CHAPTER II

MATERIAL AND METHODS

2.1. Study subjects

Blood samples were collected from lung cancer patients admitted to Maharaj Nakorn Chiang Mai hospital during September 2006 to May 2007 (n=227). All of the cases were newly diagnosed, previously untreated (chemotherapy or radiotherapy), and histologically confirmed. Blood samples were also collected from the control group which was healthy volunteers who came for check-up at laboratory center of Faculty of Associated Medical Sciences (AMS), Chiang Mai University, Thailand. At the time of collecting sample, they have no history and symptom of lung cancer such as cough, shortness of breath, wheezing, weight loss, weakness, and fatigue. All the patients and controls were in 17 provinces of northern region, Thailand. Controls were matched to cases based on gender, ethnicity and age. The characteristics of a total of 173 studied subjects comprising 91 lung cancer patients and 82 healthy volunteers are summarized in Table 2.1. As shown in the table, there were 58 males (64%), 33 females (36%) of cases and 38 males (46%), 44 females (54%) of controls. The average age of the case and control groups was (mean ±SD) 59.77±9.73 (ranged from 38 to 80) and, 55.77 ±13.39 (ranged from 22 to 88). The case and control subjects were significantly different in term of age, gender and smoking status ($p=0.028$, 0.023 and <0.001 respectively). The human subject protocol for this study received certificate of ethic clearance by the Human

Experimental Committee for the Faculty of AMS and the Human Experimental Committee for Faculty of Medicine, Chiang Mai University, Thailand. After informed consent was obtained, study subjects were personally interviewed using a questionnaire to obtain epidemiology information on sociodemographic characteristics and smoking status. Five milliliters of venous blood was collected into EDTA (disodium salt) tubes from each patient and control.

Table 2.1. Characteristic of lung cancer patients and control subjects

Variable	Cases n (%)	Controls n (%)	p
All subjects (n = 173)	91	82	
Age (Mean±SD) (Min= 22, Max=88)	59.8 ± (9.73) (38-80)	55.8 ± (13.39) (22-88)	0.028[a]
Gender			0.023[b]
Males (n = 96)	58 (64)	38 (46)	
Females (n = 77)	33 (36)	44 (54)	
Smoking Status			<0.001[b]
Smokers (n = 101)	73 (80)	28 (34)	
Males (n = 76)	54	22	
Females (n = 25)	19	6	
Non-smokers (n = 72)	18 (20)	54 (66)	
Males (n = 20)	4	16	
Females (n = 52)	14	38	

[a]un-paired t-test, [b]chi-square test

2.2. Extraction of DNA (Johns, 1989)

EDTA blood sample was centrifuged at 2,500, rpm for 10 minutes in order to remove blood plasma and red blood cells (RBCs) was lysed by adding 2.5 ml of steriled water and mix throughly by inversion the pellet of white blood cells (WBCs) was obtained by centrifugation at 2,200, rpm (KUBOTA 5200, Japan), for 15 minutes and discarding the red supernatant .

Three ml of reagent B (see appendix) containing proteinase K (10µl/ml, Sigma) was added into a 15 ml Falcon tube containing WBC pellets. The deproteinisation was continued overnight in an roller mixer (Stuart scientific, UK) at room temperature. The sample was examined the next morning to ensure complete digestion. After ensuring complete digestion, the sample was mixed with 750 µl of sodium perchlorate (see appendix) and stood at roon temperature for 15 minutes. An equal volume (4 ml) of chloroform (BDH) was added to the sample and mixed at room temperature for 30 minutes followed by centrifugation at 2,500, rpm (KUBOTA 5200, Japan) for 5 minutes. The upper aqueous phase was transferred into a steriled 15 ml Falcon tube containing an equal volume of propanol-2-ol mixture. The sample was inverted several times; the DNA should be seen at this stage as very fine white threads. The DNA was spun down at 2,500, rpm (KUBOTA 5200, Japan) for 3-5 minutes and washed twice with with 75% ethanol (BDH). The DNA was then allow to air dry for up to 30 minutes at the bottom of the Falcon tube before being dissolved in 100 µl of steriled distilled water. The DNA was stored at -20°C until use.

Table 2.2 List of primers used in this study

Polymorphisms	Primers sequences (5'-3')	References
CYP1A1(Ile462Val)	Fw: 5'CCT ACC TGA ACG GTT TCT CAC CCC-3' Rv :5'CAG GTA GAC AGA GTC TAG GCC TCA G-3'	(Cascorbi, 1996)
hOGG1(Ser326Cys)	Rv (P1) :5'CTG CTT CCC TAC CAC TCC TCA C-3' Fw (P2) :5'-CTC CCT AGG TTT CCT CTC CTC C-3' Fw (S1) :5'-TGC CGA CCT GCG CCA TTG-3' Rv(S2) :5'-GCT CCT GAG CAT GGC CGG-3'	(Liang, 2005)
CYP1A1 (MspI)	Fw:CAG TGA AGA GGT GTA GCC GCT Rv:TAG GAG TCT TGT CTC ATG CCT	(Ma, 2006)
CYP2E1 (PstI)	Fw:CCA GTC GAG TCT ACA TTG TCA Rv:TTC ATT CTG TCT TCT AAC TGG	(Kim, 1995)
CYP2E1 (DraI)	Fw:TCG TCA GTT CCT GAA AGC AGG Rv:GAG CTC TGA TGC AAG TAT CGC A	(Kim, 1995)
MPO(AciI)	Fw:CGG TAT AGG CAC ACA ATG GTG Rv:GCA ATG GTT CAA GCG ATT CTT	(London, 1997)
MMP-1 (AluI)	Fw:TGA CTT TTA AAA CAT AGT CTA TGT TCA Rv:TCT TGG ATT GAT TTG AGA TAA GTC ATA GC	(Zhu, 2001)
p53(Arg72Pro)	Fw (P1) :5'GGA TGA TTT GAT GCT GTC CCC GGA CGA-3' Rv (P2) :5'GCC CAG ACG GAA ACC GTA GCT GC-3' S1: 5'TGC CAG AGG CTG CTC CCC GCG-3' S2: 5'CTG GTG CAG GGG CCA CGG GG-3'	Own designed
GSTM1	Fw:GAA CTC CCT GAA AAG CTA AAG Rv:GTT GGG CTC AAA TAT ACG GTG G	(Arand, 1996)
GSTT1	Fw:GTT GGG CTC AAA TAT ACG GTG G Rv:TCA CCG GAT CAT GGC CAG CA	(Arand, 1996)
Albumin	Fw:GCC CTC TGG TAA CAA GTC CTA C Rv:GCC CTA AAA AGA AAA TCG CCA ATC-3'	(Arand, 1996)

2.3. Genotyping

Polymerase chain reaction (PCR) based restriction fragment length polymorphism (RFLP) was used to examine the polymorphisms of CYP1A1 (Ile462Val), CYP1A1(MspI), CYP2E1 (PstI), CYP2E1 (DraI), MPO (AciI) and MMP-1.(AluI). DNA isolated from peripheral blood of lung cancer patient and healthly volunteers were submitted to separate amplifications followed by digestion with appropriate restriction enzymes. In the case of detecting missense mutation in, p53 (Arg72Pro), and hOGG1 (Ser326Cys) genes a newly developed technique called diASA-AMP (di-allele-specific amplification with artificially modified primers) was utilized. For genotyping of the GSTM1 and GSTT1 null genotype, a multiplex PCR utilizing a set of primers specific for detected sequence of GSTM1 and GSTT1 was performed (summarized in tables 2.3). Primers specific for albumin gene was also included as an internal controls to avoid misreading of simple PCR amplification failure as a null genotype.

Table 2.3. Summary of genotyping methods used to detect each polymorphisms

Polymorphisms	Detection methods
CYP1A1 (Ilu462Val)	PCR-RFLP
CYP1A1 (M*sp*I)	PCR-RFLP
CYP2E1 (PstI)	PCR-RFLP
CYP2E1 (DraI)	PCR-RFLP
MPO (AciI)	PCR-RFLP
MMP1 (AluI)	PCR-RFLP
GSTM1	Multiplex PCR
GSTT1	Multiplex PCR
hOGG1 (Ser326Cys)	diASA-AMP
p53 (Arg72Pro)	diASA-AMP

2.3.1. PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism)

Polymerase Chain Reaction (PCR) is capable of producing enormous amplification of a short DNA sequence from a single molecule of starter DNA. It is used to amplify a specific DNA sequence lying between known positions (flanks) on

a double-stranded (ds) DNA molecule. The amplification process is mediated by oligonucleotide primers that, typically, are 20-30 nucleotides long. The primers are single-stranded (ss) DNA that have sequences complementary to the flanking regions of the target sequence. Primers anneal to the flanking regions by complementary-base pairing (G=C and A=T) using hydrogen bonding. Then the amplified product was digested with specific restriction endonucleases (RE). The digested products were analyzed on agarose gel electrophoresis.

(1) Detection of CYP1A1(Il462Val) polymorphism

The 25 µl PCR reaction mixture contained approximately 50-100 ng of genomic DNA, 1.25 units of Taq polymerase (Fermentas, USA), 0.4 µM of a pair of primers, and 200 each of 4 dNTP (Fermentas, USA) in 1x of PCR buffer (Fermentas, USA). The amplification reaction (Table 2.4) was performed using a Thermal Cycler (Eppendorf, Germany). Ten µl of PCR product was digested with 2.5 units of BsrDI (Fermentas, USA) at 55 °C for overnight. After digestion, the products were subjected to 2.5% agarose gel electrophoresis, followed by ethidium bromide staining. The gels were photographed using an ultraviolet light transilluminator. Homozygous wild-type alleles show 209, 123 base pair (bp) fragments, while heterozygous alleles show three bands at 293, 209, 123 bp, respectively. Homozygous rare-allele individuals show only 293 bp band.

(2) Detection of CYP1A1(MspI) polymorphism

PCR amplification of a 340 bp DNA fragment containing an MspI restriction site was performed using the specific primers (Table 2.2).

A total of 50-100 ng DNA was amplified in a total volume of 25 µl containing 1x buffer, 1.25 units of Taq polymerase, 0.3 µM of each primer and 200 µM deoxynucleotide triphosphates. PCR reaction was performed according to condition described in table 2.4. A 10 µl aliquot of PCR product was digested with 10 units MspI restriction enzyme (Fermentas, USA) at 37°C overnight, and the digested PCR product was then resolved by electrophoresis on a 2.5% agarose gel. Homozygous wild-type individuals show 340 (bp) fragments, while heterozygous individuals show four bands at 340, 200, 140 bp, respectively. Homozygous rare-allele individuals show only 200,140 bp bands.

(3) Detection of CYP2E1(DraI) and CYP2E1 (PstI) polymorphism

CYP2E1 gene was performed using polymerase chain reaction (PCR) amplification with the primer set of CYP2E1(PstI) and CYP2E1 (DraI) (Table 2.2). The reaction mixture contained 50-100 ng DNA, 200 µM of each deoxynucleotide triphosphate, 2 mM MgCl2, 0.48 µM primer and 1.25 units DNA polymerase (Fermentas, USA) in a volume of 25 µl under condition in table 2.3. Amplified 995 base pair DraI PCR products were digested with DraI (Fermentas, USA), and the amplified 410 base pair PstI PCR products were digested with PstI (Fermentas, USA). Digested PCR products were analyzed using agarose gel electrophoresis. The wild-type DraI polymorphism (D/D) was characterized by 572, 302 and 121 bp fragments. A homozygote variant (C/C) was characterized by 874 and 121 bp fragments and the heterozygote variant (C/D) was characterized by 874, 572, 302 and 120 bp fragments. The wild-type PstI polymorphism (c1/c1) was characterized by

410 bp fragments after enzyme digestion. A homozygote variant (c2/c2) was characterized by 290, 120 bp fragments. The heterozygote variant (c1/c2) was characterized by 410, 290 and 120 bp fragments.

(4) Detection of MPO (AciI) polymorphism

25 µl PCR reaction contained 6 pmol of each of the MPO(AciI) primers (Table 2.2) 50-100 ng of genomic DNA in 1x buffer, 1.25 units of Taq polymerase, and 200 µM deoxynucleotide triphosphates. The amplification product was then digested with 20 units of AciI restriction enzyme (Fermentas, USA) overnight at 37°C. After restriction, the DNA products were analyzed electrophoresis in a 2.5% agarose gel. Two bands at 289 and 61 bp distinguished the homozygous AA variant genotype. Four bands at 289, 169, 120 and 61 bp represented the heterozygous AG and three bands at 169, 120 and 61 bp indicated the wild-type GG genotype.

(5) Detection of MMP1 (AluI) polymorphism

PCR amplification of a 269 bp DNA fragment was performed using the specific primers (Table 2.2) 0.5 µM. The PCR reactions were performed in a 25ul volume as the same mixer and condition as describe in the section of MPO(AciI) polymorphism detection. An 10 ul aliquot of PCR product was digested overnight 37°C with 10 U of Alu I (Fermentas, USA). After digestion, the products were subjected to electrophoresis on a 4% agarose gel at 50 volts 90 minutes. The MMP-1(AluI) 2G alleles (mutant) were represented by a DNA band with size at 269bp, the 1G alleles (wild type) were represented by two DNA bands with size 241 and 28bp, whereas the heterozygotes displayed a combination of the both alleles (269, 241 and 28bp).

Table 2.4 Conditions of PCR used to amplify DNA in order to detect polymorphism by PCR-RFLP

Polymorphisms	**PCR Reaction**					
	Initial temperature	Denature temperature	Annealing temperature	Extension temperature	Elongation temperature	No. of cycles
CYP1A1 (Ile462Val)	94°C 45 sec	94°C 1 min	55°C 1 min	72°C 1min	72°C 10 min	35
CYP1A1 (M*sp*I**)**	95°C 5 min	95°C 1 min	61°C 1 min	72°C 1 min	72°C 10 min	30
CYP2E1(**Pst**I**)**	95°C 5 min	95°C 45 sec	56°C 1 min	72°C 1 min	72°C 10 min	35
CYP2E1(DraI)	95°C 5 min	95°C 1 min	60°C 1 min	72°C 2 min	72°C 10 min	35
MPO(AciI)	94°C 5 min	94°C 1 min	56°C 1min	72°C 1 min	72°C 7 min	35
MMP1(AluI)	94°C 5 min	94°C 1 min	56°C 1min	72°C 1 min	72°C 7 min	35

Table 2.5 Lists of restriction enzymes and digestion conditions utilized in this study

Polymorphisms	Restriction Enzymes	Temperature Reaction	Time
CYP1A1(Ile462Val)	BsrDI	55°C	overnight
CYP1A1 (M*sp*I**)**	MspI	37°C	overnight
CYP2E1 (P**stI)**	PstI	37°C	overnight
CYP2E1 (DraI)	DraI	37°C	overnight
MPO (AciI)	AciI	37°C	overnight
MMP1 (AluI)	AluI	37°C	overnight

Table 2.6. Sizes of PCR products and RFLP pattern after restriction enzyme digestion

Polymorphisms	PCR Products (base-pair)	RFLP-Fragment Pattern (bp)		
		Homozygous wild-type	Heterozygous variant	Homozygous variant
CYP1A1(Ile462Val)	293	209, 123	123, 209, 293	293
CYP1A1 (M*spI*)	340	340	340, 200, 140	200, 140
CYP2E1(PstI)	410	410	410, 290, 120	290, 120
CYP2E1 (DraI)	995	572, 302, 121	874, 572, 302, 121	874, 121
MPO(AciI)	350	169, 120, 61	289, 169, 120, 61	289, 61
MMP1(AluI)	269	241, 28	269, 241, 28	269

2.3.2. Multiplex PCR

Genotypes for the GSTM1 and GSTT1 were determined by multiplex PCR, using GSTM1- and GSTT1- specific primer pairs together with a third pairs of primer for albumin as an internal control. The reaction mixture contained 50-100 ng DNA, 200 µM of each deoxynucleotide triphosphate, 2 µM $MgCl_2$, 1 µM of GSTT1 primer, 1µM of GSTM1 primer, 0.6 µM of albumin primer and 1.25 units DNA polymerase (Fermentas, USA) in a volume of 25 µl according to the protocol given in Table 2.7. The PCR product from GSTM1 gene was 215 bp in size, GSTT1 gene was 480 bp in size and albumin gene was 350 bp in size. The absence of the GSTM1 and/or GSTT1 specific PCR product indicated the corresponding null genotype, whereas albumin-specific fragment confirmed proper functioning of the reaction.

Table 2.7. The condition of multiplex PCR used to determine GSTM1 and GSTT1 genotypes

Step	Temperature	Duration	Number of cycles
Primary denaturation	95°C	2 min	
Denaturation	94°C	1 min	
Annealing	64°C	1 min	→ 30
Extension	72°C	1 min	
Final elongation	72°C	5 min	

2.3.3 Di-allele-specific amplification with artificially modified primers (diASA-AMP)

This method was first described by Liang G, 2005. Two pairs of primers (P1, P2, S1, S2), as complementary to the non-coding strand and coding strand of template DNA respectively, were designed to determine SNP type. One pair (S2 and P2) produced a band representing the wild-type allele (representing as A allele in Figure 8) (S2P2) and the other pair (S1 and P1) produced a band representing the mutant allele (representing as G allele) (S1P1). Outer primers (P1 and P2) produced a common band (P1P2). The 3' end of the allele-specific primers (S1 and S2) is just on the position of SNP, which controls the extension reaction of each specific primer. To enhance allelic specificity, an artificially modified mismatch (indicated by an asterisk) at position 3 from the 3' end was incorporated in the inner primers (S1 and S2). By positioning the two outer primers (P1 and P2) at different distances from the SNP site, the two allele specific bands (S1P1 and S2P2) differed in length.

Discrimination of wild-type allele and mutant-type allele was achieved by analyzing the length of the resulting amplification fragments using gel electrophoresis.

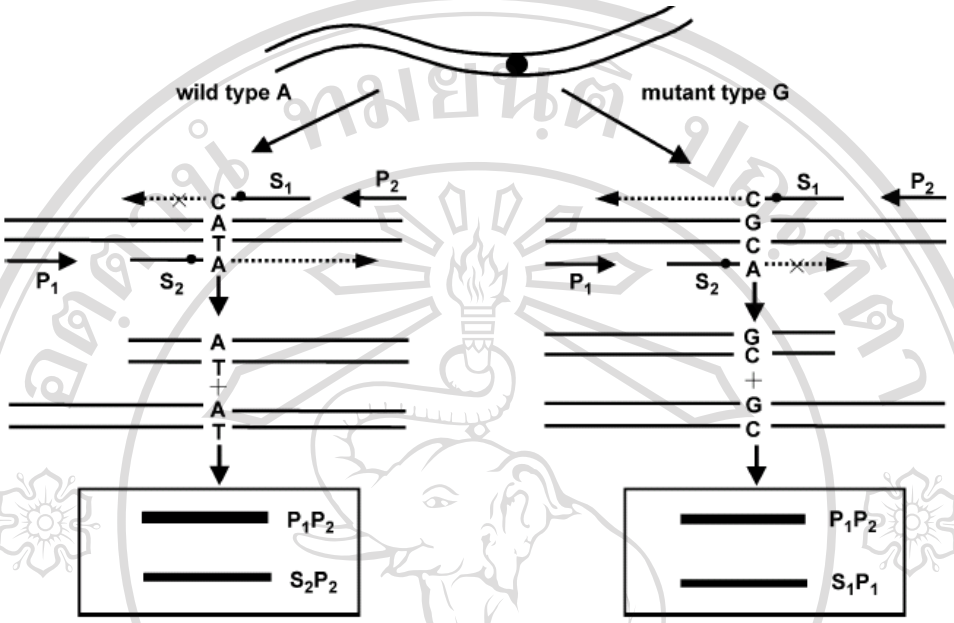

Figure 2.1. Logic of SNP typing method by di-allele-specific-amplification with artificially modified primers (Figure from Liang, 2005)

(1) Detection of p53 (Arg72Pro) polymorphism

The reaction mixture contained 25 ng of genomic DNA, 200 μM of each deoxynucleotide triphosphate, 10xPCR buffer (including Mg^{2+}), 10 pmol of each primer, and 0.25 units of HotstarTaq DNA polymerase in a volume of 25 μl. After initial denaturation at 94° for 5 min, PCR reaction was carried out using 28 cycles at 94°C for 30 s, 63°C for 30°s, and 72° C for 40 s. The amplified products were analysed by electrophoresis on a 4.0% agarose gel at 50 volts 90 minutes. The wild type (Arg/Arg), the homozygote variant (Pro/Pro) the heterozygote (Arg/Pro) was characterized by 140 (S2P2) and 217bp (P1P2) fragments, 113(S1P1) and 217 bp (P1P2) fragments, 113 (S1P1), 140 (S2P2) and 217 bp (P1P2) fragments, respectively.

(2) Detection of hOGG1 (Ser326Cys) polymorphism

The reaction mixture contained 25 ng DNA, 200 mM of each deoxynucleotide triphosphate, 10xPCR buffer (including Mg^{2+}) 10 pmol of each primer, and 0.25 units of HotstarTaq DNA polymerase in a volume of 25 µl. After initial denaturation at 95°C for 15 min, PCR reaction was carried out using 30 cycles at 95°C for 30 s, 58°C for 30 s, and 72°C for 40 s. The amplified products were analysed by electrophoresis on a 2.5% agarose gel. The wild type (Ser/Ser), the homozygote variant (Cys/Cys) the heterozygote (Ser/Cys) was characterized by 271 (S2P2) and 598bp (P1P2) fragments, 362(S1P1) and 598 bp (P1P2) fragments, 271 (S1P1), 362 (S2P2) and 598 bp (P1P2) fragments, respectively.

2.4. Agarose gel electrophoresis

Agarose powder at various concentrations (1.5-4%) was dissolved in 1X Tris-Borate-EDTA (TBE) buffer (see appendix) at a boiling temperature. After cooling down, it was poured into the tray and left for polymerization. The gel was put submarine in 1X TBE buffer. A 5 µl of PCR product or digested DNA was mixed with 1 µl of 6X loading dye (Fermentas, USA) before loading into each well. Electrophoresis was carried out at 50-80 volts until the loading dye reach the bottom of the gel, depend on the product length. After that, the gel was stained with 0.002% ethidium bromide for 30 minutes and detected by Gel Documentation (Bio-rad, Italy)

2.5. Statistical analysis

The X^2 tests were used to test the association between lung cancer risk and genetic polymorphisms in CYP1A1 (MspI), CYP1A1 (Ilu462Val), CYP2E1 (PstI), CYP2E1 (DraI), GSTM1, GSTT1, hOGG1(Ser326Cys), p53 (Arg72Pro), MPO(AciI), MMP-1(AluI) genes. The odds ratios (ORs) and CI were used to describe the strength of the association and using the following formulae:

OR=AC/BD using a 2x2 table

95%CI=ln (OR)±1.96$(1/A+1/B+1/C+1/D)^{0.5}$

Logistic regression was used to obtain age, gender and smoking-adjusted ORs. All statistical analyses were performed using SPSS version (version 10.0) software (SPSS Inc., USA).