CHAPTER IV

RESULTS

1. Patient characteristics

Childhood hematologic diseases have been diagnosed by clinicians among 73 patients inform of acute leukemias. We collected all bone marrow samples, 6 from King Chulalongkorn Memorial Hospital, and 67 from the Children's Hospital, from children under 15 years of age. Of these patients, 40 were boys and 33 were girls (mean age 7.15$\pm$4.07 years). The immunophenotype was determined by flow cytometry using a panel of monoclonal antibodies, including those against CD10, CD19, CD5 CD20, CD3, CD22, CD7, CD34, HLA-DR, CD13, CD14, GPA, CD33 and CD71. Among 73 children with newly-diagnosed acute leukemia, 6 were T-cell ALL, 2 were mature B-cell ALL, 26 were acute myeloid leukemia and 39 were B-precursor ALL, which co-expressed CD10 and CD19. B-precursor ALL would express B-cell markers which are CD20, CD22, CD19, HLA-DR and some ALL patients have dimly expressed myeloid cell marker; CD13 or CD33.

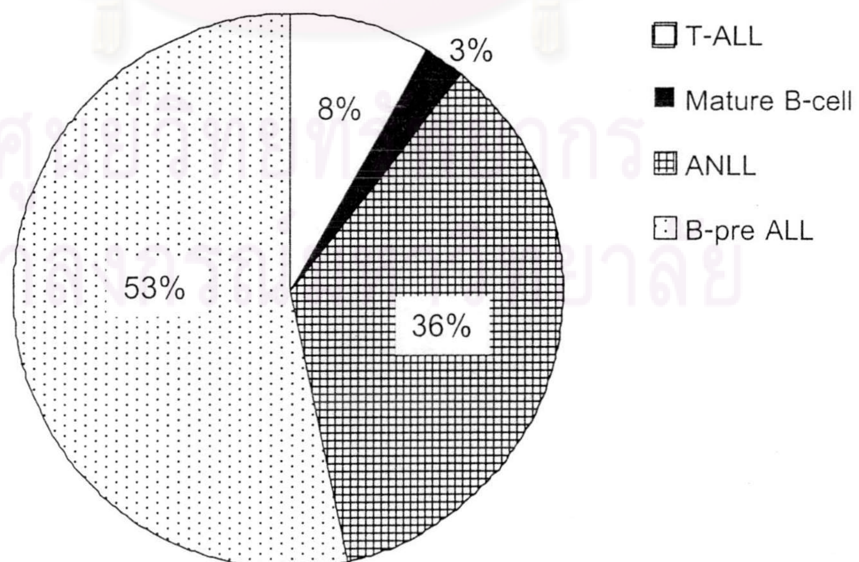

Figure 10 Pie chart representing prevalence of children acute leukemia

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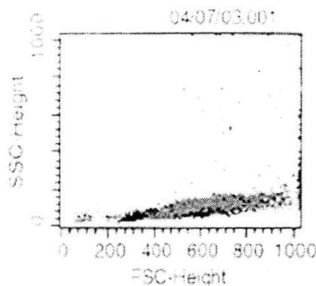

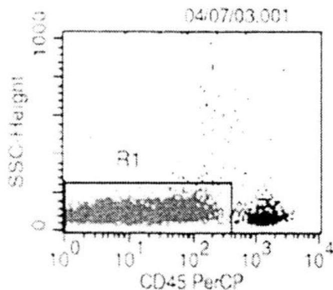

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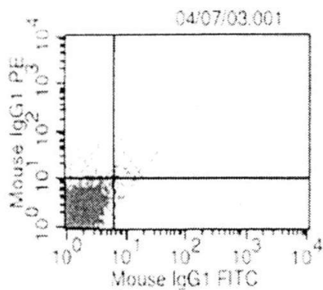
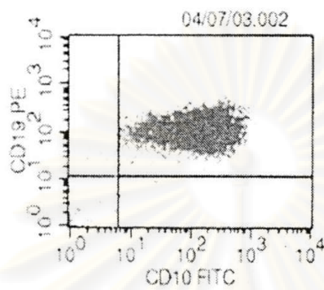
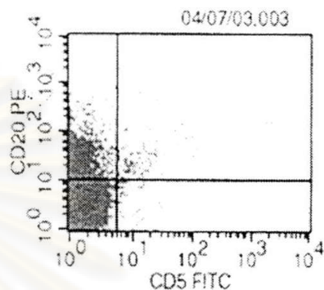






File: 04/07/03.001
Gate: G1
Gated Events: 8960

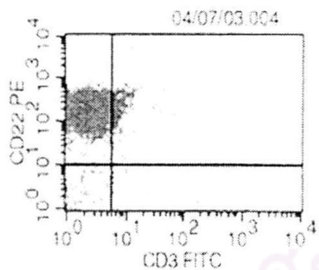
Quad	Events	% Gated	% Total
UL	45	0.50	0.45
UR	49	0.55	0.49
LL	8837	98.63	88.37
LR	29	0.32	0.29

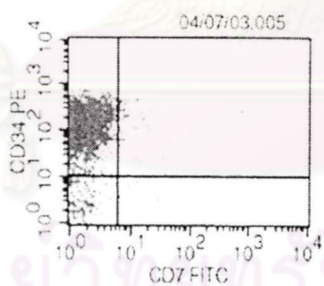
File: 04/07/03.002
Gate: G1
Gated Events: 8928

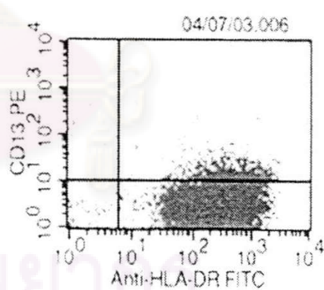
Quad	Events	% Gated	% Total
UL	31	0.35	0.31
UR	8827	98.87	88.27
LL	62	0.69	0.62
LR	8	0.09	0.08

File: 04/07/03.003
Gate: G1
Gated Events: 8913

Quad	Events	% Gated	% Total
UL	1673	18.77	16.73
UR	124	1.39	1.24
LL	7072	79.34	70.72
LR	44	0.49	0.44







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Gated Events: 8917

Quad	Events	% Gated	% Total
UL	8377	93.94	83.77
UR	447	5.01	4.47
LL	78	0.87	0.78
LR	15	0.17	0.15

File: 04/07/03.005
Gate: G1
Gated Events: 8915

Quad	Events	% Gated	% Total
UL	8613	96.61	86.13
UR	118	1.32	1.18
LL	175	1.96	1.75
LR	9	0.10	0.09

File: 04/07/03.006
Gate: G1
Gated Events: 8763

Quad	Events	% Gated	% Total
UL	14	0.16	0.14
UR	916	10.45	9.16
LL	63	0.72	0.63
LR	7770	88.67	77.70

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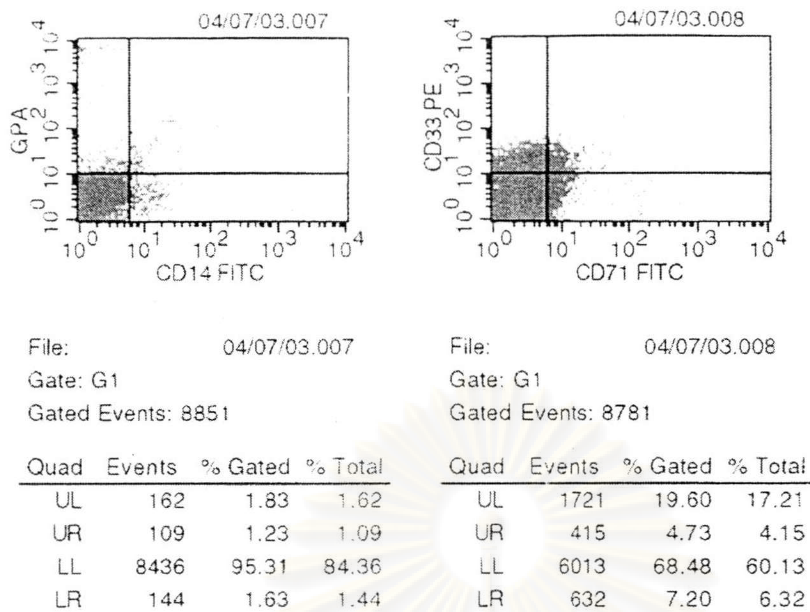

File: 04/07/03.007
Gate: G1
Gated Events: 8851

Quad	Events	% Gated	% Total
UL	162	1.83	1.62
UR	109	1.23	1.09
LL	8436	95.31	84.36
LR	144	1.63	1.44

File: 04/07/03.008
Gate: G1
Gated Events: 8781

Quad	Events	% Gated	% Total
UL	1721	19.60	17.21
UR	415	4.73	4.15
LL	6013	68.48	60.13
LR	632	7.20	6.32

Figure 11 Flow cytometry representing Immunophenotype of an ALL patient.

Immunophenotyping in figure 11 shows an analysis gate (R1) is set on the leukemic population. Antigen displays on the R1-gated population showing bright expression of the B-cell marker; CD10, CD19, CD20, CD22, HLA-DR and positivity for the immature marker CD34. Coexpression of CD10 and CD19 population is the key diagnostic finding of a B-precursor ALL.[82]

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2. *TEL-AML1* translocation

To determine the prevalence of TEL-AML1 translocation in B-precursor ALL of Thai children, a semi-nested RT-PCR was performed using TEL-AML1 specific primers in ALL samples. The PCR product of TEL-AML1 translocation was 158 base pairs. TEL-AML1 transcript was detected in 9 of 39 (23%) of B-precursor ALL childhood patients.

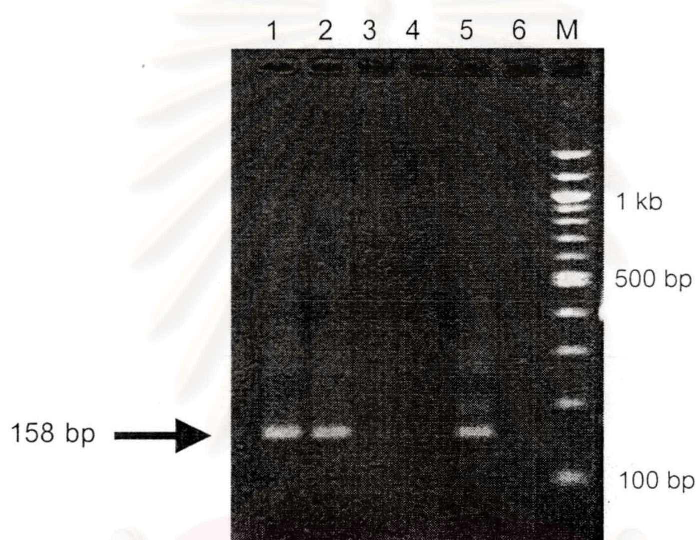

Figure 12 RT-PCR amplification of *TEL-AML1* transcript in B-precursor ALL samples. Lanes 1-4 are ALL patient samples; lane 5 is a positive control; lane 6 is a negative control. M is a 100 bp DNA ladder marker.

We analyzed the relationships of age, white blood cell (WBC) and gender with B-precursor ALL patients which did or did not have TEL-AML1 expression. In Table 3, the interrelationships of age, WBC (T-test) and gender (Fisher's exact test) in B-precursor ALL do not shown a relationship to TEL-AML1 expression. All TEL-AML1 positive cases ranged in age from one to ten years and the majority of TEL-AML1 negative patients also have the same age range. The TEL-AML1 positive patients also tended to have lower WBC counts at diagnosis, although one patient had counts greater than 50 x 10^9/L. There was no difference in the distribution of males and females.

Table 3 Comparison of the clinical and laboratory features of patients with or without TEL-AML1 gene expression

Factor	TEL-AML1 positive (n=9)	TEL-AML1 negative (n=30)	p-value
Age			
< 1 year	0	0	P=0.12*
1-10 years	9	22	
> 10 years	0	8	
Median	3.9	4.92	
range	2-9.75	1-14	
WBC			
< $50x10^9$/L	8	25	P=0.14*
>$50x10^9$/L	1	5	
Median	11.7	13.3	
range	4.5-54	0.5-175	
Gender			
Male	5	18	P=1.00
female	4	12	

* using t-test analysis

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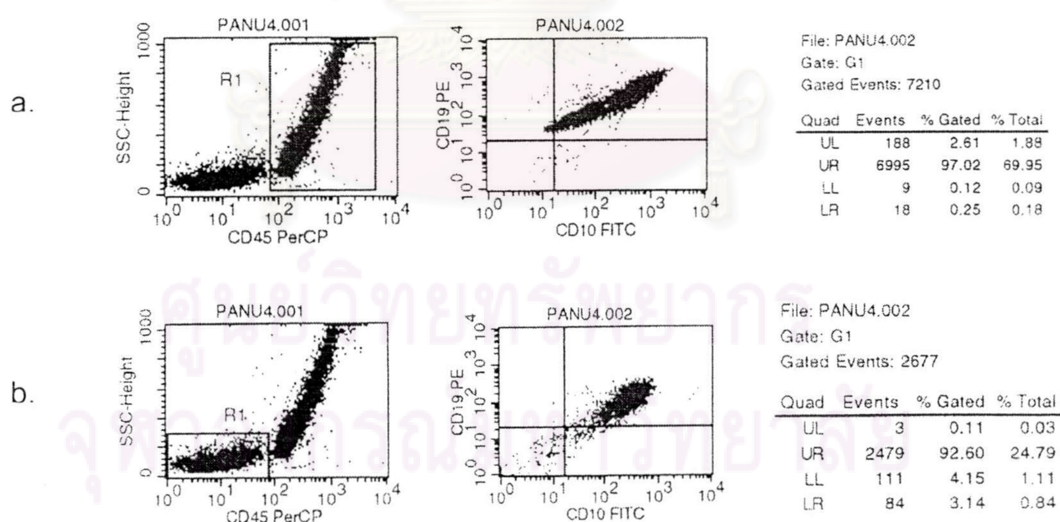
3. Target gene expression

TEL-AML1 fusion protein functions as transcription factor. The expression of five target genes was assessed, all of which had AML1 binding sites on their promoter or enhancer. It was hypothesized that the target gene expressions are different in TEL-AML1 positive and TEL-AML1 negative groups. Because some target genes are known to be expressed in normal hematopoietic cells, the leukemic cells were isolated using immunomagnetic beads. Immunomagnetic selection was performed to sort B-precursor cells. First, CD19 monoclonal antibody coated to dynabeads was used to select from other hematopoietic cells. Then the CD10 coated with dynabeads was used to select only the leukemic cells. Immunomagnetic selection was performed to exclude other cells that were not B-precursor cells, so the result of target gene expression is the real result of TEL-AML1 expression in B-precursor cells. This method of selection could select pure CD10^{+} and CD19^{+} ALL, as demonstrated by flow cytometry. Approximately 70% of ALL blast were recovered.



Quad	Events	% Gated	% Total
UL	188	2.61	1.88
UR	6995	97.02	69.95
LL	9	0.12	0.09
LR	18	0.25	0.18

Quad	Events	% Gated	% Total
UL	3	0.11	0.03
UR	2479	92.60	24.79
LL	111	4.15	1.11
LR	84	3.14	0.84

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Figure13 Flow cytometry representing CD10^{+} and CD19^{+} using immunomagnetic selection. (a). Immunophenotyping shows an analysis gate (R1) is set on the leukemic population was sorted with CD10 and CD19 immunomagnetic beads. (b). Immunophenotyping shows an analysis gate (R1) is set on the leukemic population lost from sorting with immunomagnetic beads.

3.1 *IL-3* expression

RT-PCR was performed to detect *IL-3* mRNA transcript in patient cell samples by using specific primers. Nine (all) of TEL-AML1 positive ALL samples, all had detectable IL-3 expression. Among 30 TEL-AML1 negative ALL samples, 24 had detectable IL-3 expression, but only 6 were not detectable. However, this is not statistically different p=0.30 (Fisher's exact test)(Table 3)

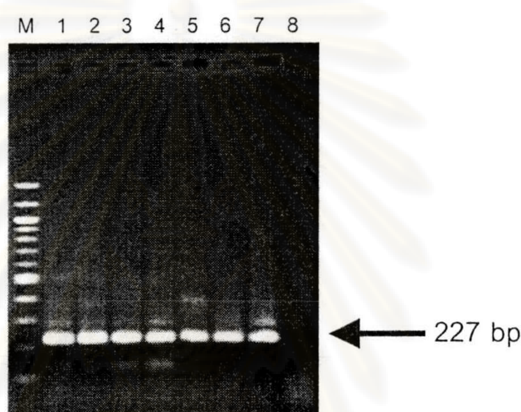

Figure 14 RT-PCR pattern of IL-3 transcript. Lane 1-6 are *IL-3* mRNA. Lane 7 is positive control (Con-A stimulated spleen cells). Lane 8 is negative control. M is 100 bp DNA ladder marker.

3.2 *TCRγ* expression

RT-PCR was performed to detect *TCRγ* mRNA in patient cell samples by using specific primers. Of nine TEL-AML1 positive ALL samples, only 2 had detectable *TCRγ* expression, while 7 did not. Among 30 TEL-AML1 negative ALL samples, 8 had detectable *TCRγ* expression, while 22 were not detectable. There was not statistical difference (p=1.00) (Fisher's exact test)(Table 3)

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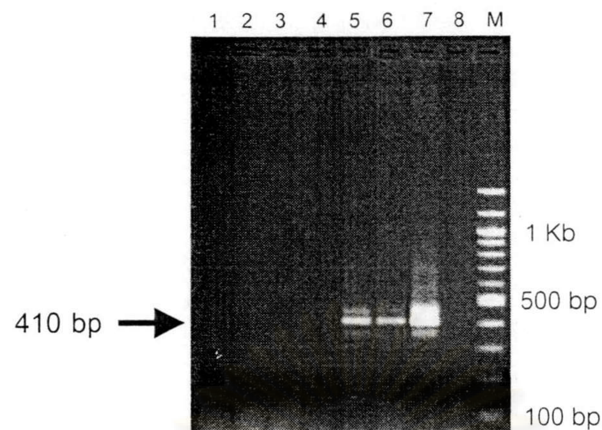

Figure 15 RT-PCR pattern of *TCRγ* transcript. Lanes 1-6 are patient samples; Lane 7 is positive control; Lane 8 is negative control. M is 100 bp DNA ladder marker.

3.3 *CR1* expression

RT-PCR was performed to detect *CR1* mRNA in patient cell samples by using specific primers. Of a TEL-AML1 positive ALL sample, 6 had detectable *CR1* expression, while 3 did not. Among 30 TEL-AML1 negative ALL samples, 17 had detectable *CR1* expression, while 13 did not. However, this is not statistically different p=0.71 (Fisher's exact test)((Table 3)

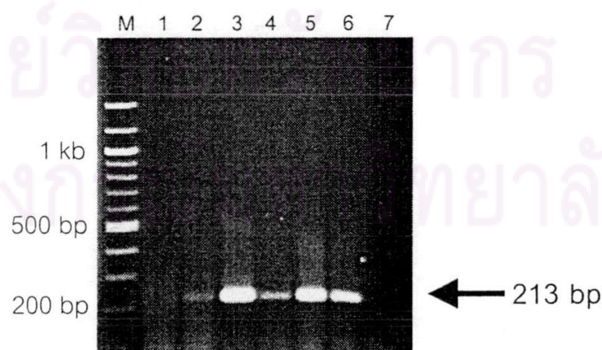

Figure 16 RT-PCR pattern of *CR1* transcript. Lanes 1-5 are patient samples, lane 6 is positive control and lane 7 is negative control. M is 100 bp DNA ladder marker.

3.4 *PKC expression*

RT-PCR was performed to detect *PKC* mRNA in patient cell samples by using specific primers. Of 9 TEL-AML1 positive ALL samples, 8 had detectable *PKC* expression, while only 1 did not. Among 30 TEL-AML1 negative ALL samples, 24 had detectable *PKC* expression while 6 did not. This was not statistically different (p=1.00) (Fisher's exact test) (Table 3)

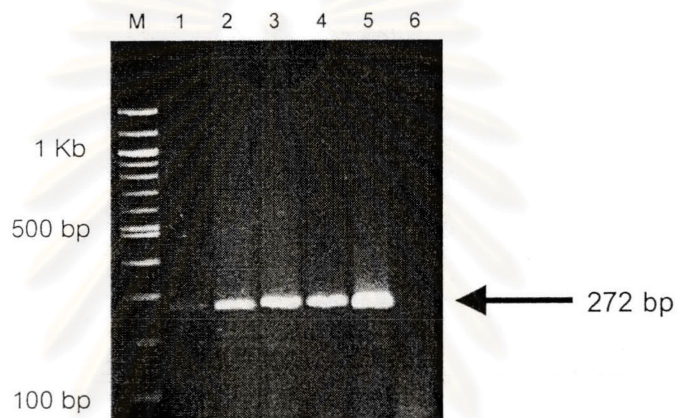

Figure 17 RT-PCR pattern of *PKC* transcript. Lanes 1-4 are patient samples; Lane 5 is positive control; Lane 6 is negative control. M is 100 bp DNA ladder marker.

3.5 *RAG1 expression*

Figure 18 was performed to detect *RAG1* mRNA in patient cell samples by using specific primers. Of 39 B-precursor ALL samples, both TEL-AML1 positive and TEL-AML1 negative had detectable *RAG1* expression. This is not statistically different.

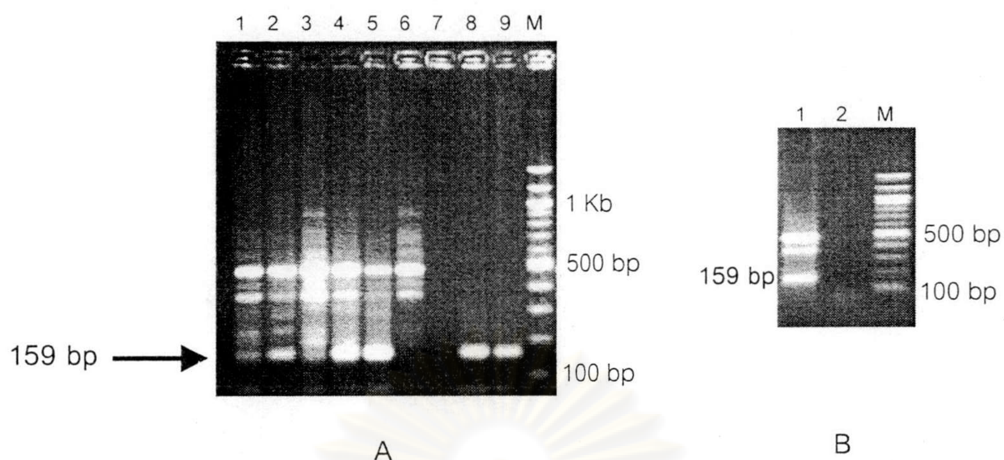

Figure 18 RT-PCR pattern of *RAG1* transcript. A; Lane 1-5,8,9 are patient samples; lane 6 is fibroblast; lane 7 is distilled water (negative control), M is 100 bp DNA ladder marker. B; Lane 1 is RAG1 positive control from thymus cells; Lane 2 is negative control. M is 100 bp DNA ladder marker.

Because of the problem of a non-specific primer, we confirmed RAG1 transcript with Southern blot analysis.

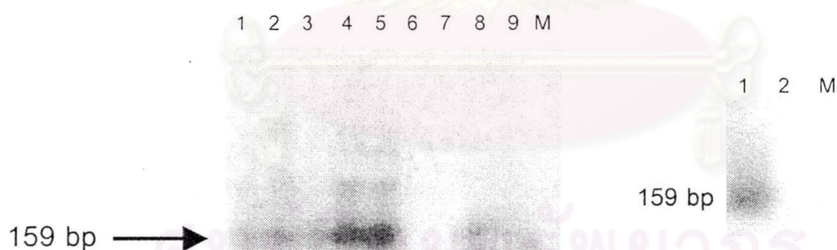

Figure 19 Southern blot analysis of *RAG1* transcript that was transferred from RT-PCR gel electrophoresis (figure 18) into nylon membrane.

Table 4 Relationship between TEL-AML1 expression and target gene expression in B-precursor ALL.

Expression	TEL-AML1 positive (n=9)	TEL-AML1 negative (n=30)	p-value
IL-3 positive	9	24	0.30
IL-3 negative	0	6	
TCRγ positive	2	8	1.00
TCRγ negative	7	22	
CR1 positive	6	17	0.71
CR1negative	3	13	
PKC positive	8	24	1.00
PKC negative	1	6	
RAG1 positive	9	30	-
RAG1negative	0	0	

To determine the relationships between TEL-AML1 expression and target gene expression in B-precursor ALL patients, Fisher's exact tests were performed. The results of these tests are summarized in Table 4, which shows the interrelationships of target gene expression with and without TEL-AML1 expression. Of the investigated target genes, *IL-3* (p=0.30), *TCRγ* (p=1.00), *CR1* (p=0.71), *PKC* (p=1.00) and *RAG1* showed no relationship to TEL-AML1 expression (Fisher's exact tests).

To find out the association of target genes in TEL-AML1 positive ALL, TEL-AML1 negative ALL and all B-precursor ALL patients. Fisher's exacts test were performed. The results of these tests are summarized in Table 5.

Table 5 The association of target genes in TEL-AML1 positive ALL, TEL-AML1 negative ALL and all B-precursor ALL.

Gene	p-value (TEL-AML1 positive)	p-value (TEL-AML1 negative)	p-value (B-precursor ALL patients)
IL-3 and TCRγ	-	1.00	1.00
IL-3 and CR1	0.08	1.00	0.67
IL-3 and PKC	-	0.07	0.058
TCRγ and CR1	-	0.69	0.26
TCRγ and PKC	1.00	1.00	0.65
CR1 and PKC	0.33	0.67	1.00

The results show no relationship between each target gene in each group. IL-3 gene in TEL-AML1 positive ALL and RAG1 gene in all samples cannot determine the association because they express in all of samples.

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