# CHAPTER III

# MATERIAL AND METHODS

## 1. Materials

### 1.1. Population Study

In this study volunteers from pediatric hematology clinic at King Chulalongkorn Memorial Hospital, Bangkok who consented for peripheral blood samples or bone marrow samples. One hundred and thirty-nine acute lymphoblastic leukemia samples were collected from January 2007-November 2007. One hundred and thirty-nine control samples were collected from January 2007 - November 2007 who were not having cancer history, match case and control subjects by age and sex.

### 1.2. Collecting Specimen

After parental or patient consent, bone marrow or peripheral blood were obtained from children , who were having acute leukemia at Chulalongkorn Memorial Hospital, by a pediatric hematologist under sterile conditions. About 3 ml of bone marrow or peripheral blood was mixed with ethylene diaminetetraacetic (EDTA) immediately after collection for DNA extraction. The samples were stored at 4°C until used.

### 1.3. DNA Extraction

Phenol-chloroform DNA extraction method

### 1.4 Identification of Xenobiotic-metabolizing enzymes gene polymophisms

#### 1.4.1 Synthetic Oligonucleotides (or primers)

Table 3 Oligonucleotides and their descriptions

| Name | Sequence (5'-3') | $T_m$ (°C) | Description |
|---|---|---|---|
| F46 | GCCCTGGCTAGTTGCTGAAG | 59 | Oligonucleotides for PCR amplification of a fragment of *GSTT1* gene of exon 6 nt 2293 (Katoh *et al.*, 1996) |
| R137 | GCATCTGATTTGGGGACCACA | 58 | |
| P1 | CGCCATCTTGTGCTACATTGCCCG | 62 | Oligonucleotides for PCR amplification of a fragment of *GSTM1* gene of exon 5 nt 1578 (Zhong *et al.*, 2001) |
| P3 | TTCTGGATTGTAGCAGATCA | 58 | |
| M3F | GGCTGAGCAAT CTGACCCTA | 57 | Oligonucleotides for PCR amplification of a fragment of *CYP1A1* gene of nt 3801 T→C (Cascorbi *et al.*, 1996) |
| P80 | TAGGAGTCTTGTCTCATGCCT | 55 | |
| M2F | CTGTCTCCCTCTGGTTACAGGAAGC | 61 | Oligonucleotides for PCR amplification of a fragment of *CYP1A1* gene of nt 2455 A→G (Cascorbi *et al.*, 1996) |
| M2R | TTCCACCCGTTGCAGCAGGATAGCC | 65 | |
| F609 | TCCTCAGAGTGGCATTCTGC | 57 | Oligonucleotides for PCR amplification of a fragment of *NQO1* gene at region in intron 5 and exon 6 (Martin *et al.*, 2005) |
| R609 | TCTCCTCATCCTGTACCTCT | 53 | |

Note $T_m$ was calculated from the formula 2 (A+T) + 4 (G+C)

### 1.4.2 Enzymes

Taq DNA polymerase (Fermentas)

Restriction endonucleases (Fermentas)

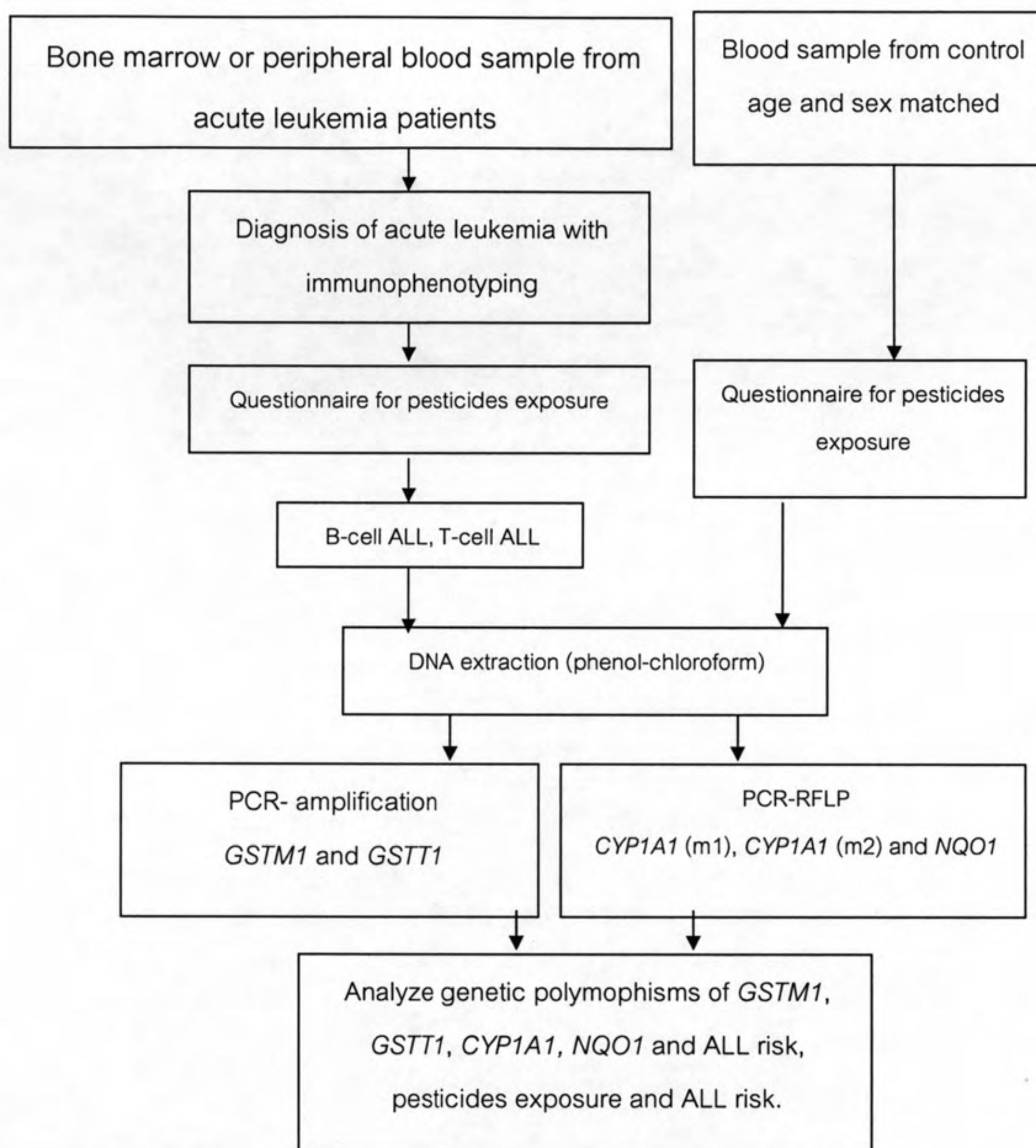
Table 4 Restriction enzymes and buffer

| Enzymes | Recognition sequence | Buffer | Manufacturer |
|---|---|---|---|
| *Msp*I | 5'---C↓CGG---3'<br>3'---GGC↑C---5' | Buffer Tango | Fermentas |
| *BsrD*I | 5'---GCAATGN ↓ ---3'<br>3'---CGTTAC↑NN ---5' | Buffer R | Fermentas |
| *Hinf*I | 5'---G ↓ ANTC---3'<br>3'---CTNA ↑ G---5' | Buffer R | Fermentas |

## 1.5 Chemicals

All other chemicals used in this work were either analytical or molecular biology grades purchased from many suppliers (Sigma; BIO-RAD; Phamacia Biotech AB; USB; Fermentas and Merck).

1.6 Work Outline



## 2. Methods

### 2.1 Extraction of Genomic DNA from sample

Genomic DNA Extraction by standard phenol chloroform method. EDTA blood was centrifuged at 4,000 rpm for 10 minutes and buffy coat was removed into a 15 ml tube to which 15 ml of lysis reagent buffer I was added then centrifuged at 4,000 rpm for 10 minutes. The supernatant was discarded lysis buffer I 3.4 ml then centrifuge the mixture at 4,000 rpm for 10 minutes. The supernatant was discarded from 15 ml tube, added 900 μl of lysis buffer II, 50 μl of 10% SDS and 10 μl of Proteinase K (20 mg/ml), then incubated at 55° C overnight. 500 μl of phenol-chloroform was added into the mixture, vortexed and centrifuged at 6,000 rpm for 5 minutes. The lower was discarded supernatant and this step was repeated again. The upper supernatant was transferred into 1.5 microcentrifuge tube, add 50 μl of sodium acetate and 500 μl of cold 100% ethanol or frozen at -20°C around 2 hours or overnight at 4°C. The mixture was then centrifuged at 14,000 rpm for 30 minutes in 4°C. The supernatant discard from mixture, pellet was washed with 500 μl cold 70% ethanol, centrifuged at 14,000 rpm for 5 minutes in 4°C. Genomic DNA pellet was air dried overnight at 37°C, dissolved in 100 μl of the TE buffer or $dH_2O$ at 37°C overnight and stored at 4°C.

### 2.2 Identification of *CYP1A1* (m1) and *CYP1A1* (m2) polymorphism

*CYP1A1* mutations 3801T→C (m1) and 2455A→G (m2) were characterized by the PCR-RFLP approach of Cascorbi (Cascorbi et al., 1996). A DNA fragment of 899-bp was amplified in 20 μL contained 1X PCR buffer, 0.2 mM of each dNTPs, 1.5 mM $MgCl_2$ Mix, 0.05 units Taq DNA polymerase (Fermentas), and 0.1 μM of each primers M3F, 5'-GGCTGAGCAATCTGACCCTA-3' and P80, 5'-TAGGAGTCTTGTCTCATGCCT-3'. PCR was performed for 35 cycles of 30 seconds at 94°C, 1 minute at 63°C, and 1 minute at 72°C. The PCR product (10 μL) was digested with *Msp*I (1 U, 37°C), resulting in smaller fragments (693 and 206 bp) in case of the mutation, and subjected to electrophoresis on a 1.5% agarose gel. Mutations m2 were

detected by amplifying a 204 bp fragment with primers M2F, 5'-CTGTCTCCC TCTGGTTACAGGAAGC-3' and M2R, 5'-TTCCACCCGTTGCAGCAGGATAGCC-3' as described above, followed by digestion with *Bsr*DI (1 U, 65°C) respectively. This restriction sites were lost in the case of mutation and the resulting restricted fragments were evaluated on a 2.0% agarose gel. These mutations were then used to define two distinct alleles, *CYP1A1*2A* (presence of m1 only), *2B (both m1 and m2).

### 2.3 Identification of *NQO1* polymrphism

*NQO1* mutation C609T was characterized by the PCR-RFLP approach of Buffler (Buffler et al, 2004). A DNA fragment of 211 bp was amplified in 20 μL contained 1X PCR buffer, 0.2 mM of each dNTPs, 1.5 mM $MgCl_2$ Mix, 0.05 units Taq DNA polymerase (Fermentas), and 0.1 μM of each primers F609, 5'-TCCTCAGAGTGGCATTCTGC-3' and R609, 5'-TCTCCTCATCCTGTACCTCT-3'. PCR was performed for 35 cycles of 30 seconds at 95°C, 1 minute at 63°C, and 1 minute at 54°C. The PCR product (10 μL) was digested with *Hinf*I (1 U, 37°C), resulting in smaller fragments (204 and 7 bp) in case of the mutation, and subjected to electrophoresis on a 2.0% agarose gel. The fragment 211 bp of *NQO1* gene included of last 7 base at intron 5 and first 204 bp of exon 6. This restriction sites were lost in the case of normal.

### 2.4 Identification of *GSTM1* Polymorphic deletion

The polymorphic deletion of the *GSTM1* gene was genotyped using the multiplex PCR approach described by Zhong (Zhong *et al.*, 1993). The primers used to amplified *GSTM1* were as followed: P1, 5'-CGCCATCTTGTGCTACATTGCCCG-3' and P2, 5'-ATCTTCTCCTCT TCTGTCTC-3' and P3, 5'-TTCTGGATTGTAGCAGATCA-3'. P1 and P3 amplified a 230 bp product that specific to *GSTM1*, whereas P1 and P2 anneal to *GSTM1* and *GSTM4* genes, yielding a 157 bp fragment that serve as an internal control. The amplification reaction was performed in 20 μl containing 1X PCR buffer, 0.2 mM of each dNTPs, 1.5 mM $MgCl_2$ Mix, 0.05 units Taq DNA polymerase (Fermentas), and 0.1 μM of each primer. The PCR was performed for 35 cycles of 15 seconds at

94°C, 30 seconds at 58°C, and 45 seconds at 72°C. The last elongation step was extended to 7 minutes. The presence of one or both *GSTM1* alleles, identified by a 230 bp fragment, or its complete deletion (null genotype), was analyzed by electrophosesis on a 1.5% agarose gel and detect the band under ultraviolet light. The absence amplifiable GSTM1 (in the presence of the *GSTM4* coamplified control) indicate a null genotype.

### 2.5 Identification of *GSTT1* Polymorphic deletion

The polymorphic deletion of the *GSTT1* gene was determined by a modification of the PCR protocol described by Katoh (Katoh *et al.*,1996). The amplification reaction was performed in 20 μl containing 1X PCR buffer, 0.2 mM of each dNTPs, 1.5 mM $MgCl_2$ Mix, 0.05 units Taq DNA polymerase (Fermentas), and 0.1 μM of each primer. The primers used to amplified *GSTT1* were F46, 5'-GCCCTGGCTAGTTGCTGAAG-3' and R137, 2225'-GCATCTGATTTGGGGACCACA-3'. A 157-bp fragment of *GSTM4* gene was coamplified with the primers P1, 5'-CGCCATCTTGTGCTACATTGCCCG-3' and P2, 5'-ATCTTCTCCTCTTCTG- TCTC-3' as a control. The PCR was performed for 35 cycles of 15 seconds at 94°C, 30 seconds at 59°C, and 45 seconds at 72°C. The last elongation step was extended to 7 minutes. The presence of one or two *GSTT1* alleles, identified by a 112 bp fragment, or its complete deletion (null genotype), was analyzed by electrophosesis on a 1.5% agarose gel and detect the band under ultraviolet light.

## 3. Data Collection

Collection data at Pediatric Hematology clinic in King Chulalongkorn Memorial hospital, we obtains informed consent and collected bone marrow or peripheral blood from acute lymphoblastic leukemia cases and control subjects (hospital control that not malignancy history). Control subjects are match to cases on date of birth, gender and maternal race. Cases and controls are interviewed by researcher. Data collections were collected from parents using questionnaire. The

questionnaire was developed by the North California Childhood Leukemia Study (NCCLS) (Smith T et al., 2005). The information obtained includes demographic data, relevant medical history, a detailed history of pesticide use in the before hospital follow-up, and the importance of the timing exposures relative to a child's development, in addition to asking systematically about types of pesticides (e.g. insecticides spray, insect repellents), a list of trade names of commonly used products is enquired about to maximize recall. If this contact is unsuccessful, either by telephone or by visits to the participating hospitals at least every other week. The data of pesticide exposure is asked to complete an in-person interview, which takes about 10 minutes. Using a computer aided personal interview.

## 4. Data analysis

Statistic analysis was done using the Statistical Package for Social Sciences (SPSS) statistical software (SPSS Windows Version) trial version. The descriptive statistic (mean, frequency and standard deviation) were characterized the genetic polymorphisms, characteristics of leukemia subtypes, age and sex of ALL group. The genetic polymorphisms of *CYP1A1, NQO1, GSTM1* and *GSTT1* were analyzed by Hardy-Weinberg Equilibrium (HWE) in both cases and controls. The association between genotypes and ALL risk were analyzed by calculating the crude odds ratio (95% confidence intervals) and McNemar's test. *P*-value reported in the study are based on two-side probability test with a significance level of $P<0.05$.