CHAPTER IV
RESULTS

1. Patient characteristics

1.1 Characteristics of leukemia subtype

Childhood hematologic diseases have been diagnosed between January to November 2007. Bone marrow or peripheral blood samples from 139 patients inform of acute lymphoblastic leukemia, who under 21 years of age at King Chulalongkorn Memorial Hospital. Of these patients, 68 were male and 71 were female (mean age 9.08±4.13 years). 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, and CD71. Among 139 childhood acute lymphoblastic leukemia (ALL), 71% were precursor B, 6% were precursor T-cell, 1% were mature B-cells, 1% were infantile and 21% were unclassified (Figure 12).

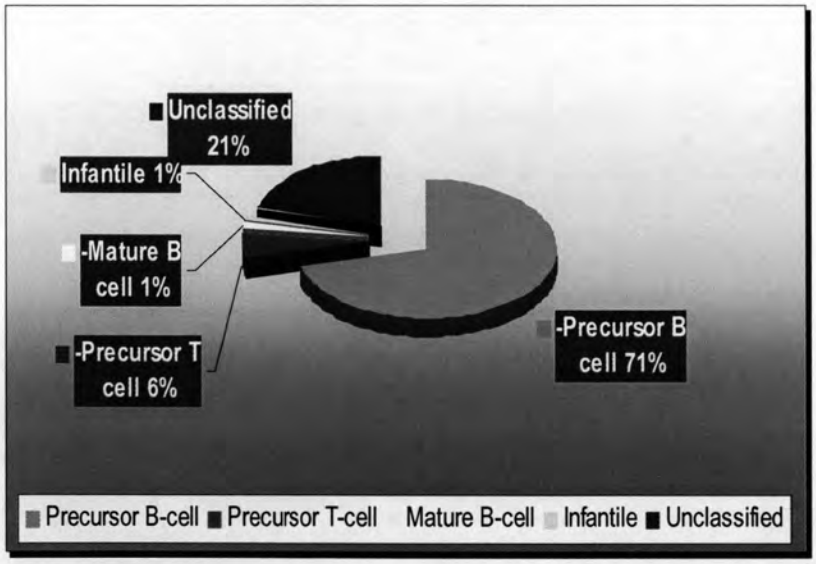

Figure 12 Pie charge representing prevalence of childhood acute lymphoblastic leukemia.

1.2 Characteristics xenobiotic-metabolizing enzyme gene polymorphisms.

The genetic variants of *CYP1A1, NQO1, GSTM1* and *GSTT1,* which were shown in Table 5, were characterized. The distribution of each allele of *CYP1A1 and NQO1* polymorphisms were compatible with the Hardy-Weinberg Equilibrium (HWE) (Table 6 and 7). The Hardy-Weinberg Equilibrium (HWE) equation;

$$p^2+2pq+q^2$$

Where p= frequency of alleleA

q= frequency of allele a

p^2 = frequency of genotype AA

2pq = frequency of genotype Aa

q^2 = frequency of genotype aa

p^2, 2pq, and q^2 frequencies of all polymorphisms were counted, as allele frequencies of allele 1 and allele 2 were calculated as follow;

Allele frequency of allele A= $[[p^2]^2+pq]/ [p^2+2pq+q^2]^2$

Allele frequency of allele a= $[pq+[q^2]^2]/ [p^2+2pq+q^2]^2$

Then, the expected genotype frequencies of p^2, 2pq, and q^2 were calculated using the formula as follow;

Expected p^2 = [Allele frequency of allele A]^{2}x $[p^2+2pq+q^2]$

Expected 2pq = 2 x [Allele frequency of allele A] x [Allele frequency of allele a] x $[p^2+2pq+q^2]$

Expected q^2 = [Allele frequency of allele a]^{2}x $[p^2+2pq+q^2]$

Next, the difference between the observed and expected value of genotype frequencies were tested using Ficher's exact x^2 test.

Where H_o = The observed value = the expected value (HWE)

H_1 = The observed value ≠ the expected value (HWE)

After Fisher's exact x^2 testing, the deviation of genotype frequency were not significant that mean the genotype frequency results of *CYP1A1 and NQO1* are under the HWE theory in both cases and controls.

Table 5 Characteristics of the genetic variants.

Gene	Polymorphisms	Variant
Phase I *CYP1A1*	Wild type	*1
	3801T→C (m1)	*2A
	3801T→C (m1) 2455A→G (m2)	*2B
NQO1	609 C→T	*2
Phase II *GSTM1*	Deletion	Null
GSTT1	Deletion	Null

Table 6 Genotype frequencies of *CYP1A1* (m1), *CYP1A1* (m2), and *NQO1* polymorphisms in cases-controls.

Genotype	Cases (%) (n=139)	Controls (%) (n=139)	OR (95% CI)	*P*-value
CYP1A1 (m1)				
T/T	42 (30.2)	57 (41.0)	1	
T/C	67 (48.2)	54 (38.8)	1.68 (0.99-2.88)	0.056
C/C	30 (21.8)	28 (21.1)	1.45 (0.76-2.79)	0.259
CYP1A1 (m2)				
A/A	34 (24.5)	33 (23.7)	1	
A/G	61 (43.9)	66 (47.7)	0.90 (0.50-1.62)	0.719
G/G	44 (31.7)	40 (28.8)	1.07 (0.56-2.03)	0.842
NQO1				
C/C	41 (29.5)	51 (36.7)	1	
T/C	63 (45.3)	58 (41.7)	1.35 (0.78-2.33)	0.278
C/C	35 (25.2)	30 (21.6)	1.45 (0.77-2.75)	0.252

1.2.1 *CYP1A1* polymorphisms

The *CYP1A1* polymorphisms were characterized by PCR-RFLP, m1 were digested by *Msp*I, as m2 were digested by *BsrD*I, results in smaller fragments of DNA (Figure 15). Among 139 children with ALL, 42 cases (30.2%) were the wild type, 67 cases (48.2%) were of the heterozygous mutant and 30 cases (21.6%) were homozygous mutant for *CYP1A1*2A* (m1) (Table 6), For the control group, there were less proportion of heterozygote (54 of 139, 38.8%), but similar proportion of homozygote (28 cases, 20.1%) (Table 6). The increasing in proportion of heterozygous mutant in ALL cases compared with controls. However, not statistically significant (OR= 1.68, 95% CI 0.99-2.88) (Table 6).

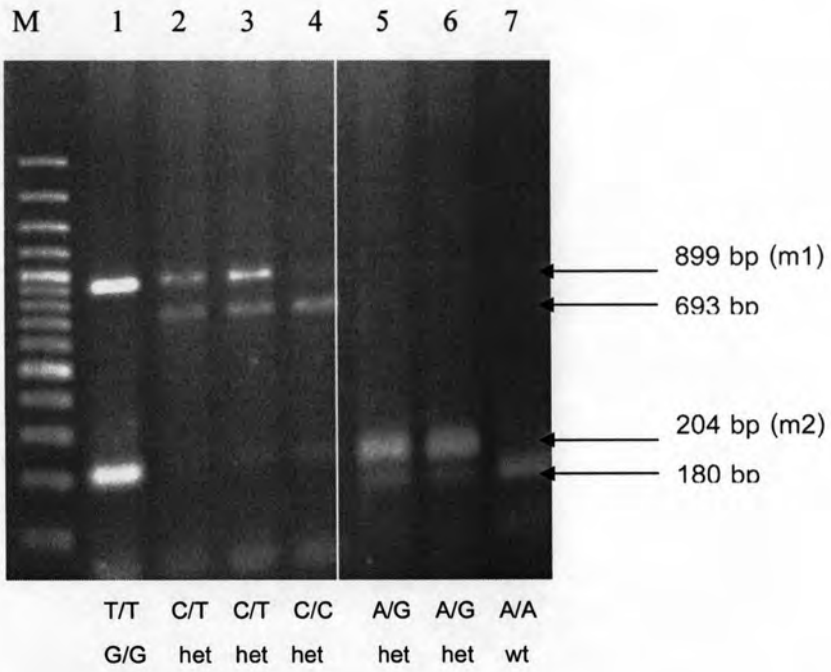

Figure 15 PCR-RFLP of *CYP1A1* T3801C (m1) and A2455G (m2). Lane 1 presents 899 bp of *CYP1A1* (m1) T/T (wild type) and 204 bp of *CYP1A1* (m2) G/G (homozygous mutant). Lanes 2 and 3; *CYP1A1* (m1) T/C (heterozygous mutant), lane 4; *CYP1A1* (m1) T/T (homozygous mutant). Lanes 5 and 6; *CYP1A1* (m2) A/G (heterozygous mutant), lane 7; *CYP1A1* (m2) A/A (wild type).

The allele frequencies of *CYP1A1* (m1) is slightly less common in cases than in controls. The difference was not statistically significant (OR= 1.29, 95% CI 0.92-1.79) (Table 7).

Table 7 Allele frequencies of *CYP1A1* (m1), *CYP1A1* (m2), *NQO1* polymorphisms in cases-controls.

Allele	Cases (%) Allele (n=278)	Controls (%) Allele (n=278)	OR (95% CI)	*P*-value
CYP1A1 (m1)				
T	127 (45.7)	110 (39.6)	1	
C	151 (54.3)	168 (60.4)	1.29 (0.92-1.79)	0.152
CYP1A1 (m2)				
A	155 (55.8)	132 (47.5)	1	
G	123 (44.2)	146 (55.5)	1.04 (0.75-1.46)	0.791
NQO1				
C	145 (51.2)	160 (57.6)	1	
T	133 (47.8)	118 (42.4)	1.24 (0.89-1.74)	0.202

Of 139 *CYP1A1 (*m2*)* genotype of ALL cases, 34 cases (24.5%) were the wild type, 61 cases (43.9%) were the heterozygous mutant and 44 cases (31.7%) were the homozygous mutant for *CYP1A1 (*m2*)* The allele frequencies of *CYP1A1 (*m2*)* were not the statistically significant from controls (Table 6).There was no difference in allele frequency between cases (44.2%) and controls (55.5%) (OR= 1.04, 95% CI 0.75-1.46) (Table 7).

Table 8 Distribution of *CYP1A1* genotypes in ALL patients and controls.

Genotype	Cases (n=139) n	Controls (n=139) n	OR (95% CI)	*P*-value
CYP1A1				
*1/*1	15	17	1	
*1/*2A	11	8	0.53 (0.20-1.34)	0.211
*1/*2B	31	27	1.80 (0.92-3.64)	0.085
*2A/*2A	8	8	0.53 (0.21-1.34)	0.214
*2A/*2B	14	11	0.73 (0.30-1.71)	0.563
*2B/*2B	8	9	0.60 (0.23-1.46)	0.311

The distribution of *CYP1A1* genotypes was shown in Table 8. CYP1A1*1/*2B was the most common genotype in this population, 31 cases and 27 controls, among 8 cases and 8 controls were *2A/*2A genotype, among 14 cases and 11 controls were *2A/*2B genotype, among 8 cases and 9 controls were *2B/*2B genotype. There were no statistical difference of these genotypes between cases and controls.

1.2.2 *NQO1* polymorphisms

The *NQO1* were characterized by PCR-RFLP, the restriction site were digested by *Hinf*I, which results in smaller fragments of 211 bp and 204 bp (heterozygous mutant), or only 204 bp fragment in homozygote of 609 (Figure 16). For *NQO1* genotyping, among 41 cases (29.5%) were the wild type, 63 cases (45.3%) were the heterozygous mutant and 35 cases (25.2%) were the homozygous mutant in the ALL group. For the control group, 51 cases (36.7%) were of the wild type, 58 cases (41.7%) were the heterozygous mutant and 30 cases (21.6%) were the homozygous mutant. There was not found the statistically significant difference in cases and controls, and not found the significant increased risk of ALL (OR= 1.35 95% CI 0.78-2.33; OR 1.45 95%

CI 0.77-2.75) (Table 6). The allele frequencies of *NQO1*, among 47.8% of cases and 42.4% of controls, there was no difference in allele frequency between cases and controls (OR= 1.24 95% CI 0.89-1.74) (Table 7).

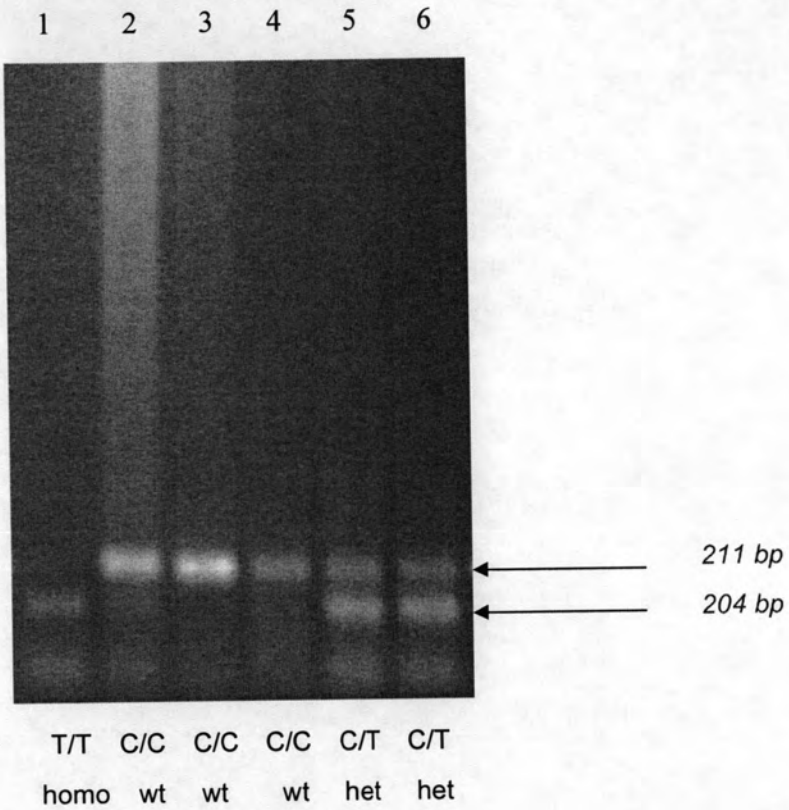

Figure 16 PCR-RFLP of *NQO1* C609T. Lane 1 presents only 204 bp of *NQO1* T/T (homozygous mutant). Lanes 2, 3 and 4 present only 211 bp (wild type). Lanes 5 and 6 present 204 bp and 211 bp of *NQO1* C/T or T/C (heterozygous mutant).

1.2.3 *GSTM1* polymorphisms

The *GSTM1* deletion were characterized by the PCR amplification, the present of *GSTM1* allele was identified by a 230 bp and 157 bp bands, the deletion of *GSTM1* or null genotype identified by only 157 bp (Figure 13). The frequency of *GSTM1* was demonstrated in table 9. As regards *GSTM1* genotyping, among 65 cases (46.8%) were expressed and 74 cases (53.2%) were null (homozygous deletion) genotype. Among the control group, 79 cases (56.8%) were expressed and 60 cases (43.2%) were the null genotype. This data showed that about half of childhood ALL were absence of the *GSTM1* gene. However, in childhood controls also were mostly found *GSTM1* null genotype. There was no significantly difference in frequency between cases and controls (Table 9). In addition, the association between *GSTM1* null genotype and risk of ALL was not found (OR= 1.22 95% CI 0.96-1.56) (Table 9).

Table 9 Frequencies of *GSTM1* and *GSTT1* polymorphisms in cases-controls.

Genotype	Cases (%) (n=139)	Control (%) (n=139)	OR (95% CI)	*P*-value
GSTM1				
Present	65 (46.8)	79 (56.8)	1	
Null	74 (53.2)	60 (43.2)	1.22 (0.96-1.56)	0.722
GSTT1				
Present	89 (64.0)	95 (68.3)	1	
Null	50 (36.0)	44 (31.7)	1.10 (0.34-0.72)	0.981

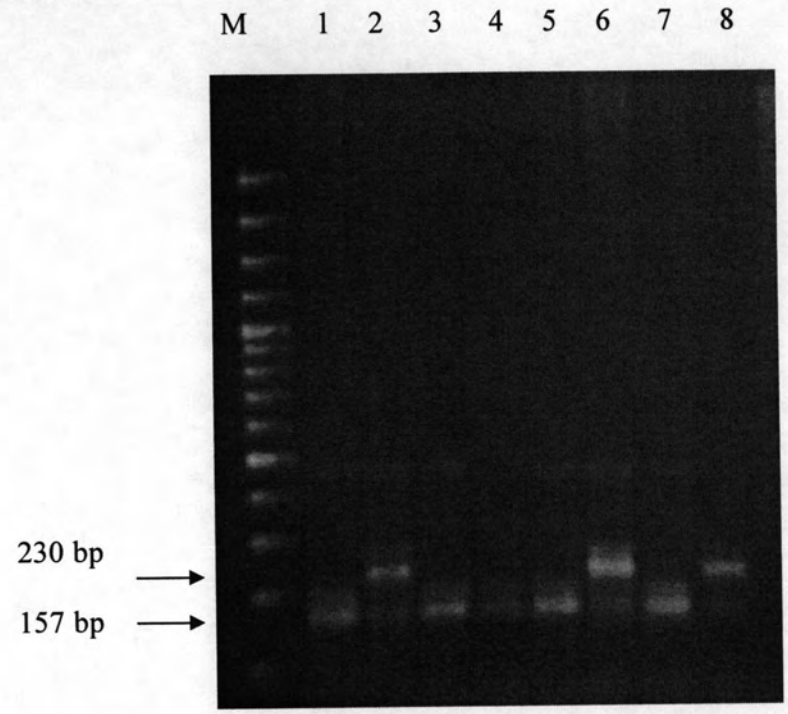

Figure 13 PCR amplification of *GSTM1* gene in ALL samples. Lanes 1, 3, 4, 5, 7; *GSTM1* deletion or null genotype (only 157 bp) and lane 2, 6, 8 *GSTM1* present (both 157 bp and 230 bp).

1.2.4 *GSTT1* polymorphisms

The *GSTT1* deletion were characterized by the PCR amplification, the present of *GSTT1* allele identified by 112 bp and 157 bp, the deletion of *GSTT1* or null genotype identified by only 157 bp (Figure 14). The frequency of *GSTT1* was demonstrated in table 9. The results of *GSTT1* genotyping among the acute lymphoblastic leukemia group (ALL), 89 cases (64.0%) were expressed and 50 cases (36.0%) were null genotype. Among the control group, 95 (68.3%) were expressed and 44 (31.7%) were of the null genotype. There was no significantly difference in frequency between cases and controls. And the association between *GSTT1* null genotype and risk of ALL was not found (OR= 1.10 95% CI 0.34-0.72) (Table 9).

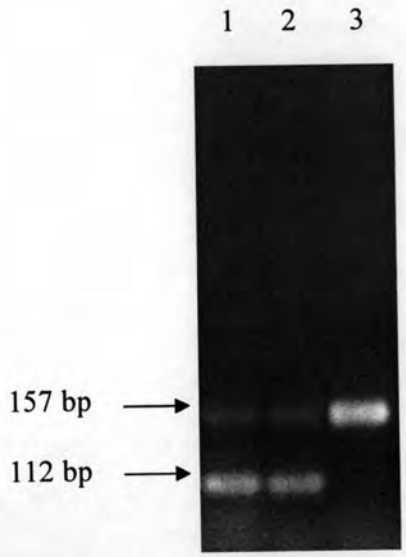

Figure 14 PCR amplification of *GSTT1* gene in ALL samples. Lanes 1 and 2 *GSTT1* present (112 bp and 157 bp), lanes 3; *GSTT1* deletion or null genotype present only 157 bp.

The frequencies of the combination between *GSTM1* and *CYP1A1**2A genotypes were demonstrated in Table 10. Among 20 cases and 27 controls of the combined *GSTM1* null and *CYP1A1* T/T genotype were not significant increased risk of ALL (OR=1.01 95% CI 0.45-2.24). For the combination of *GSTM1* present and *CYP1A1* T/C, C/C genotypes, among 37 cases and 49 controls were not significant increased risk of ALL (OR= 1.03 95% CI 0.51-2.07). As the combination of *GSTM1* null and *CYP1A1* T/C, C/C genotypes, among 60 cases and 33 controls were significant difference between cases and controls. Moreover, they increased risk of ALL (OR=2.48 95% CI 1.24-4.97).

Table 10 Combined effects of *GSTM1* and *CYP1A1*2A* allele in cases-controls.

Genotype at risk	*GSTM1*	*CYP1A1*2A*	Cases (n=139)	Controls (n=139)	OR (95% CI)	*P*-value
None	Present	T/T	22	30	1.0	
One	Null	T/T	20	27	1.01(0.45-2.24)	1.000
	Present	T/C, C/C	37	49	1.03 (0.51-2.07)	1.000
Two	Null	T/C, C/C	60	33	2.48 (1.24-4.97)	0.014

1.3 Characteristics of the pesticides exposure.

For the pesticide exposure among 102 cases (73.4%) were exposed and 37 cases (26.6%) were unexposed. Among the controls, 85 cases (61.2%) were exposed and 54 cases (38.8%) were unexposed. The incidence of the pesticide exposure show significant difference between cases and controls, the pesticide exposure associated with an increased risk of ALL (Table 11).

Table 11 Demographic data of pesticide exposure for the childhood ALL cases and controls.

Category	Cases (%) (n=139)	Controls (%) (n=139)	OR (95% CI)	*P*-value
Pesticide use				
No	37 (26.6)	54 (38.8)	1	
Yes	102 (73.4)	85 (61.2)	2.29 (1.54-3.47)	0.001

Characteristics the type of pesticides exposure for ALL group compared with control group shown that the most common of used pesticides were mosquito/insect spray, among 87 cases (85.3%) of ALL group and 58 cases (68.5%) of control group. There was significant associated with risk of childhood ALL (OR= 2.29 95% CI 1.54-3.47). But the mosquito coil/stick/others types, among 15 cases (14.7%) of ALL group and 27 cases (31.5%) of control group, the most of used pesticides in control group were mosquito coil/stick and others, there was no significant increased risk of ALL (OR=0.31 95% CI 0.19-0.48) (Table 12).

Table 12 Type of pesticides exposure distribution for the childhood ALL cases and controls.

Category	Cases (%) (n=139)	Controls (%) (n=139)	OR (95% CI)	*P*-value
Type				
Mosquito/insect spray	87 (85.2)	58 (68.5)	3.86 (2.16-7.34)	0.001
Mosquito coil/stick/others	15 (14.7)	27 (31.5)	0.31 (0.19-0.48)	0.540
Total	102	85	1	

For the timing of pesticides exposure, the childhood ALL group had more prevalent than control group. The timing of pesticides exposure distribution in cases and controls were demonstrated as follow; during 3 months before pregnancy (75 cases and 11 controls), during pregnancy (61 cases and 12 controls), after pregnancy 1 year (55 cases and 24 controls) and after pregnancy 2-3 year (52 cases and 38 controls).

Table 13 Timing of pesticides exposure distribution for the childhood ALL cases and controls.

Timing	Cases n	Controls n
3 month before pregnancy	75	11
During pregnancy	61	12
After pregnancy 1 year	55	24
After pregnancy 2-3 years	52	38

History of pesticides exposure of ALL patients was more frequent than that of the control group, there was not shown the significant difference between case and control groups due to the limitation of statistics. Moreover, the overlapping of subjects in the durations of timing made the multivariate analysis was not performed to determine all variants, which involved.

For genetic statistic analysis, only the *GSTM1* null or *CYP1A1**2A polymorphisms did not have enough potential increased risk of childhood ALL. Whereas, the combination of *GSTM1* null and *CYP1A1**2A allele were shown the significantly increased risk of childhood ALL. For the pesticides exposure especially mosquito/insect spray were significantly associated with increased risk of childhood ALL. There were not significant differences of the other synergistic or antagonist interactions of any others factors, which had been observed.