CHAPTER III

MATERIALS AND METHODS

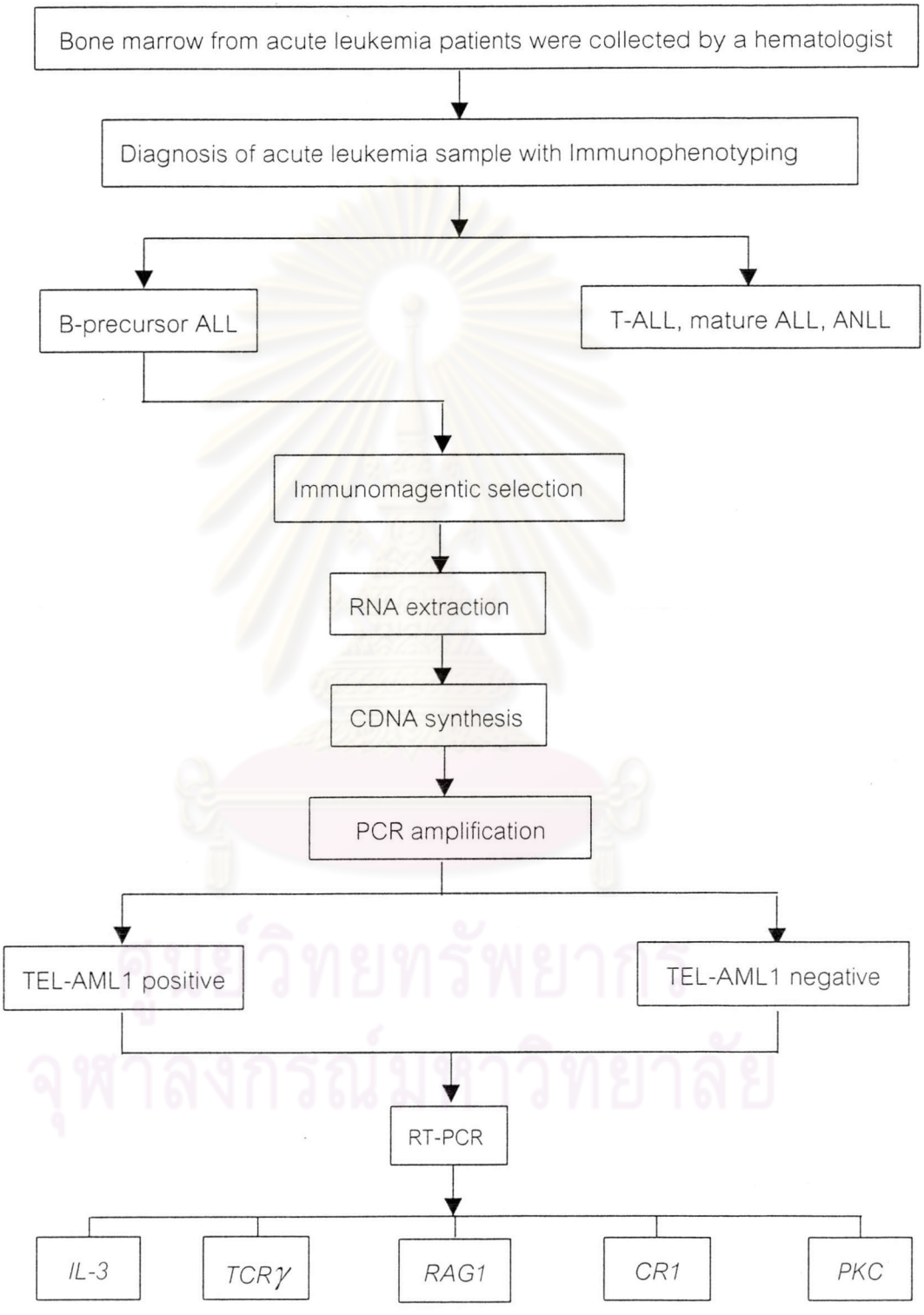

Figure 9 Schematic representation of research methodology

1. Subjects and Sample Collection

After parental consent, bone marrow samples were obtained from children, in the age range under 15 years, who were suspected of having acute leukemia at Chulalongkorn Memorial Hospital and the Children's Hospital, by a pediatric hematologist under sterile conditions. The bone marrow was mixed with ethylene diaminetetraacetic (EDTA) immediatedly after collection and shipped to the Red Cross Blood Center within 24 hours for immunophenotyping.

2. Immunophenotyping

After bone marrow samples were collected, white blood cells were separated by Ficoll-Paque™ PLUS (Amersham Phamacia, USA). The white blood cells were washed with PBS and 2% FCS, then the immunophenotype panel (See in appendix) was performed and the reaction incubated in a dark area for 20 minutes. FACS™ lysing (Becton Dickinson) was added to each tube and the incubated in a dark area for 10 minutes. All cells in each tube were washed with 1 ml of PBS and 2% FCS. The reaction tubes were centrifuged at 2,000 rpm for 2 minutes. A 1% paraformaldehyde solution was added and the result analyzed by FACScan flow cytometry (Becton Dickinson, USA).

If immunophenotypic analysis revealed CD10 and CD19 positive, the patient was diagnosed with precursor-B ALL, and the bone marrow samples were further selected for CD10 and CD19 positive blast cell using immunomagnetic beads.

3. Immunomagnetic Selection

First, white blood cells were sorted with Dynabeads M-450 CD19 (Pan B) (Dynal, Norway) coated with a primary monoclonal antibody (mAb) specific for the B-cell restricted CD19 membrane antigen. Dynabead M-450 CD19 (PanB) was coated directly to the cell sample and incubated for 20 minutes at 2-8 $^{\circ}$C on an apparatus which allows both gentle tilting and rotation. The rosetted cells were separated by Dynal MPC. Then,

CD10 monoclonal antibody (Immunotech, France) coated to Dynabead Pan Mouse IgG were used; they are specific for the B-cell CD10 membrane antigen to select only B-precursor cells.

4. RNA extraction from sorted white blood cells

The extraction method was modified from the TRIzol protocol (Life technology, GIBCO, USA). One million white blood cells were mixed with 400 μl TRIzol reagent. The mixture was shaked vigorously for 15 seconds and incubated at room temperature for 10 minutes. Chloroform 200 μl were added and centrifuged at 13,000 rpm for 30 minutes at 4°C. The supernatant was mixed with 500 μl isopropyl alcohol by inversion and incubated at 4°C for at least 1 hour, then centrifuged at 13,000 rpm for 20 minutes at 4°C. The pellet was washed with 70% ethanol and the RNA resuspended in 20-30 μl of DEPC-water. All steps were under RNase-free conditions.

5. cDNA synthesis

The first strand cDNA synthesis reaction were performed using 1 μg RNA as a template, primed with 0.5 μg $Oligo(dT)_{15}$ primer, incubated in a controlled-temperature heat block at 70°C for 5 minutes, then quick-chilled in iced water. Following cDNA synthesis, the reaction was performed in 20 μl reactions containing 1X Improm-II™ buffer, 2.5 mM Mg_2Cl, 0.5 mM dNTPs mix, 0.5 unit rRNasin® Ribonuclease Inhibitor, 1 unit ImProm-II™ Reverse transcriptase (Promega, USA). The reverse transcription reactions were first annealed at 25°C for 5 minutes and extended at 42°C for 60 minutes and at 70°C for 15 minutes to thermally inactivate the enzyme.

6. Polymerase Chain Reaction analysis

6.1 Semi-nested PCR amplification for *TEL-AML1* gene

First round amplification

The *TEL-AML1* fusion gene was amplified by PCR using oligonucleotide primers, TELS primer and AML1 primer. The PCR reaction was performed in a 20 μl reaction containing 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs Mix, 0.05 units *Taq* DNA polymerase (Fermentus), 0.15 μM of each TELS primer(5'-CAG TCC AGG CTC TCC GAG GA-3') and AML1 primer (5'-AGC GGC AAC GCC TCG CTC AT-3'), 2 μl cDNA template. After incubation at 94°C for 5 minutes, the PCR reaction was followed by 30 cycles at 94°C for 30 seconds for denaturation, 60°C for 30 seconds for annealing, 72°C for 30 seconds for extension and a final amplification cycle at 72°C for 7 minutes.

Second round amplification

The *TEL-AML1* fusion gene was amplified by PCR using oligonucleotide primers, TEL primer and AML1 primer.[83] The PCR reaction was performed in a 20 μl reaction containing 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs Mix, 0.05 units *Taq* DNA polymerase (Fermentus), 0.15 μM of each TEL primer (5'-TCC CGG CCT GAA GAG CAC GCC -3') and AML1 primer (5'-AGC GGC AAC GCC TCG CTC AT-3'), 1 μl PCR product from first round PCR. After incubation at 94°C for 5 minutes, the PCR reaction was followed by 30 cycles at 94°C for 30 seconds for denaturation, 60°C for 30 seconds for annealing, 72°C for 30 seconds for extension and a final amplification cycle at 72°C for 7 minutes.

The *TEL-AML1* positive transcript from B-precursor ALL was used as a positive control and AML patient samples were used as a negative control. A 158 bp TEL-AML1 PCR product was analyzed by running 2% agarose gel electrophoresis and detecting the band under ultraviolet light. The *TEL-AML1* fusion gene was confirmed by DNA sequencing.

6.2 PCR amplification for *β-actin* gene

The *β-actin* gene was amplified by PCR using oligonucleotide primers, β-actin forward primer and β-actin reverse primer. The PCR reaction was performed in a 20 μl reaction containing 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs Mix, 0.025 units Taq DNA polymerase (Fermentus), 0.1 μM of each β-actin forward primer (5'- GGC ACC ACA CCT TCT ACA ATG AG -3') and β-actin reverse primer (5'- CAG CTC GTA GCT CTT CTC CAG G -3'), 1 μl cDNA template. After incubation at 94°C for 5 minutes, the PCR reaction was followed by 35 cycles at 94°C for 1 minute for denaturation, 59°C for 1 minute for annealing, 72°C for 1 minute for extension with a final amplification cycle at 72°C for 7 minutes.

Distilled water was used a negative control. A 470 bp *β-actin* PCR product was analyzed by running 2% agarose gel electrophoresis and the band was detected under ultraviolet light.

6.3 Semi-nested PCR amplification for *IL-3* gene

First round amplification

The *IL-3* gene was amplified by PCR using oligonucleotide primers, IL-3 EX1 primer and IL-3 EX5 primer The PCR reaction was performed in a 20 μl reaction containing 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs Mix, 0.025 units *Taq* DNA polymerase (Fermentus), 0.5 μM each of IL-3 EX1 primer (5'- CGG ACT CCA AGC TCC CAT GAC C -3') and IL-3 EX5 primer (5'- GAT AGA ACG TCA GTT TCC TCC GG -3'), 1 μl cDNA template. After incubation at 94°C for 5 minutes, the PCR reaction was followed by 35 cycles at 94°C for 1 minute for denaturation, 59°C for 1 minute for annealing, 72°C for 1 minute for extension with a final amplification cycle at 72°C for 7 minutes.

Second round amplification

The *IL-3* gene was amplified by PCR using oligonucleotide primers, IL-3 EX2 primer and IL-3 EX5 primer. The PCR reaction was performed in a 20 μl reaction containing 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs Mix, 0.025 units *Taq* DNA polymerase (Fermentus), 0.5 μM of each IL-3 EX2 primer (5'-CCT CAA TGG GGA AGA CCA AGA C-3') and IL-3 EX5 primer (5'-GAT AGA ACG TCA GTT TCC TCC GG-3'), 1 μl PCR product from first round amplification. After incubation at 94°C for 5 minutes, the PCR reaction was followed by 30 cycles at 94°C for 30 seconds for denaturation, 58°C for 30 seconds for annealing, 72°C for 30 seconds for extension with a final amplification cycle at 72°C for 7 minutes.

IL-3 positive transcript from Concanavalin-A stimulated spleen cells was used as positive control and fibroblast was used as negative control. A 227 bp *IL-3* PCR product was analyzed by running 2% agarose gel electrophoresis and the band was detected under ultraviolet light. The *IL-3* gene was confirmed by DNA sequencing.

6.4 PCR amplification for *TCRγ* gene

The *TCRγ* gene was amplified by PCR using oligonucleotide primers, TCRγ forward primer and TCRγ reverse primer. The PCR reaction was performed in a 20 μl reaction containing 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs Mix, 0.025 units Taq DNA polymerase (Fermentus), 0.5 μM of each TCRγ forward primer (5'- GGT TAA CGG TGC CAG AAG AGT C -3') and TCRγ reverse primer (5'- GGG ACG CTT CTA GGG ACA ATA AC -3'), 1 μl cDNA template. After incubation at 94°C for 5 minutes, the PCR reaction was followed by 35 cycles at 94°C for 1 minute for denaturation, 59°C for 1 minute for annealing, 72°C for 1 minute for extension, with a final amplification cycle at 72°C for 7 minutes.

The *TCRγ* positive transcript from T-ALL patients was used a positive control and RNA from remission ALL patients was used a negative control. A 410 bp *TCRγ* PCR product was analyzed by running 2% agarose gel electrophoresis and detecting the band under ultraviolet light.

6.5 PCR amplification for *CR1* gene

The *CR1* gene was amplified by PCR using oligonucleotide primers, CR1 forward primer and CR1 reverse primer. The PCR reaction was performed in a 20 μl reaction containing 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs Mix, 0.025 units Taq DNA polymerase (Fermentus), 0.5 μM of each CR1 forward primer (5'- ATG CCG CCC TGG TTA TTC CG C -3') and CR1 reverse primer (5'- GGC AGA CGA GGA ACC AAT GAG TC -3'), 1 μl cDNA template. After incubation at 94° C for 5 minutes, the PCR reaction was followed by 35 cycles at 94°C for 1 minute for denaturation, 59°C for 1 minute for annealing, 72°C for 1 minute for extension with a final amplification cycle at 72°C for 7 minutes.

The *CR1* positive transcript from T-ALL patients was used as positive control and RNA from remission-ALL patients was used a negative control. A 213 bp *CR1* PCR product was analyzed by running 2% agarose gel electrophoresis and detecting the band under ultraviolet light.

6.6 PCR amplification for *PKC* gene

The *PKC* gene was amplified by PCR using oligonucleotide primers, PKC forward primer and PKC reverse primer. The PCR reaction was performed in a 20 μl reaction containing 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs Mix, 0.025 units Taq DNA polymerase (Fermentus), 0.5 μM of each PKC forward primer (5'-GAT CCA CAC GTA CTC CAG CCC-3') and PKC reverse primer (5'-CGT AGG GAT CTG ACA GGC CA-3'), 1 μl cDNA template. After incubation at 94°C for 5 minutes, the PCR reaction was followed by 35 cycles at 94°C for 1

minute for denaturation, 59°C for 1 minute for annealing, 72°C for 1 minute for extension, with a final amplification cycle at 72°C for 7 minutes.

The *PKC* positive transcript from T-ALL patient was used as positive control and RNA from remission-ALL patient was used a negative control. A 272 bp *PKC* PCR product was analyzed by running 2% agarose gel electrophoresis and the band was detected under ultraviolet light.

6.7 PCR amplification for *RAG1* gene

The *RAG1* gene was amplified by PCR using oligonucleotide primers, RAG1 forward primer and *RAG1* reverse primer. The PCR reaction was performed in a 20 μl reaction containing 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs Mix, 0.025 units hot start *Taq* DNA polymerase (Biolabs), 0.5 μM of each RAG1 forward primer (5'-ATC TCA ACA CTT TGG CCA GG-3') and RAG1 reverse primer (5'-ATC TCA CCC GGA ACA GCT TA-3'), 1 μl cDNA template. After incubation at 95°C for 10 minutes, the PCR reaction was followed by 40 cycles at 95°C for 45 seconds for denaturation, 51°C for 45 seconds for annealing, 72°C for 45 seconds for extension, with a final amplification cycle at 72°C 7 minutes.

The *RAG1* positive transcript from the thymus was used as positive control and fibroblast was used as negative control. A 159 bp *RAG1* PCR product was analyzed by running 2% agarose gel electrophoresis and detecting the band under ultraviolet light.

7. Southern Blot Hybridization

Southern blot hybridization was performed to confirm mRNA expression for RAG1 expression, because of the problem of the non-specific primers.

When gel electrophoresis was completed, the cDNA was denatured with denaturing solution (1.5 M NaCl, 0.5 N NaOH) and neutralized with neutralizing solution (1.5 M NaCl, 1 M Tris base). The cDNA was transferred to the nylon membrane using 20X SSC (3.0 NaCl and 0.3 M sodium citrate). The process takes about 8-24 hours. Probe (RAG1 oligonucleotide : CCA GAT GAA ATT CAG CAC CC) was labelled with radioisotope [γ-p^{32}]ATP (Amersham) with the kinase-mediated endlabelling method. The nylon membrane and probe were incubated at 42°C in a hybridization oven, with a hybridization process of at least 1 hour. The nylon membrane was washed with 5X SSC for 15 minutes at 42°C, 5X SSC for 15 minutes at 42°C and 5X SSC for 15 minutes at 45°C in the hybridization oven. Finally the membrane was transferred to filter paper, wrapped with saran wrap and exposed to a phosphorus screen. The bands were visualized using Image QuaNT software.

Statistical analysis

Fisher's exact test

Fisher's exact test was employed to test for independence between TEL-AML1 expression and expression of each target gene. It was calculated to determine the statistical significance of the data. It was used when the expected value was less than 5 in the 2x2 table.

Table 2 Contingency tables showing Fisher's exact test between group (A) and group(B)

Group	B1	B2	Total
A1	a	b	e
A2	c	d	f
Total	g	h	n

As shown in Table 2, A imply group A and B imply group B. a,b,c,d,e,f,g,h are the numbers of data and n is the total number of individuals.

Fisher's exact test was calculated according to the following formula

$$p = \frac{e!\, f!\, g!\, h!}{n!\, a!\, b!\, c!\, d!}$$

Fisher's exact test provides the p-value compared with α (significant value) to conclude the results.