CHAPTER 2
RESEARCH DESIGN AND METHODS

A. An overview

This study was divided into 2 sections, which consisted of 1) subject selection with a face-to-face interview on alcohol intake behavior, and 2) the determination of leptin gene polymorphisms.

In the first section, 200 northern Thai subjects were recruited. The alcoholics were recruited from the psychiatric OPD of Maharaj Nakorn Chiang Mai Hospital, Chiang Mai province, Thailand and non-alcoholic subjects were recruited from Maharaj Nakorn Chiang Mai Hospital staff, during April 2004 to Febuary 2005. The group comprised of 109 non-alcoholic and 91 alcohol – dependent subjects, with ages ranging from 25 to 68 years old. A description of the study and its components were provided and enrollments were solicited. All subjects answered the standard questionaire in a face-to-face interview, which included questions about demographic characteristics, city residence, occupational history, medical history and family history of the alcoholic members. Alcohol drinking status was assessed with Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV) (Thai version).

Buccal cells were collected from all subjects after informed consent was obtained. These buccal cells were used in the determination of leptin gene polymorphism. To maintain participant confidentiality throughout the study, all participant records and sample information were coded. All study protocols, questionnaires and consent forms of this study were approved by the Institutional Ethical Committee on Human Experimentation, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand.

In the second section, buccal cells from the subjects were prepared for DNA isolation. Then, the isolated genomic DNA was subjected for determining the Single Nucleotide Polymorpshim polymorphism (SNP) in 5'flanking region by a Polymerase Chain Reaction – Restriction Fragment Length Polymorphism (PCR-RFLP). The 3' Short tandem repeat polymorphism of leptin gene was determined by general PCR. Finally, the data were analyzed statistically and the summary of the research design is shown in Figure 2.

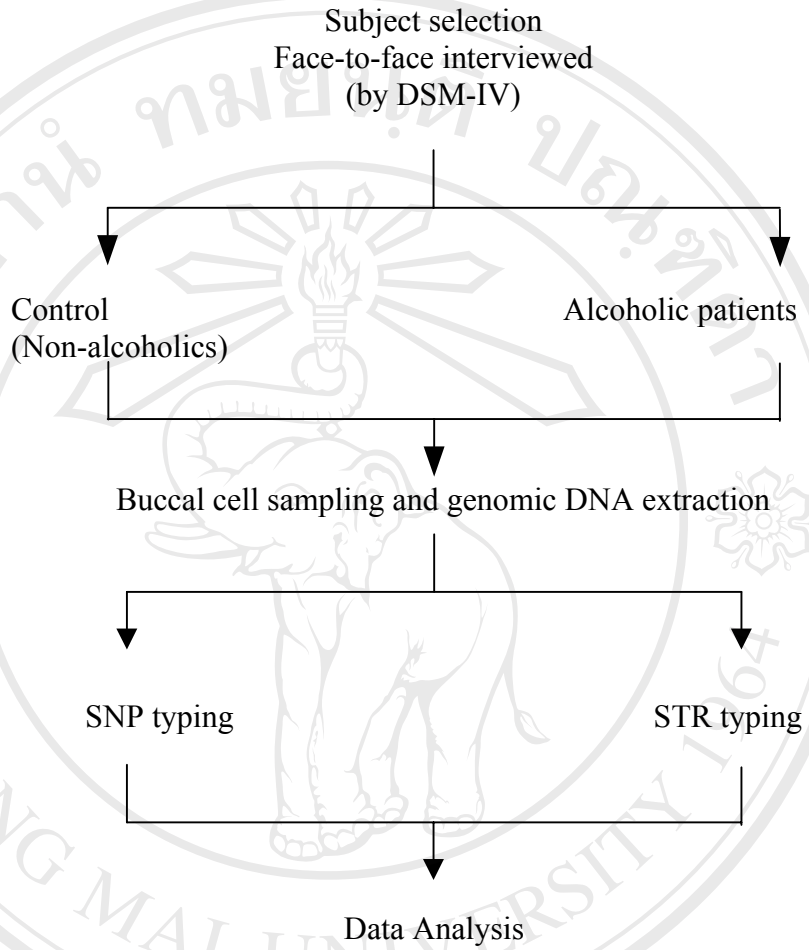

Figure 2. Summary diagram of research design

B. Method

1. Study population

Two hundred northern Thai were eligible for inclusion in this study. The number of subject was calculated from the preliminary study result. The optimum number of subject from the calculation is more than 88 subjects both alcoholics and controls. This group comprised 91 alcoholic patients and 109 non-alcoholic subjects, with age ranging from 25-68 years old. A description of the study and its components were provided and enrollments solicited. Individuals answered the standard questionnaire in a face-to-face interview, which included questions about demographic characteristics, city residence, occupational history, medical history and the family history of alcoholic members. Alcohol drinking status was assessed by DSM-IV containing 7 items and scale is useful as a screen for alcohol dependence. The individuals who met three out of seven symptoms of DSM-IV and drinking alcohol for at least 1 years were classified as alcoholics. The seven criteria of alcohol dependence in DSM-IV is

1. Tolerance, as defined by either:
   a. need for read amounts of the alcohol in order to achieve intoxication or desired effect; or
   b. markedly diminished effect with continued use of the same amount
2. Withdrawal, as manifested by either:
   a. characteristic withdrawal syndrome for the alcohol. When there is decrease or interruption of alcohol consumption, signs and symptoms of variable intensity arise. Such as sudoresis, slight tremor, nightmares, nausea, vomiting, hallucination, irritability, anxiety. or
   b. Alcohol is taken to relieve or avoid withdrawal symptoms.
3. Alcohol is often taken in larger amounts or over longer period than intended.
4. Persistent desire or unsuccessful efforts to cut down or control alcohol drinking.
5. A great deal of time is spent in activities necessary to obtain alcohol (e.g., visiting multiple doctors or driving long distances), use alcohol or recover from its effects.
6. Important social, occupational, or recreational activities given up or reduced because of alcohol drinking.
7. Continued drinking despite knowledge of having a persistent or recurrent of psychological or physical problem that is caused or exacerbated by alcohol drinking.

Individuals who met criteria of the DSM-IV were classified as alcohol-dependence. Those criteria include: present of three out of seven symptoms and drinking for at least 1 year for DSM-IV.

2. DNA extraction from buccal cells

Buccal cells were swabbed from oral mucosa, left and right cheek using wooden micro spatula. The spatulas were immediately immersed in the microcentrifuge tube containing 1.5 ml of distilled water and frozen before the DNA was extracted using the chelex method (Walsh et al., 1991).

3. DNA Extraction

Microcentrifuge tube of buccal cells was vortex, centrifuged at 7200xg for 2 minutes remove supernatant. Buccal cell pellet was rinse with 1 ml of distrilled water and centrifuged at 7200rpm for 2 minunte again then supernatant was removed. Add 180 µl of 10 mM Tris, 20 µl of proteinase K (1 µg/µl) and Chelex® beads was added until cover the cell pellet (Walsh et al., 1991). The mixture was incubated at 56°C overnight. After incubate mixture was spindown and boil in waterbath 8 minutes. Then DNA available for PCR.

4. Primer sequence

The sequences and locations of the leptin gene oligonucleotide primers are shown in Table 1. The sequence of the complete human leptin gene described in the GeneBank accession number U43589 (see Appendix E) was used as a reference.

Table 1 Sequences and locations of the leptin gene nucleotide primer used

Primers	Sequence	Amplified region
LEP18376F	5'-CAATGGTCTGATCTTGGCTCACTGC-3'	18376 – 18841
LEP18841R	5'-TTCCCCGCTTTACATGATGGGTTGG-3'	(465 bp)
LEP16768F	5'-CCTAGTGGAATGGGGAGATTCTTCC-3'	16768-17351
LEP17351R	5'-TCCTGAGGTGACGTACATCCTTAGC-3'	(583 bp)
LEP37004F	5'-CTGTATAAAAGATAACTTTGAGATGAGG-3'	37004-37154
LEP37154R	5'-ACTTCTGAGGTTGTGTCACTGG-3'	(~ 122-226)
LEP36909F	5'-ACATACCTATCAAAGGTTAGACTCC-3'	37267-36909
LEP37267R	5'-TTCTGTTTGCATCTATCACGAACGT-3'	(319-415)

LEP18376F and LEP18841R used for internal control fragment
LEP16768F and LEP17351R used for 5'G-2548A fragment
LEP37004F and LEP37154R used for STR fragment
LEP36909F and LEP37267R used for sequencing fragment

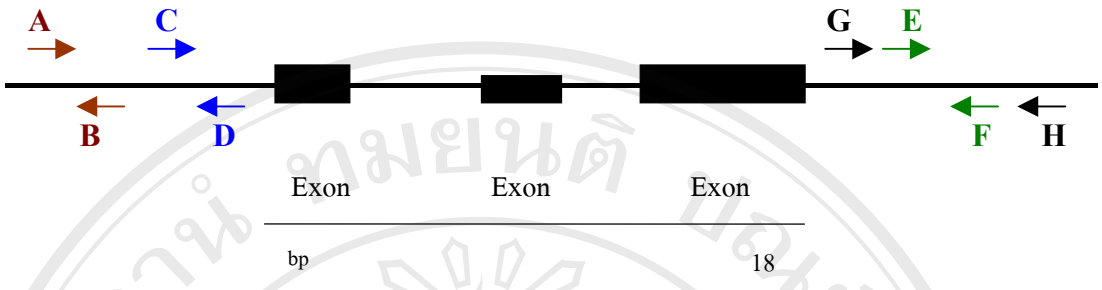

Figure 3 Location of oligonucleotide primers A:LEP16768F, B:LEP17351R, C: LEP18376F, D:LEP18841R, E:LEP37004F, F:LEP37154R, G:LEP36909F and H:LEP37267R primers.

5. Typing of the polymorphism

5.1 G/A SNP

In this study, primer was newly designed for optimal amplification of the target sequence. From figure 3, primer A and B were used to amplify G/A SNP at position –2548. The G/A SNP fragment is 584 bp in length. When G base present at polymorphic site, HhaI enzyme digest the 584 fragment to 351 and 233 bp fragments where as if A base present at polymorphic site HhaI can not digest. The primer C and D were used to amplify internal control fragment for 465 fragment which used for confirm HhaI enzyme digestion. The internal control fragment has GCGC sequence always digested by HhaI enzyme to 290 and 175 fragments (Figure 4).

5.1.2 Polymerase Chain Reaction

PCR was performed with a total volume of 30 µl. The final reaction mixture contained 0.1 mM of each deoxynucleoside triphosphate (dATP, dTTP, dGTP and dCTP); PCR buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.8); 0.25 µM of each primer and 0.6 unit of Taq DNA polymerase. The initial PCR contained 3 µl of DNA template. PCR for 5'G/A SNP fragment was conducted under the following conditions: initial denaturation at 94 ^{o}C for 2.15 min followed by 35 cycles of reaction composed of denaturation at 94 ^{o}C for 60 sec, annealing at 62 ^{o}C for 30 sec and extension at 72 ^{o}C for 1.30 min. Hot start PCR for internal control fragment were as follows: initial denaturation at 94 ^{o}C for 2.15 min then hold at 85 ^{o}C for pipette primer followed by 35 cycles of reaction composed of denaturation at 94 ^{o}C 30 sec, anealing at 58 ^{o}C 20 sec and extension at 72 ^{o}C 45 sec.

5.1.3 HhaI Digestion

The SNP was analysed by HhaI enzyme digestion. The recogniton site of HhaI enzyme is GCG!C. The G/A SNP previously reported by Mammes et al., 2000, was defined by presence (when G) or absence (when A) of HhaI restriction site (underlined): -2560 5'-CGACAGGGTT GC(G/A)CGATCCT-3'-2540. Amplified DNA was incubated at 37 °C for 12 hours with 6 units of HhaI enzyme in total reaction volume of 33 µl, 20 µl of 5'G/A SNP amplicon, 10 µl of internal control amplicon and 3 µl of HhaI enzyme (concentration 2 unit/µl). The internal control amplicon was added to confirm HhaI restriction enzyme working.

Fifteen µl of digested amplicon were separated on a 2% agarose gel with ethidium bromide in 0.5x TBE buffer at 100 volts for 45 minutes. After that DNA bands were visualized under a long wave length ultraviolet light by using the ultraviolet transilluminator.

5.2 STR typing

The primers were newly disigned for optimal resolution of the resulting amplicon on non-denaturing polyacrylamide gels.

5.2.1 Polymerase Chain Reaction

PCR was performed with a total volume of 30 µl. The final reaction mixture contained 0.1 mM of each deoxynucleoside triphosphate; PCR buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.8); 0.25 µM of each primer and 0.6 unit of Taq DNA polymerase. The initial PCR contained 3 µl of DNA template. PCR for 3' flanking STR fragment was conducted under the following conditions: initial denaturation at 94 ^{o}C for 2.15 min followed by 35 cycles of reaction composed of denaturation at 94 ^{o}C for 45 sec, annealing at 58 ^{o}C for 30 sec and extension at 72 ^{o}C for 1 min.

5.2.2 Polyacrylamide gel electrophoresis

Six µl of PCR product was mixed with 1 µl of Loading dye and loaded onto a 8.5% polyacrylamide gel in 1x TBE buffer. Electrophoresis was carried out with the BioRad^{TM} electrophoresis apparatus at 90 mA for 16.30 hours at room temperature. After electrophoresis the DNA fragments were visualized by silver-staining.

5.2.3 Silver staining

1. After electrophoresis polyacrylamide gel was soak in 10% ethanol for 10 minutes on shaker and remove solution.
2. Wash the gel for 10 minutes with1% nitric acid.
3. Wash the gel with distilled water 2-3 second two times.
4. Submerge the gel in 0.012 M silver nitrate solution (0.4 g silver nitrate aliquot with 200 ml) 35 minutes.

5. Discard silver nitrate solution and rinse the gel with two changes of distilled water for 2-3 second each.

6. Develop the gel using 0.019% formalin in 0.28 sodium carbonate with intensive shaking. It is critical that the developing solution be replaced with fresh solution once it turns yellow.

7. Terminate staining by discarding the developing solution and replacing it with 10% acetic acid.

8. Dry gel.

5.2.4 DNA Sequencing

To determine sequence of interest fragment DNA thus some of STR alleles were sequenced using Big-Dye kit and ABI 310 sequencer.

1. All of band containing specific allele were cut out of the acrylamide gel eluted with Chelex® and distrilled water.

2. PCR was performed with a total volume of 60 µl.

3. Isopropanol precipitation of DNA

The PCR amplicons were precipitated using isopropanol method to remove primer and dNTPs.

Protocol

3.1 Add 60 µl of 4 M ammonium acetate and 120 µl of isopropanol to the DNA amplicon contained in a 0.5 ml microcentrifuge tube and mixed briefly then place at room temperature for 30 minutes.

3.2 Centrifuge at 13,200 rpm for 15 min at room temperature, remove supernatant.

3.3 Wash DNA pellet with 100 µl of 70% ethanol.

3.4 Centrifuge at 13,200 rpm for 5 min at room temperature, remove supernatant.

3.5 Dry pellet at room temperature.

3.6 The amount of amplicon was checked by agarose gel electrophoresis.

4. Sequencing reaction

4.1 Mixed 5 µl of DNA template with 4 µl of terminator ready reaction mix and 1 µl of 3.2 µM LEP36909F primer vortex and spin briefly.

4.2 Place in a thermal cycler and set to run on a following program:

50 sec at 96 ^{o}C
then
10 sec at 96 ^{o}C
10 sec at 50 ^{o}C
4 min at 60 ^{o}C for 25 cycles

When cycling is complete the sequencing products were precipitate as follow.

4.3 Add 2 µl 3 M sodium acetate, 10 µl deionize water and 50 µl of 100 % ethanol. Then place in room temperature for 30 minutes or more up to 1 hour.

4.4 Centrifuge at 13,200 rpm for 30 min at room temperature, remove supernatant.

4.5 Wash pellet with 70 µl of 70% ethanol.

4.6 Centrifuge at 13,200 rpm for 5 min at room temperature, remove supernatant.

4.7 Place the microcentrifuge tube at room temperature until completely dried.

4.8 Dissolve pellet in 15 μl template suppressing reagent.
4.9 Run sequencing products on ABI310 genetic analyzer.

C. Statistics analysis

Statistical analysis was conducted with the Statistical Package for Social Sciences (SPSS) program. The statistical calculation used for describing and analyzing the data included frequency, percentage and odds ratio.

The odds ratio calculation

$$\text{Odds ratio} = \frac{\text{Ratio of risk exposure in patient group}}{\text{Ratio of risk exposure in control group}}$$

$$\text{Ratio of risk exposure in patients} = \frac{\text{risk exposure patient frequency}}{\text{non-risk exposure patient frequency}}$$

$$\text{Ratio of risk exposure in control} = \frac{\text{risk exposure subject frequency}}{\text{non-risk exposure subject frequency}}$$

To determine the significance of the difference in the distribution of genotypes alcoholic patients and control subjects were calculated by Pearson Chi-square test (χ^2). The mean values of age and BMI were compared between groups using the Independent sample t-test (t). Differences were considered statistically significant when P values were less than 0.05.