the present, the genes for alcohol metabolism are the only ones that are known to have a major impact on the development of alcoholism. One gene variant (allele) is protective and the other is a vulnerability allele. Alcohol dehydrogenase (ADH) metabolizes ethanol to acetaldehyde, a toxic intermediate, which is in turn converted to acetate by aldehyde dehydrogenase (ALDH). Approximately half the population of Asian countries such as China, Japan, and Korea have functional polymorphisms at four different genes: *ADH2, ADH3, ALDH1* and *ALDH2*. Across populations, the ALDH2-2 variant appears on a similar genetic background (haplotype) and probably has the same evolutionary origin (Peterson et al., 1999). The most important variants are ALDH2-2 (Glu_{487}-Lys_{487}) and ADH2-2 (Arg_{47}-His_{47}). ALDH2-2 dominantly inactivates *ALDH2*, the ALDH that is mitochondrially localized and responsible for most acetaldehyde metabolism in cells. ADH2-2 is a superactive variant. ADH2-2 and ALDH2-2 raise the levels of acetaldehyde by increasing the rate of synthesis, decreasing the rate of metabolism, and by interacting additively, but not synergistically (Thomasson et al., 1991). This results in ingestion of small amounts of ethanol, which produces an unpleasant reaction characterized by facial flushing, headache, hypotension, palpitations, tachycardia, nausea, and vomiting (Harada et al., 1982). The allele frequency of the dominantly acting ALDH2-2 is 0.3 in Japanese and Chinese, hence, about one in two individuals experience flushing after alcohol consumption. Their risk of alcoholism is reduced about four- to tenfold. Approximately 10% of Japanese are ALDH2-2/ALDH2-2 homozygotes. Thus far, only one alcoholic ALDH2-2/ALDH2-2 homozygote has been observed across a series of studies in which several hundred alcoholics have been genotyped, and that individual is the focus of a report (Chen et al., 1999). Other studies about genetics, which effect susceptibily to alcoholism, are focused on neurotransmitter pathways implicated in ethanol use. Ginoulakis et al. (1998) also found lowered Beta-endorphin levels in non-alcoholic subjects with strong family histories of alcoholism (high risk), relative to those with no family history of alcoholism (low risk), and also in alcoholics who had abstained for at least 6 months. These results suggest that high-risk subjects have an inherited deficiency in the basal activity of the endogenous opioid system (Farren et al., 1999). The polymorphism of the mu-opioid receptor causes a difference in vulnerability of alcoholism. One polymorphism of interest involves a common A118G nucleotide exchange in exon 1 of the mu-opioid receptor. A alleles were more frequently found in alcoholics than in the control. Likewise, dopamine is one neurotransmitter that impacts on the risk of heavy drinking and alcoholism through potentially diverse mechanisms including the reinforcing effects of alcohol. The A1 allele of the D_2 Dopamine receptor (DRD2) is linked to alcoholism, and was significantly higher in alcoholics compared with non-alcoholics. The other axis effecting vulnerability to alcoholism is the HPA axis, which indicates that hormonal status of high risk and low risk are different in some studies. Dai et al. (2002) studied HPA axis response to alcohol in a high risk and low risk group, and found that the low risk group presented a higher increase of plasma ACTH and cortisol in response to alcohol than the high risk group. Moreover, the basal plasma ACTH level in the high risk group was significantly lower than the low

risk group (Dai et al., 2002). All of these data represent the association of genetic effect on alcoholism in both genetic of enzymatic and neurocircuit system.

DNA polymorphism and disease

DNA polymorphism is a common phenomenon of differentiation in the sequence of DNA or protein form. There is also a considerable variety of polymorphism types now amenable to analysis, and each one has special merits. The most common are single nucleotide polymorphisms (SNPs or "snips"), i.e. replacement, loss or addition of one nucleotide. Another polymorphism is a repetitive DNA sequence frequently found in non-coding region. A polymorphism may have no effect, or it may be considered functional if it results in an altered protein function, stability, or amino acid substitution depending on its location and polymorphism form. The polymorphism at the 5' and 3' end effect the expression of DNA because they regulate gene expression (Kelada et al., 2003).

If the sequence of exactly the same region of DNA located at a particular position on a chromosome is determined in a large number of chromosomes carried by many different individuals from around the world, a remarkably high level of similarity is observed. In fact, any given segment of human DNA about 1,000 base pairs in length contains, on average, only one base pair that varies between two individuals in the population. As previously studied, different versions of a particular DNA sequence at one particular chromosomal location (locus) are called alleles. When alleles are so common that they are found in more than 1 percent of chromosomes in the general population, the alleles constitute what is known as genetic polymorphism. Some alleles that represent a change in the sequence of the DNA located between genes or within introns, are inconsequential to the functioning of any gene, and can be detected only by direct DNA analysis. Other sequence changes are located in the coding sequence of genes themselves and may result in different protein variants that possible lead, in turn, to sharply distinct phenotypes. Most (but not all) deleterious mutation that lead to genetic disease are rare variants. Mutant alleles that lead to severe genetic disease are often the most obvious form of genetic diversity; and on examination, many proteins have been found to exist in different populations in several relatively common, distinguishable forms. (Thompson et al., 2001)

Although all polymorphisms are ultimately the result of differences in DNA sequence, some polymorphic loci have been studied by examining the variation in the proteins encoded by the alleles rather than examining the differences in DNA sequence of the alleles themselves. Studying variation in proteins rather than the DNA that encodes them has real utility: after all, it is the protein product of a polymorphic allele, rather than the DNA sequence change itself that is often responsible for different phenotypes and, therefore, is likely to dictate how some genetic variations affect the interaction between an individual and the environment. Polymorphic alleles in regulatory regions may also be important in determining phenotypes by affecting transcriptional regulation of genes (Thompson et al., 2001).

The 5'flanking region of a gene usually contains the promoter which regulates gene expression (transcription) by controlling whether the gene will be trancribed and if so, the corresponding level of expression. Many regulatory regions in eukaryotes contain several conserved sequences such as the TATA box [TATA(A or T)A(T or

A)] and the CCAAT motif [GC(C or T)CAATCT], which are believed to play and important role in binding other components (including RNA polymerase and transcription factors) necessary for transcription (Carporaso 1999).

Repetitive DNA is another polymorphism form, which is an interested point for the researcher. Intensive research on these sequences has inevitably produced a large number of concepts and theories about their nature. Repetitive DNA was distinguished in tandem into three classes: satellites, minisatellites and microsatellites. Satellites comprise repeat units of several thousand base pairs each with repetition grades of 10^3-10^7 at each locus and are usually located in the heterochromatin, mainly in centromeres. Minisatellites and microsatellites are distinctly different from satellites in that each cluster has only a moderate degree of repetition, the length of the repeat unit is shorter, and repetitive loci tend to be more dispersed throughout the genome. They are usually distinguished according to the size of the repeats. Microsatellites, also termed “simple sequence satellites” or “short tandem repeats (STR)”, consist of repeat tracts of 1-13 bp, and minisatellites (also called variable number tandem repeats, VNTR) usually contain repeats of greater length (Debrauwere et al., 1997).

The association of repetitive sequences with human disease has made a large impact. It now appears likely that repeat sequence mutations are the cause of human diseases, particularly for those disorders that exhibit a dominant mode of inheritance. It is currently accepted that instability of simple repetitive tracts reflects replicative errors, which occur either during the mitotic divisions or meiotic division of the gametogenesis. Several arguments strengthen this model: i) replication slippage on short repeat tracts has been observed in *in vitro* experiments; ii) orientation of repeat tracts with regard to the replication origin affects the rate of mutation; iii) mutations in genes affecting DNA mismatch repair dramatically elevate microsatellites instability; and iv) mutations that eliminate most types of recombinations do not affect the frequency of tract alteration. The replication slippage rate of different dinucleotide and trinucleotide repeats in an *in vitro* system is found to be independent of the fragment length and lower for trinucleotides than dinucleotides. Amongst trinucleotides, the rate of slippage is sequence-specific. Surprisingly, disease-associated repeats have lower slippage rates than other trinucleotides analyzed, a result which contradicts the current observations made *in vivo*.

The relationship between genetic polymorphism and disease is accepted among sciencetists and researchers. Many diseases which relate to gene polymorphism had identified alcoholism as one of these that polymorphism affect the vulnerability of this disease. Leptin is a peptide hormone in the mesolimbic pathway, previous studies have shown sufficient evidence of the association between leptin and alcoholism.

Leptin

Leptin, a 167 amino acid protein transcribed from the ob gene, was originally cloned in mice during a research directed at identifying the molecular defect in an obesity-prone strain, the ob/ob mouse (Meinders et al., 1996). The name leptin derived from the Greek leptos, which means thin. The human leptin gene is on chromosome 7q31 and locates in the interval between D7S514 and D7S530 (Shintani et al., 1996). The leptin gene has more than 15,000 base pairs in length and there are three exons and two introns (Figure 1). Leptin is mainly produced in white adipose tissue; and very small amounts were found in brown adipose tissue (Auwerx et al. 1998). Mutations in the leptin gene lead to defective leptin production and cause recessively inherited early onset of obesity in mice (Mammes et al., 2000). In man, two families with a genetic deficiency in leptin levels have been described (Mammes et al., 2000). Their serum leptin levels were very low, a homozygous frame-shift mutation involving the deletion of a single guanine nucleotide in codon 133 of the gene for leptin was found (Montague et al., 1997). However, mutations in the translated part of the leptin gene cannot explain the high prevalence in normal populations (Mammes et al., 2000).

Leptin is a circulating hormone that the best known as a regulator of food intake and energy expenditure (Sweeney 2002). Evidence supporting the claim that leptin was an adipostat was provided by the decrease in bodyweight and the improvement in metabolic control in rodents with genetic (Weigle et al., 1995) or diet-induced (Campfield et al., 1995) obesity that were injected with leptin. These effects of leptin are from lipid metabolism regulation and satiety control. The effect on lipid metabolism is leptin directly inhibits intracellular lipid concentrations by reducing fatty-acid and triglyceride synthesis and concomitantly increasing lipid oxidation (Shimabukuro et al., 1997). This effect on lipid metabolism may be mediated by an inhibitory effect of leptin on acetyl-CoA carboxylase activity, the rate-limiting enzyme in fatty-acid synthesis (Bai et al., 1996). Inhibition of this enzyme leads to a reduction in malonyl-CoA, an inhibitor of carnitylacyltransferase I and mitochondrial β-oxidation. Inhibition of acetyl-CoA carboxylase will thus block fatty-acid synthesis and favour mitochondrial fatty-acid uptake and oxidation, resulting in lower intracellular fatty-acid and triglyceride concentration. Therefore, the higher leptin level the higher fatty-acid metabolism. Moreover, leptin regulates food intake. The *ob* mRNA in adipose tissue of normal animals increases in response to overfeeding, while it decreases following starvation (Fredrich et al., 1995). Leptin has been shown to alter the gene expression of corticotropin releasing hormone (CRH) and pro-opiomelanocortin (POMC) in the hypothalamus, which suggests a role both in regulating the HPA-axis and a possible role in the endorphinergic modulation of the drug reward systme (Inui et al., 1999). Fasting and leptin deficiency (in *ob*-mice) were shown to be associated with decreased POMC mRNA in the rostral arcuate nucleus (Schwartz et al., 1997) whereas leptin treatment increased POMC mRNA levels in the hypothalamous of fasting rats and *ob*-mice (Mizuno et al., 1998). By stimulating the POMC activity and enhancing β-endorphine, leptin could be able to interact with the mesolimbic brain system as one common pathway of both food and drug reward (Kiefer et al., 2001). These evidences show that the leptin protein associates obesity by regulate lipid metabolism and food consumption.

Mammes et al. (2000) determined a 5' flanking region polymorphism of the leptin gene by Restriction Fragment Length Polymorphism (RFLP) technique, which revealed that G alleles frequently found in a higher body weight than A alleles. In contrast, study conducted in Finnland and Greece using the Single Strand Conformation Polymorphism (SSCP) technique found that the distribution of G and A alleles among obesity and normal weight were equal (Karvonen et al., 1998; Yiannakouris et al., 2003). The polymorphism in the 5'flanking region of leptin gene causes the differentiation in plasma leptin level, and subjects with G allele have a higher plasma leptin level than those with A allele (Stunff et al., 2000). These results were opposite from those reported by Mammes et al. (2000) that A allele had lower plasma leptin level than G allele. Moreover, Hoffstedt et al. (2002) study was found that obesity with AA carriers had 50% higher plasma leptin level than in GA/GG carriers and mRNA of leptin gene was 60% higher in obesity with AA genotype than GA and GG genotypes.

Ethnics variation of leptin G/A SNP was reported in which G allele 53% and A allele 47% in Greek subjects, G allele 56.38% and A allele 43.62% in France subjects, G allele 67.47% and A allele 35.52% in Finn subjects.

Hypertension is one of leptin related diseases. A chronic increase in circulating leptin was reported to cause a sustained increase in arterial pressure in rats (Shek et al., 1998). Moreover transgenic skinny mice overexpressing leptin were reported to exhibit elevated blood pressure, suggesting a direct effect of leptin on the pathogenesis of hypertension (Casto et al., 1998). The mechanism by which leptin increases blood pressure is thought to be through sympathetic activation (Shintani et al., 2002). Moreover, the study of correlation between hypertension and leptin gene polymorphism have been shown. The polymorphism in the 3'flanking region which differ in tetranucleotide repeat units (CTTT) was studied in hypertension subjects (Shintani et al., 2002). This 3' STR allele was seperated in 2 group first group is class I size 121-145 base pairs (9-15 repeat units) and second group is class II size 197-225 base pairs (26-32 repeat units). The frequency of class I/class I genotype was much higher in hypertensive subjects than in controls (13.5% vs 3.4% P=0.0027). Preclamsia, a hypertensive disorder of pregnancy, is another leptin-related disease. The class I/class II genotype increased risk of preclamsia (OR=3.8; 95% CI 0.8-18.0) compare with class I/class I genotype (Muy-Rivera et al., 2005).

At first, leptin was seen as an adipocyte-derived signaling molecule, which limits food intake and control energy balances. It reduces appetite by decreasing expression of the orexegenic peptides such as neuropeptide Y and agouti-related peptide (Vernon et al., 2001; Sandoval et al., 2003; Meinders et al., 1996; Montzoros et al., 1999). When food or alcohol is consumed enough adipocyte secretes leptin to the blood circulation and on to the brain. Leptin receptors are in the hypothalamus, cerebellum, hippocampus, thalamus, choroids plexus, brain capillary endothelium and POMC neurons (Mantzoros et al., 1999), and they are members of class I cytokine receptor family that activate JAK (Janus Kinase). After circulating to the brain leptin binds to its receptor, resulting in transcription and modulate expression of POMC in an arcuate nucleus (Harris 2000). Then, the POMC was modified to Beta-endorphin and ACTH. Beta-endorphin and ACTH circulate to many sites in the brain and finally modulate the mesolimbic system, which is the drug addiction modulator area (Gianoulakis 2001). Thus defect in leptin protein or polymorphism of leptin gene may also affect mesolimbic system and also affect susceptibility to alcoholism.

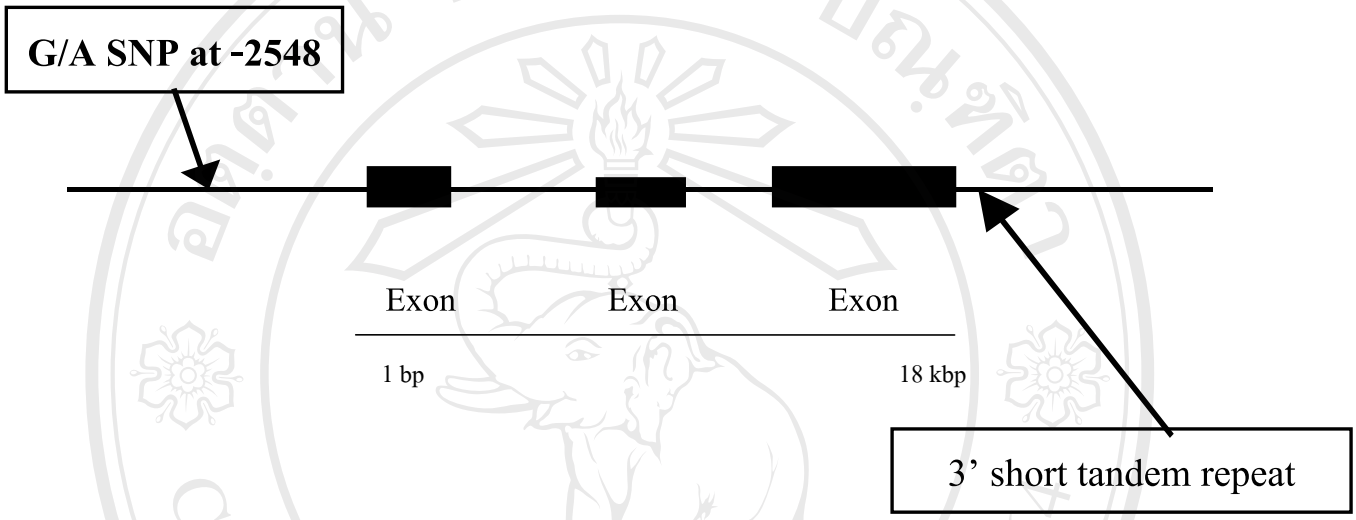

Figure 1. Human leptin gene structure and polymorphic site

Leptin and alcoholism

The association of leptin and alcoholism is due to leptin control the HPA axis and opiodergic system, which affect the dopaminergic system in the mesolimbic system. The differences in these systems cause varied vulnerability to alcoholism. In 2001, Kiefer et al. found the association between leptin and craving, the sign of wanting alcohol caused by abstinence in alcoholic patients. Increased leptin may also be responsible for withdrawal-induced alcohol craving (Kiefer et al., 2001). Alcoholic patients with a higher plasma leptin level have a higher self-rate craving scale. These results are in accordance with that of another study, which was conducted with 30 male alcoholics to determine the relationship between plasma leptin level, tumor necrosis factor-alpha and alcohol craving. Elevated plasma leptin levels at the onset of withdrawal significantly correlated with self-rated craving, a higher plasma leptin level and higher self-rated craving (Kiefer et al., 2002). Similarly, female alcoholics with a higher plasma leptin level were also found to have higher alcohol craving than control group (Kraus et al., 2004). In animal models, mice were habituated to alcohol consumption for 3 months and applied with leptin (1g/kg IP). The application of leptin resulted in an increased free-choice alcohol intake after a 3-day period of alcohol withdrawal (Kiefer et al., 2001). These results show the association of plasma leptin level and alcohol craving in both humans and animals. A study of the neurocircuit level of intracerebroventricular infused leptin in rats was shown that the rewarding effect was activated by leptin (Fulton et al., 2000). Moreover, the level of leptin in cerebrospinal fluid was found to correlate with dopamine level, may be because of leptin enhanced dopamine secretion (Hagan et al., 1999). These results support the assumption that leptin may be the modulator of alcoholism. To date, there is no study of the correlation between leptin gene polymorphism and alcoholism etiology has been carried out.

Purpose of this study

1. To identify leptin gene polymorphisms in alcoholic patients and control subjects.
2. To determine the association between leptin gene polymorphism and alcoholism risk.