CHAPTER 3

RESULTS

3.1 Method validation

3.1.1 Primers set testing

Primer set 1 in Table 3 was newly designed and used in pilot tests, but gave many unspecific products (data not shown). Primer set 2 resulted in specific products of the expected size and was hence used for the genotyping of the subjects.

3.1.2 Optimal annealing temperature

The different annealing temperature was conducted to optimize the PCR condition according to the following protocol:

Initial denaturation at 94^{o}C for 2 minutes

Denaturation at 94^{o}C for 30 seconds

Annealing temperature ranging from 50 to 57 ^{o}C for 30 seconds

Extension at 72^{o}C for 1 minute 30 seconds

The annealing temperature range in this study was shown in Table 4. The optimal temperature for annealing was around 56 ^{o}C as shown in Figure 6.

Table 4 The annealing temperature range use in this study.

lane	1	2	3	4	5	6	7	8	9	10	11	12
T ^{o}C	50.1	50.3	50.7	51.3	52.0	52.8	53.7	54.6	55.4	56.0	56.5	56.8

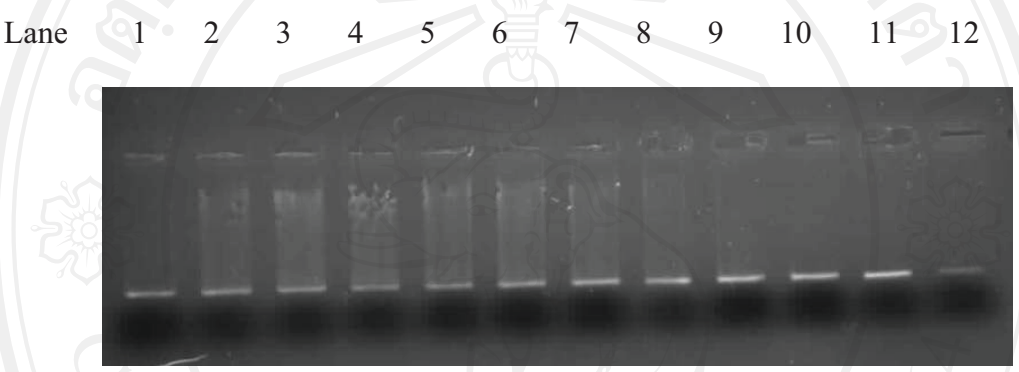

Figure 5 PCR products with different annealing temperature ranging from 50-57 ^{o}C. The brightest band with the lowest signal of background was found in lane 10 (56 ^{o}C).

3.1.3 DNA Quantitation

The PCRs were performed using the DNA extracts from 6 volunteers. The PCR products were quantitated by UV spectrophotometry. The total 250 µl mixture (1:25 dilution, dilution factor = 25) were contained 10 µl of amplicons and 240 µl of ultrapure water. The mixture was filled in cuvette and measured the absorption at 260 nm (A260). The concentration of PCR product was approximately 200 ng/ µl (Table 5 and Figure 6). The absorbance at 260 nm of approximately 1.0 OD equals to 50 ng/ µl of DNA concentration. The amplicon concentration was calculated using this formula:

DNA concentration (ng/ µl) = A260 x dilution factor x 50

The PCR products concentration from 6 volunteers were measured and shown in Table 5. The 310 bp products were checked by 2% agarose gel electrophoresis compared to 100 bp ladder as shown in Figure 7.

Table 5 Concentration of amplified DNA from 6 volunteers.

Sample	Absorbance at 260 nm (A260)	Concentration (ng/µl)
S1	0.166A	207.5
S2	0.153A	191.25
S3	0.149A	186.25
S4	0.162A	202.5
S5	0.168A	210.0
S6	0.160A	200.0

Figure 6 PCR products from 6 volunteers on 2% agarose gel electrophoresis.

3.1.4 Genotyping

We used the amplicons of A2A2 (CC) genotype as the pilot sample to test the completeness of the restriction enzyme digestion. The four concentrations of *Taq* I restriction enzyme (8, 4, 2 and 1 Unit/µl) was used to digest 1,600 ng of amplicon in the reaction mixture volume of 30 µl. The mixture tubes were incubated overnight at 65^{o}C under paraffin oil. The digested product was separated in 8.5% acrylamide gel electrophoresis as in Figure 8. If the product was completely digested, there would be two bands of 130 and 180 bp products. The minimal concentration of restriction enzyme that could digest 1,600 ng of amplicon was about 1 Unit/µl .

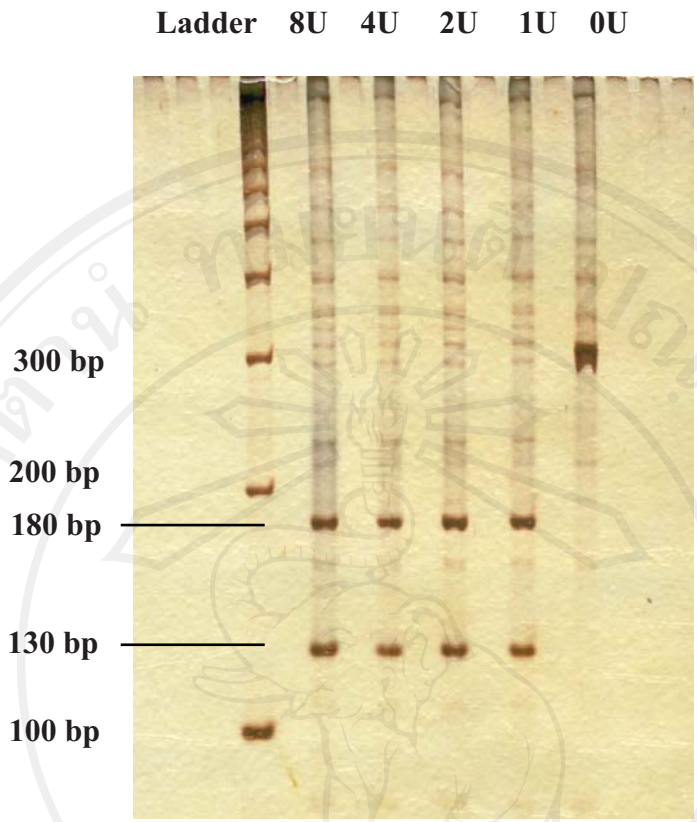

Figure 7 Digested product separated in 8.7% acrylamide gel electrophoresis with different *Taq* I restriction enzyme concentrations (8, 4, 2, 1 and 0 Unit/µl).

3.2 Population characteristics

The study was conducted in 90 Thai subjects which separated into 3 groups (2 alcohol dependence groups and non-alcohol dependence controls: 30 persons/group). All subjects were asked do the self-reported questionaires and interviewed face-to-face. The data collected from the subjects include their demographic data, city of residence, occupation, medical history, family history and drinking status.

The alcohol dependence groups (moderate and severe dependence) were recruited from the detoxification unit department of psychiatry. These groups met the

diagnostic criteria from the checklist at least three of seven items in module J: alcohol abuse and dependence for Mini International Neuropsychiatric Interview (M.I.N.I) Thai version 5.0.0. Their severity of dependence were assessed using Severity of alcohol Dependence Questionnaires (SADQ). The control subjects were collected from the non-alcohol dependence and non-alcohol psychoactive substance use disorders assessed by module J and K from M.I.N.I. Thai version 5.0.0.

The demographic characteristics of the subject are shown in Table 6. The mean age of moderate alcohol dependence, severe alcohol dependence and control subjects were not significantly different (df = 2, F= 0.405, p = 0.668).

Table 6 Demographic data of moderate dependence (n=30), severe dependence (n=30) and control (n=30)

Variables	Moderate dependence	Severe dependence	Control subjects
Age range (year)			
21-30	1	1	6
31-40	9	9	7
41-50	10	12	9
51-60	10	8	4
> 61	0	0	4
Mean age (year)	44.37 ± 8.65	44.93 ± 8.17	42.73 ± 12.18
BMI	21.61 ± 3.35	21.67 ± 2.86	23.70 ± 4.36
Mean SADQ score	19.90 ± 6.37	38.70 ± 6.27	0
Underlying diseases			
none	12	10	15
Diabetes	4	3	1
Cardiovascular diseases	2	0	0
Skin diseases	2	0	0
Hypertension	2	5	7
Hypercholesterol	0	0	1
Hepatic diseases	9	11	2
Peptic ulcer	1	7	1
Allergy	1	0	1
Gout	0	0	1
Thyroid	0	1	0
Kidney diseases	1	1	0
Bone diseases	0	0	1
Education			
Primary school	6	17	4
Secondary school	15	7	10
Bachelor's degree	8	6	13
Higher than bachelor's degree	1	0	3
Occupation			
Merchant	1	1	2
Government officer	16	7	12
Employee	12	18	8
Farmers	0	2	1
Others	1	2	7

3.3 Polymorphism Detection

The SNP polymorphism of the study site was typed by PCR-RFLP technique using *Taq*I restriction enzyme for digestion. The test of Hardy-Weinberg equilibrium was performed. The distribution of the genotypes was determined using Pearson's chi squared test by comparing the observed genotype frequencies and the expected genotype frequencies. The calculation of allele frequencies found that the number of observed genotype frequencies and expected genotype frequencies were not significantly different (p = 0.138). As a result, the distribution of genotype in this study was in agreement of Hardy-Weinberg equilibrium.

The total 90 subjects were participated in this study. The allele frequencies of rs 1800497 C/T SNP are shown in Table 7. The frequencies of allele in moderate dependence, severe dependence and controls revealed no significant difference. (χ^2 = 0.952, df =2, $p > 0.05$)

In Table 8, the distribution of rs 1800497 C/T SNP genotypes were shown. The percentage of TT genotype in severe patients (33.3%) was higher than moderate dependence and controls (13.3 and 20.0% respectively). However, we did not found the difference of genotype frequency (χ^2 = 3.792, df =4, $p > 0.05$) among each group.

The severe patients seemed to have the percentage of A1+ allele carriers (TC and TT genotype) higher than in moderate and control group. However, we found no significant difference among these three groups (p = 0.621) as shown in Table 9.

The odds ratio for A1+/A1- allele in moderate compare to control group was 1.0 (95% CI = 0.342-2.926). To compare between severe and moderate group was also in the severe and control group comparison of 1.643 (95% CI = 0.527-5.120) as

shown on Table 5. To compare the level of association we use rho-spearman as in Table 10.

In Table 11, when we compared the significant difference of mean of standard drink. We found that the standard drink was 17.00 ± 10.34 in moderate dependence, 29.46 ± 14.19 in severe dependence and 6.93 ± 7.69 in control group. The mean comparison shown that there was significant difference of standard drink between 3 groups ($p = 0.00$)

To compare the numbers of positive family history between patients (moderate and severe patients) and controls, we found that there was 23, 28 and 6 positive family history subjects in moderate, severe and control group respectively. There was significant difference between dependence and control subjects ($p = 0.00$). However, there was no significant difference between moderate and severe dependence ($p = 0.71$) but there was significant difference in positive family history between severe and control group at $p = 0.00$ as shown in Table 12.

Table 7 Percentage of rs 1800497 C/T SNP alleles in subjects: moderate dependence (n = 30), severe dependence (n = 30) and controls (n = 30)

Subjects	% of C allele (n)	% of T allele (n)
Moderate	60.0 (36)	40.0 (24)
Severe	45.0 (27)	55.0 (33)
Control	57.0 (34)	43.0 (26)

Pearson Chi-Square 2.996, df = 2, $p = 0.224$

Table 8 Percentage of rs 1800497 C/T SNP genotypes in subjects: moderate dependence (n = 30), severe dependence (n = 30) and controls (n = 30)

Subjects	%CC (n)	%TC (n)	%TT (n)
Moderate	33.3 (10)	53.3 (16)	13.3 (4)
Severe	23.3 (7)	43.3 (13)	33.3 (10)
Control	33.3 (10)	46.7 (14)	20.0 (6)

Pearson Chi-Square 3.792, df = 4, $p = 0.435$

Table 9 Numbers of A1+ allele (TC and TT genotype) and A1- allele (CC genotype) carriers in all subject groups

Subjects	%A1+ carriers (n)	%A1- carriers (n)	Total
Moderate	66.7(20)	33.3(10)	100.0(30)
Severe	76.7(23)	23.3(7)	100.0(30)
Control	66.7(20)	33.3(10)	100.0(30)

Pearson Chi-Square 0.952, df = 2, $p = 0.621$

Table 10 Odds ratio of A1+/A1- allele found in subjects

A1+ allele ratio in groups	OR	95% CI	Rho spearman correlation
Moderate/control	1.0	0.342-2.926	1.0
Severe/moderate	1.643	0.527-5.120	0.399
Severe/Control	1.643	0.527-5.120	0.399

Table 11 Mean of standard drinking in 3 groups of subjects

Subjects	Mean of standard drinks	SD	Variance
Moderate	17.00	10.34	106.97
Severe	29.46	14.19	201.36
Control	6.93	7.69	59.24

F = 31.19, $p = 0.00$

Table 12 Numbers of positive family history in 3 groups of subjects

Subjects	%positive history (n)	%negative history (n)	Total (n)
Moderate	76.67 (23)	23.33 (7)	100 (30)
Severe	93.33 (28)	6.67 (2)	100 (30)
Control	20 (6)	80 (24)	100 (30)

Pearson Chi-Square 36.388, df = 1, $p = 0.00$