CHAPTER 3
RESULTS

A. Population characteristics

The study of polymorphism flanking the leptin gene was conducted in 200 Northern Thai men. The 91 alcoholic subjects were patients admitted to the psychiatric department for detoxicification. They fulfilled at least three of seven diagnosis criteria for dependence according to the Desk Reference to the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV). 109 healthy control subjects were recruited by interview. They were non-alcoholics according to the DSM-IV and had no alcoholic relatives. Demographic characteristics of the alcoholic and non-alcoholic subjects are shown in Table 2. The mean age of the alcoholic patients and control subjects were not significantly different ($t=1.153$; $df=198$; $p=0.250$).

Table 2 Demographic of alcoholic patients and control subjects

Variables	Alcoholic patients	Control subjects
Total (n)	91	109
Age (year)		
25 – 34	9	19
35 – 44	32	46
45 – 54	41	28
55 – 64	7	16
$\geq$ 65	2	0
Mean age (years)	44.72 $\pm$ 8.46	43.33 $\pm$ 8.56
BMI	22.11$\pm$3.08	24.29$\pm$3.33
Under lying diseases		
None	55	76
Hypertension	13	9
Diabetes	1	4
Lung disorder	1	1
Hypercholesterol	1	3
Hepatic diseases	5	2
Peptic ulcer	4	9
Cardiovascular diseases	4	1
Kidney stone	1	0
Allergy	1	4
Gout	4	0
Piles	1	0
Education (n)		
Primary school	33	29
Secondary school	24	44
Diploma	23	27
University	11	9
Occupation (n)		
Unemployed	12	0
Employed	35	95
Farmer	8	2
Merchant	7	0
Government officer	26	12
Others	3	0

B. Typing of the polymorphisms flanking the leptin gene

1. Typing of the 5' G/A SNP

The 5' G/A SNP was typed by PCR amplification, followed by digestion with HhaI restriction enzyme. The pattern of HhaI digestion is shown in Figure 4. The Hardy-Weinberg equilibrium of 5' G/A SNP genotype was determined by comparing between the number of expected genotype carrier, which was calculated from the observed allele frequency, and the number of observed genotype carrier using Pearson Chi-square test. The number of observed genotype carriers was not significantly different from the number of expected genotype carriers. Therefore, the distribution of genotypes was in Hardy-Weinberg equilibrium.

The frequencies of alleles were investigated in a total of 200 northern Thai subjects (91 alcoholics and 109 non-alcoholics). The allelic frequencies of G/A SNP are shown in Table 3. The frequencies of alleles observed in the alcoholics and non-alcoholics did not differ significantly ($\chi^2 = 0.162$; df=1; $p > 0.05$).

The distribution of the 5' G/A SNP genotypes are shown in Table 4. The majority genotype was AA. The genotype frequencies of GA and AA in non-alcoholics were higher than alcoholics, on the other hand, the GG genotype frequency among alcoholics was higher than non-alcoholics. However, the frequency of all of genotypes was found quite similar in both groups.

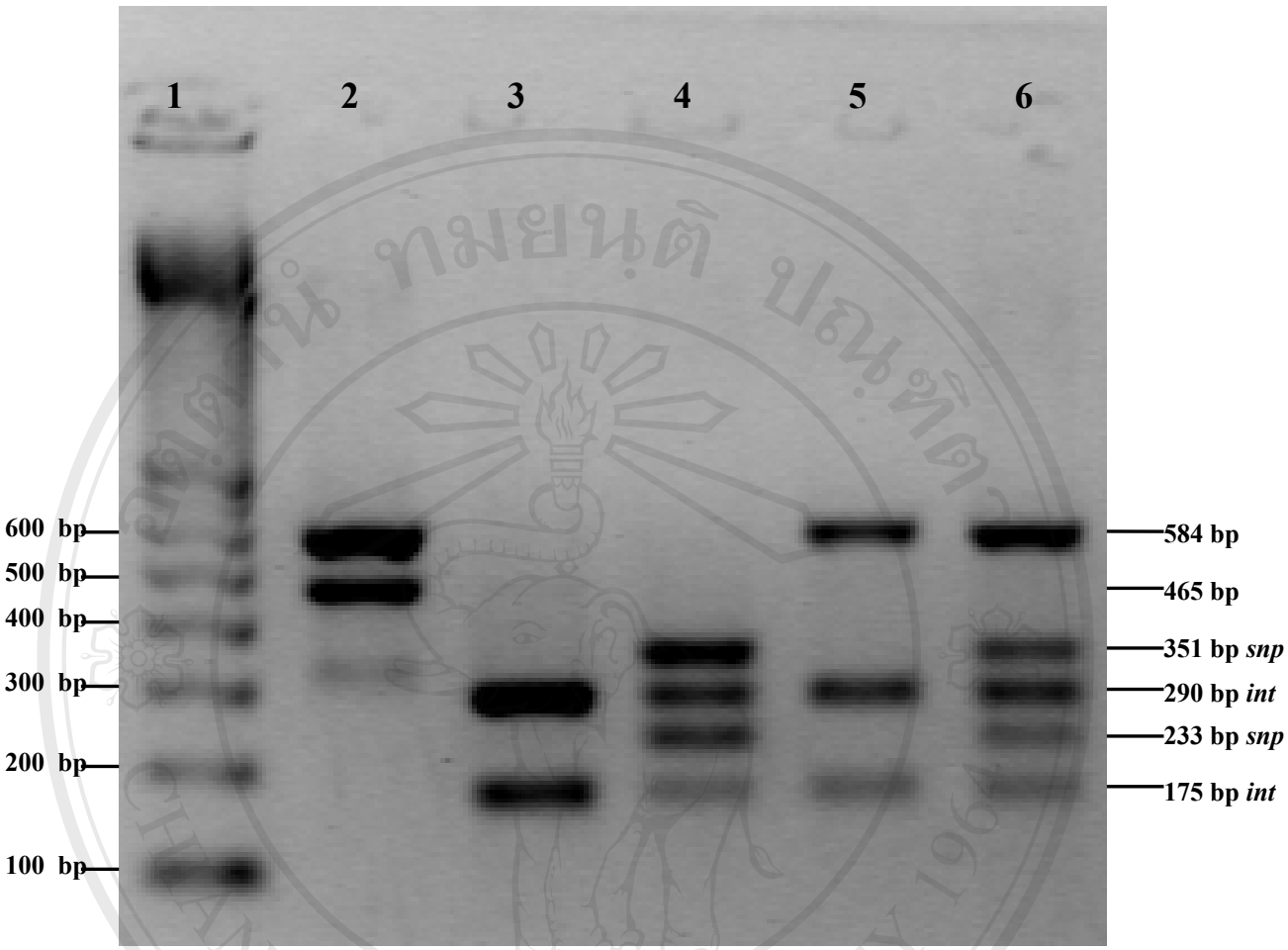

Figure 4 Analysis of the G/A SNP. Lane 1: 100 bp ladder; Lane 2: SNP + internal control non-digested amplicon; Lane 3: HhaI digested internal control; Lane 4: homozygous alleles G; Lane 5: homozygous alleles A; Lane 6: heterozygous (G and A alleles). The amplicon of G/A SNP and internal control were used for HhaI digestion in lane 4, 5 and 6. The G/A SNP amplicon size is 584 bp when it is digested by HhaI enzyme seperating in 351 and 233 bp fragment. In addition, the internal control amplicon size is 465 bp when it is digested by HhaI enzyme seperating in 290 and 175 bp fragment.

snp: fragment derived from G/A SNP amplicon.

int: fragment derived from internal control amplicon.

Table 3 Distribution of 5' G/A SNP alleles in alcoholic (n=91) and non-alcoholic (n=109) Northern Thai

	G(%)	A(%)
Alcoholics	50 (27.5)	132 (72.5)
Non-Alcoholics	56 (25.7)	162 (74.3)

Pearson Chi-Square 0.162 p=0.687

Table 4 Distribution of 5'G/A SNP genotype in alcoholic (n=91) and non-alcoholic (n=109) Northern Thai

	GG (%)	GA (%)	AA (%)
Alcoholics	8 (8.8)	34 (37.4)	49 (53.8)
Non-Alcoholics	6 (5.6)	44 (40.4)	59 (54.1)

2. Typing of the 3' flanking STR

Primers were newly disigned for optimal resolution of allele in polyacrylamide gel (Figure 5). Alleles were defined by the number of repeat units according to forensic genetics convention. Each allele of 3'flanking STR differed from the adjacent allele by 4 bp, thus, the amplicon size must be small enough to be separated in polyacrylamide gel. Therefore, newly designed primer position located close to position of repeat sequence (Figure 5). These primers give amplicon 131-231 bp of size which is easily to be seperated on native polyacrylamide gel. The DNA samples of all subjects could be amplified. An allelic ladder of 3' flanking STR, which was be found out to have 17 alleles, was constructed for allele typing. Then, 4 short and 3 long alleles were sequenced, in order to determine their sequence and number of repeat units. The smallest allele had 11 repeats size of 131 bp (allele 11). The 3 other short alleles had 12, 13, and 15 repeats size of 133, 138 and 145 bp, respectively. The 3 long allele had 25, 29 and 30 repeats size of 189, 207 and 211 bp, respectively. The electropherogram for allele 11 is shown in Figure 6 and the others are shown in Appendix F. The alignment of allele sequence structures is shown in Figure 7. The short alleles were called class I allele, while the long alleles were called class II allele. The class I group had 5 alleles (allele 11 - 15), whereas, the class II one had 12 allele (allele 24 - 35)(Figure 8). The allele size is shown in Table 5.

LEP37004F

gttcaaatag aggtccaaat caa**CTGTATA AAAGATAACT TTGAGATGAG G**aaaatttaa

atgggggctc tgtttt**CTTT** CTTT**CTTT**CT TT**CTTT**CTTT **CTTT**CTTT**CT TT**CTTT**CTTT**

LEP37154R

ctaacttttt tg**CCAGTGAC ACAACCTCAG AAGT**tcctga gaacatgtgt cccaattcca

tttcagatgc tgataagaaa ttctagttaa ttttactagg tataatgatg

Figure 5 Sequence of the 3' flanking STR. The sequences in bold capitals (LEP37004F and LEP37154R) are the positions of newly designed primers, they flank eleven CTTT repeat units.

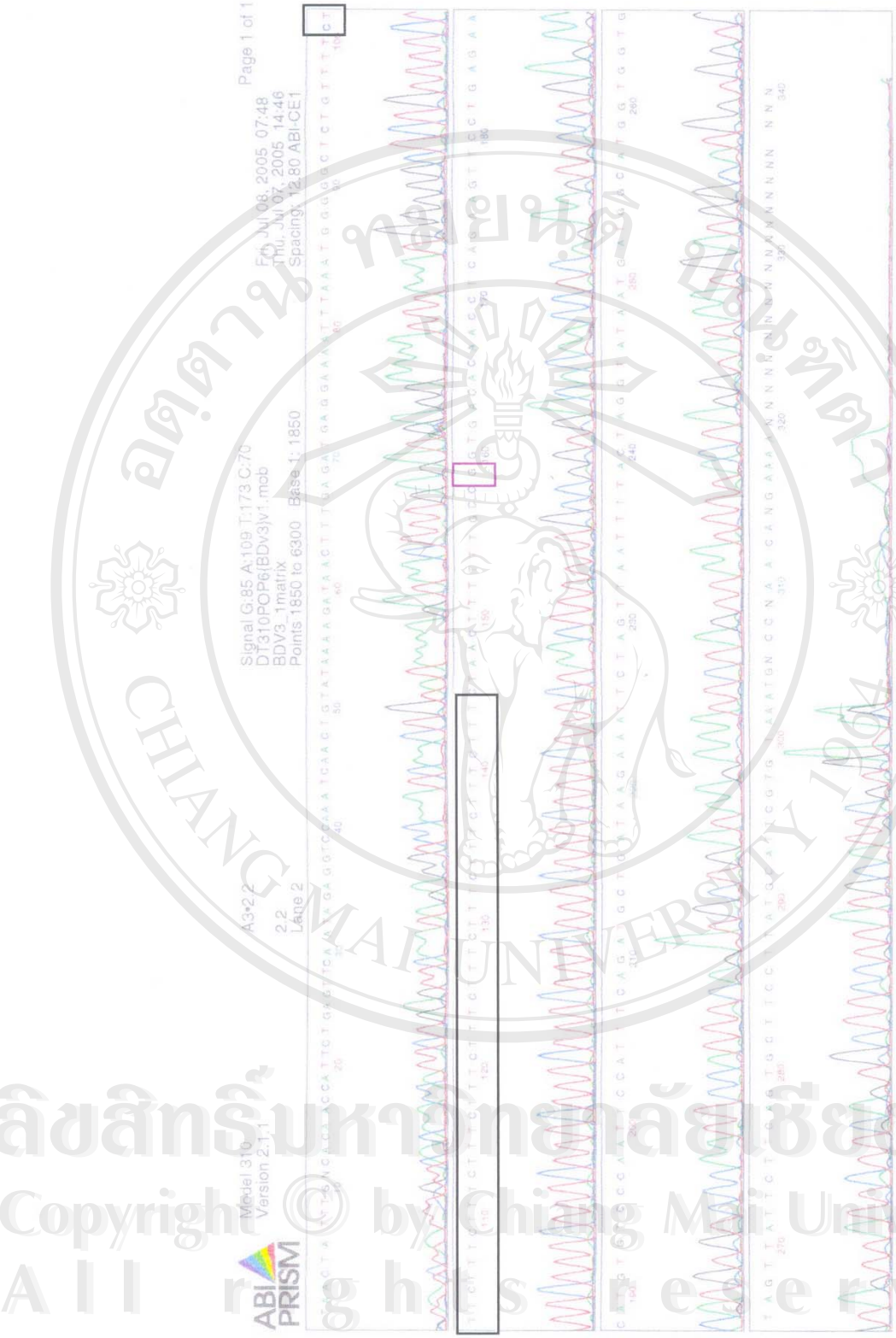

Figure 6 Electropherogram for allele 11. Black box: 11 CTTT repeat units; Purple box: G/A base substitution.

Figure 7 Alignment of allele sequence structures

Reference $(CTTT)_{16}CT$ AACTTTTTTGCCAGT

Allele 11 $(CTTT)_{11}CT$ AACTTTTTTGCC**G**GT

Allele 12 $(CTTT)_{12}C$**–** AACTTTTTTGCCAGT

Allele 13 $(CTTT)_{13}CT$ AACTTTTTTGCC**G**GT

Allele 15 $(CTTT)_{15}C$**–** AACTTTTTTGCCAGT

Allele 25 $(CTTT)_{12}$**T**$(CTTT)_{11}$**TT**$(CTTT)_{2}CT$**T**AACTTTTTTGCC**G**GT

Allele 29 $(CTTT)_{14}$**T**$(CTTT)_{13}$**TT**$(CTTT)_{2}CT$**T**AACTTTTTTGCCAGT

Allele 30 $(CTTT)_{14}$**T**$(CTTT)_{14}$**TT**$(CTTT)_{2}CT$**T**AACTTTTTTGCCAGT

The 7 alleles of the allelic ladder of 3' flanking STR were sequenced. The sequence of each allele was aligned to compare with the reference sequence obtained from GeneBank accession number U43589. Allele 12 and 15 have T base deletion (**–**); allele 25, 29 and 30 have T base insertion (**T**); allele 11, 13 and 25 have G base substitution (**G**).

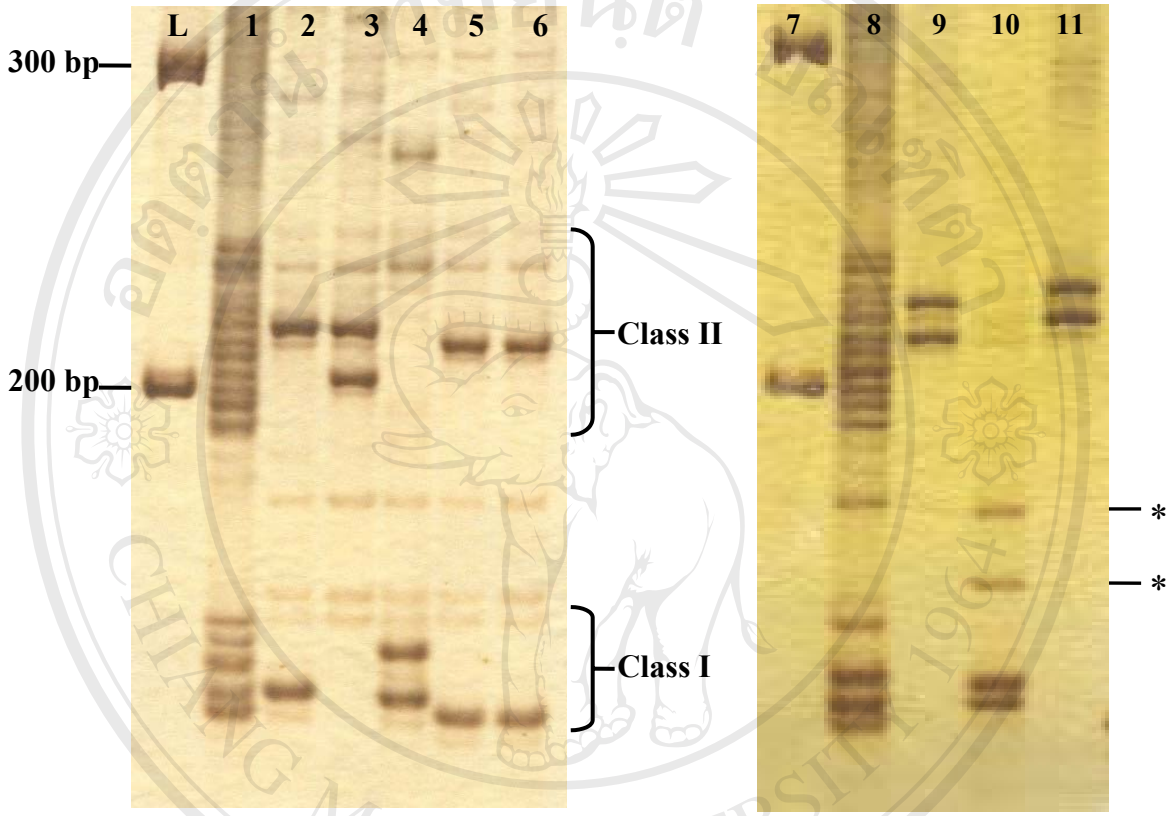

Figure 8 Typing of the 3' flanking STR by polyacrylamide gel electrophoresis. L and 7: 100 bp ladder; Lane 1 and 8 allelic ladder; Lane 3, 9 and 11: homozygous class II; Lane 4 and 10: homozygous class I; Lane 2, 5 and 6: heterozygous class I/class II.

* : heteroduplex bands

Table 5 Allele size, alleles are defined by their number of repeat units.

Allele	Group	Size
11	Class I	131 bp
12	Class I	133 bp
13	Class I	138 bp
14	Class I	142 bp
15	Class I	145 bp
24	Class II	185 bp
25	Class II	189 bp
26	Class II	193 bp
27	Class II	197 bp
28	Class II	202 bp
29	Class II	207 bp
30	Class II	211 bp
31	Class II	215 bp
32	Class II	219 bp
33	Class II	223 bp
34	Class II	227 bp
35	Class II	231 bp

The allele frequencies of the alcoholic patients were significantly different from the non-alcoholics (χ^2 = 4.607; df=1; p=0.032). Alcoholic patients were found to have a higher frequency of class I than class II allele (Table 6). The distribution of the 3' flanking STR genotypes is shown in Table 7. The distribution of genotypes was in Hardy-Weinberg equilibrium. The major genotype of both alcoholic and non-alcoholic subjects was class II/class II. The genotype frequencies of class I/class I and class I/class II in alcoholics were found higher than non-alcoholics. In contrast, the genotype frequency of class II/class II was found higher among non-alcoholics than alcoholics (Table 7).

The odds ratio for class I/class I and class I/class II carriers versus class II/class II was 1.66 (95% CI 1.10-2.22) (Table 8).

Table 6 Distribution of 3' flanking STR alleles in alcoholic (n=91) and non-alcoholic (n=109) Northern Thai.

	Class I (%)	Class II (%)
Alcoholics	62 (34.1)	120 (65.9)
Non-Alcoholics	53 (24.3)	165 (75.7)

Pearson Chi-Square 4.607 p=0.032

Table 7 Distribution of the 3' flanking STR genotypes in alcoholic (n=91) and non-alcoholic (n=109) Northern Thai.

	Class I/class I(%)	Class I/class II(%)	Class II/class II(%)
Alcoholics	12 (13.2)	38 (41.8)	41 (45.0)
Non-Alcoholics	7 (6.4)	39 (35.8)	63 (57.8)

Table 8 Odds ratio of class I/class I and class I/class II carriers compare with class II/class II carriers.

Genotype	Alcoholics	Non-alcoholics	Total	OR	95%CI
ClassI/ClassI ClassI/ClassII	50	46	96	1.66	1.10-2.22
ClassII/ClassII	41	63	104		
Total	91	109	200		