CHAPTER III

MATERIALS AND METHODS

1. Materials

1.1 Population study

In this study volunteers from each ethnic group (control and patients) who consented for blood sampling were included. People blood samples from children with acute lymphoblastic leukemia in Thai children were included for polymorphisms analysis.

A total of one hundreds and thirty nine children diagnosed with childhood ALL at Department of Pediatrics, King Chulalongkorn Memorial Hospital, Bangkok, Thailand, between January – December 2007 were included in the study. The diagnosis of ALL was made by bone marrow morphology and immunophenotype. One hundreds and sixty one children coming for treatment for minor ailments at Department of Pediatrics, King Chulalongkorn Memorial Hospital with no evidence of cancer and matched for age and sex, were selected as controls. All cases and controls were of age $\leq$ 15 years. Only Thai population for at least two generation up was recruited for the cases and controls. The research protocol was approved by the Research Ethics Committee on Human Experimentation of King Chulalongkorn Memorial Hospital, Faculty of Medicine, Chulalongkorn University. Informed consent was obtained from each of these subjects.

1.1.1 Criteria for diagnosis of ALL

Diagnosis by bone marrow morphology had blast cell over than 25% of bone marrow cells and immunophenotype of blood samples had abnormal blast cells CD19+, CD10+/-, CD20+/-, CD22+, HLA-DR+ and CD3-, CD5-, CD7- by flow cytometry method.

1.1.2 Criteria for controls

Children treatment for minor diseases at Department of Pediatrics, King Chulalongkorn Memorial Hospital with no evidence of cancer and matched for age and sex, were selected as controls.

1.2 Specimen collection

Venous blood was obtained from consented patients at remission and controls. Blood samples were collected in tube containing 0.3 ml of ethylene diaminetetraacetic acid (EDTA) and stored at 4°C for genomic DNA extraction.

1.3 Genomic DNA Extraction

Genomic DNA was extracted from leukocytes using a standard phenol chloroform method.

1.4 Genotyping of *XRCC1*

Genotyping of *XRCC1* Arg194Trp (C>T), 280 Arg to His (G>A), and 399 Arg to Gln (G>A) polymorphisms were performed using PCR-RFLP technique (Lee et al., 2001). Negative controls in all PCR assays consisted of a similar reaction mixture with template replaced with sterile water. The primers used were as follows: F: GTT CCG TGT GAA GGA GGA GGA and R: CGA GTC TAG GTC TCA ACC CTA CTC ACT for codon 194 and F2: TTG ACC CCC AGT GGT GCT AA and R2: AGT CTG CTG GCT CTG GGC TGG for codon 280 and 399. The PCR product was visualized using a UV transilluminator after ethidium bromide staining.

The PCR products were digested with specific restriction enzymes for detecting the codon 194, 280 and 399 polymorphisms of *XRCC1* gene. Ten microliters of the PCR products were digested separately with 2 units of *Pvu* II for codon 194, *Rsa* I for codon 280, and *Msp* I for codon 399 (Biolabs) at 37°C for overnight. The products were then resolved on 2% agarose gels. DNA molecular weight marker of 100 bp was used to assess the size of the PCR-RFLP products.

1.5 Data collection

Data collections were collected from parents using questionnaire. The questionnaire was developed by The Northern California Childhood Leukemia Study (NCCLS) (Martyn T *et al.*, 2005) and translated to Thai language by back-to-back translation method. The parents of cases and controls were interviewed by researcher.

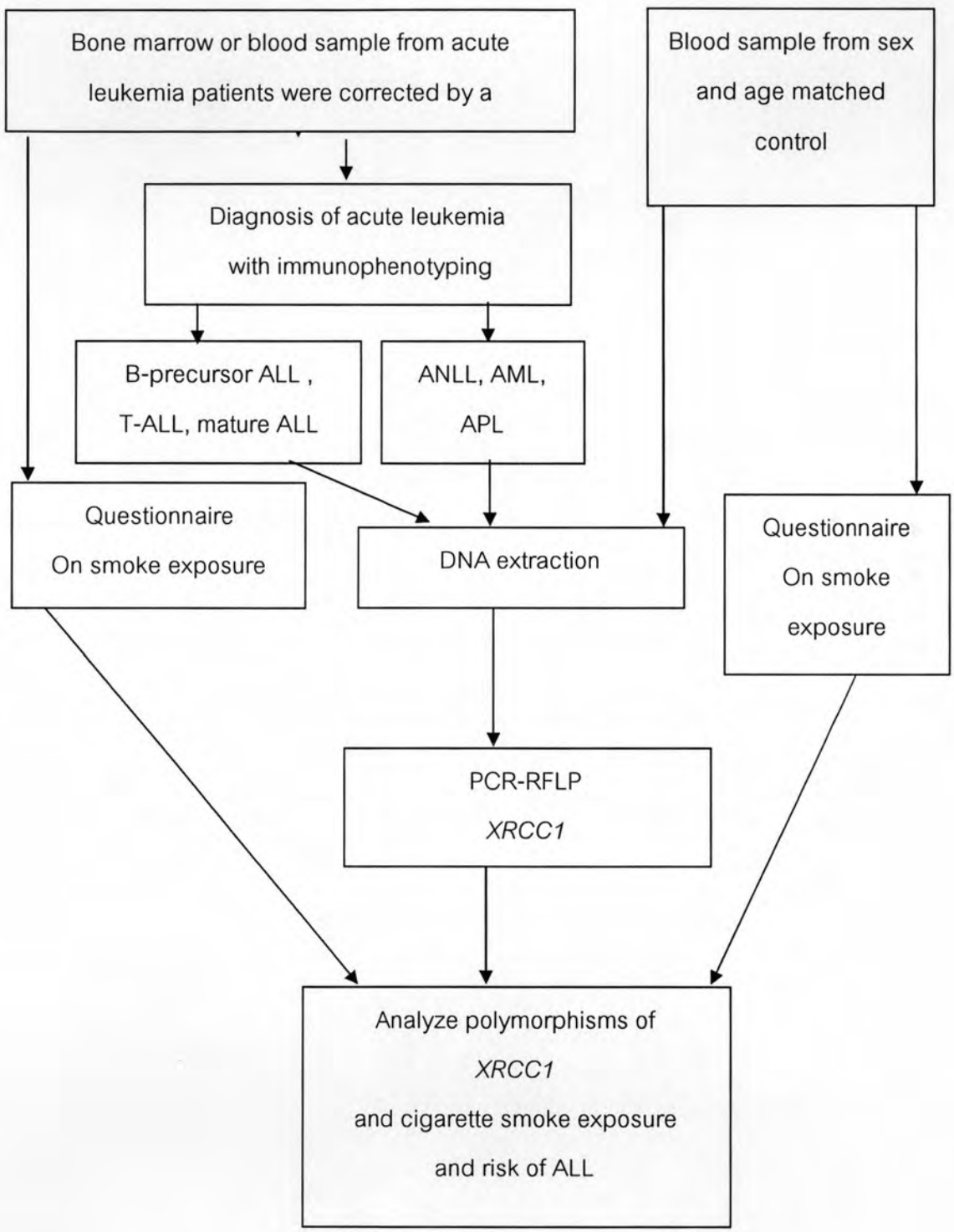
1.6 Statistical analysis

Statistical analysis was done using the Statistical Package for Social Sciences (SPSS) statistical software (SPSS Windows Version) trial version. Risk of ALL was analyzed using Chi square test. The association between genotypes and ALL risk were analyzed by calculating the crude odds ratios (OR) and 95% confidence intervals (95% CI). *P*- values reported in the study are based on two-sided probability test with a significance level of $P<0.05$.

1.7 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).

1.8 Work flow


Bone marrow or blood sample from acute leukemia patients were corrected by a
Blood sample from sex and age matched control
Diagnosis of acute leukemia with immunophenotyping
B-precursor ALL , T-ALL, mature ALL
ANLL, AML, APL
Questionnaire On smoke exposure
DNA extraction
Questionnaire On smoke exposure
PCR-RFLP XRCC1
Analyze polymorphisms of XRCC1 and cigarette smoke exposure and risk of ALL

2. Methods

2.1 Immunophenotyping by Three-color flow cytometry

Bone marrow was separated by Ficoll-Paque™PLUS (Amersham Phamacia, USA). It was rinsed it down with phosphate buffered saline (PBS) with 2% fetal calf serum (FCS). Separated leukocytes to CD panels and incubated in dark side for 20 minutes. Added FACS™ lysing and incubated in the dark side for 10 minutes. It was rinsed with PBS with 2% FCS 1 ml again. Take to centrifuge at 2,000 rpm for 2 minutes. Added 1% paraformaldehyde in all tube and analyzed with FCAScan flow cytometry (Becton Dickinson, USA). The immunophenotype was determined by flow cytometry using a panel of monoclonal antibodies, including those against CD10, CD19, CD20, CD22, CD3, CD5, CD7, CD34, HLA-DR, CD13, CD14, GPA, CD33, CD71.

2.2 Genotyping of *XRCC1*

2.2.1 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.

2.2.2 Genomic DNA amplification (Wiemels J et al., 2002)

2.2.2.1 Primers

Table 6 Base sequences of primers for PCR polymorphisms assay. (Joseph *et al.*, 2005)

Polymorphisms	Primer
codon 194	F: 5'-GTT CCG TGT GAA GGA GGA GGA-3'
	R: 5'-GGA GTC TGA GTC TCA ACC CTA CTC ACT-3'
Codon 280 and	F: 5'-TTG ACC CCC AGT GGT GCT AA-3'
Codon 399	R: 5'-AGT CTG CTG GCT CTG GGC TGG-3'

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

2.2.2 PCR conditions

A typical PCR reaction was carried out in a 25 μl reaction containing 2.5 μl 10X PCR buffer, 1.5 U of Taq DNA polymerase (Fermentas), 50 ng of each primer, 2.5 mM $MgCl_2$, 0.2 M of each dNTPs, and approximately 100 ng genomic DNA Template. For codon 194, after incubation at 94°C for 4 minutes, amplification was carried out for 35 cycles with the following temperature cycling parameters; 94°C for 40 seconds of denaturation, 55°C for 30 seconds of annealing and 72°C for 40 seconds of extension. The final amplification cycle included an addition of a 10 minutes extension at 72°C. For codon 280 and 399, after incubation at 95°C for 5 minutes, amplification was carried out for 35 cycles with the following temperature cycling parameters; 95°C for 30 seconds of denaturation, 56°C for 30 seconds of annealing and 72°C for 30 seconds of extension. The final amplification cycle included an addition of a 7 minutes extension at 72°C.

2.2.3 Restriction Fragment Length Polymorphism (RFLP)

Ten microliters of PCR product was digested with 2 U of each restriction enzyme (The type of restriction enzyme was used in digestion depend on *XRCC1* polymorphisms that represent in table) according to manufacturer's protocols (New England Biolabs), 10X reaction buffer (provided) and sterile distilled water added to a final volume of 20 µl. The digestion was incubated at 37° for 13-16 hours.

Table 7 *XRCC1* polymorphisms, restriction enzymes and results for *XRCC1* polymorphisms.

	codon 194	codon 280	codon 399
Restriction enzyme	*Pvu*II	*Rsa*I	*Msp*I
Size	Wild type: 138 bp Heterozygous: 75, 63 และ 138 bp Homozygous: 75 และ 63 bp	Wild type: 597, 201 และ 63 bp Heterozygous: 63, 201, 597 และ 660 bp Homozygous: 660 และ 201 bp	Wild type: 285, 461, 576 bp Heterozygous: 115, 285, 461 และ 576 bp Homozygous: 285 และ 576 bp

Note bp represent base pair (size of PCR product)

2.2.3.1 Enzymes

Taq DNA polymerase was purchased from Fermenta, USA.

Restriction endonucleases *Pvu*II, *Rsa*I, *Msp*I were purchased from Fermentas, USA.

2.2.4 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to detect *XRCC1* polymorphisms that their result produced large DNA fragments. Agarose gel of 1.5% was prepared by completely dissolving the gel powder upon heating in 1X TAE (89 mM Tris-HCl pH 7.4, 89 mM acetic acid, 2.5 mM EDTA). The solution was boiled in a microwave oven until it was completely dissolved and allowed to cool to around 50°C before pouring into an electrophoresis chamber set, with comb inserted. Twenty microliters of digestion's product was mixed with 1/6 volume of Loading Dye (0.25% bromophenol blue, 40% (W/V) sucrose in water) and loaded into gel slots in a submarine condition. Electrophoresis was performed at 85 volts for 1 hour. The DNA bands in the gel were visualized by staining with 2.0 µg/ml ethidium bromide and photographed under UV light at 302 nm.

2.3 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. The information obtained includes demographic data, relevant medical history, a detailed history of smoke exposure, which takes about 10 minutes and data were record in the case record form (CRF, Appendix C)

2.4 Data analysis

The descriptive statistics (mean, frequency) were applied to genetic polymorphism frequencies and characteristics of leukemia subtypes. Mean and standard deviation used for age, and frequency used for sex. For the inferential statistics, the proportions of genetic polymorphisms of XRCC1 gene were analyzed by Hardy-Weinberg Equilibrium (HWE) in both cases and controls. For genetic polymorphisms, smoke exposure and risk of ALL were analyzed by crude Odds Ratio

(95% confidence intervals) and Mc Nemar's test by Statistical Package for Social Sciences (SPSS) statistical software (SPSS Windows Version) trial version.