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E2A/PBX1, *MLL/AF4*, *BCR/ABL (M-BCR)*, *BCR/ABL(m-BCR)* Gene Rearrangements in Acute Lymphoblastic Leukemia in Iranian Children

Ahmad Reza Rahnemoon,[1,*] Leila Koochakzadeh,[2,3] Shahla Ansari,[4] Anna Boyajyan,[5] and Arsen Arakelyan[5]

[1]Allied Medical School, Cellular and Molecular Research Center, Iran University of Medical Sciences, Tehran, IR Iran
[2]Children's Medical Center, Pediatrics Center of Excellence, Tehran, IR Iran
[3]Department of Pediatrics, Tehran University of Medical Sciences, Tehran, IR Iran
[4]Department of Pediatrics, Iran University of Medical Sciences, Tehran, IR Iran
[5]Institute of Molecular Biology NAS RA, Yerevan, Armenia

[*] *Corresponding author*: Ahmad Reza Rehnemoon, Allied Medical Schoool, Cellular and Molecular Research Center, Tehran University of Medical Sciences, Tehran, IR Iran.
E-mail: ar.rahnemoob@gmail.com

Abstract

Objectives: The following observation was primarily based on the study of gene fusion in blood and bone marrow cells taken from 68 Iranian children with acute lymphoblastic leukemia (ALL), to compare with healthy population.

Methods: Peripheral blood and bone marrow samples obtained from patients with ALL were immunophenotyped to determine the lineage and the level of differentiation. With reverse transcriptase-polymerase chain reaction (RT-PCR), the RNA molecules were analyzed according to Van Dongen et al protocol to detect fused genes in cell population.

Results: Leukemic cell type was identified by cytochemical stains and classified on the basis of FAB classification. Nonetheless the frequencies of *E2A/PBX1*, *MLL/AF4*, *BCR/ABL (M-BCR)* and *BCR/ABL(m-BCR)* gene transcripts were 1.5%, 0%, 0% and 4.4% respectively. The positive case of *E2A/PBX1* fusion gene had an early pre B and 3 *BCR/ABL (m-BCR)*. Positive cases had an early pre B and pre-B ALL immunophenotype.

Conclusions: Early pre-B cells were the most common types in our patients. The RT-PCR was shown to be an ideal method for detecting hybrid transcripts and to estimate the prevalence of the fusion genes in ALL patients. The frequency of these fusion genes in Iranian pediatric ALL patients were found to be similar to some developed countries. Thus, their presence does not seem to be predictive of increasing malignancy, but rather it can challenge the prognostic significance of these rearrangements.

Keywords: Childhood Acute Lymphoblastic Leukemia, Immunophenotype, Genetic Alterations

1. Background

Acute lymphoblastic leukemia (ALL) in children is a heterogeneous disease with different subtypes based on their cellular and molecular characteristics. ALL accounts for approximately 80% of all acute leukemias in childhood, contrasting with about 20% of the cases in adults (1).

Genetic molecular analysis on leukemia cell has provided the basic knowledge of pathogenesis and prognosis in ALL. Since the translocation discovery of the first fusion gene, BCR-ABL resulting from a t (9;22) translocation, many fusion transcripts that occur in leukemia, such as t (12;21), t (4;11), and t (1,19), have subsequently been detected (2, 3). Research has shown that normally-fused translocated genes play a crucial role in the development and function

of lymphocytes and bone marrow cells (4). It has therefore been suggested that the fusion genes may be closely correlated with the onset of leukemia. The presence of *MLL/AF4* is associated with a very poor prognosis the same as *E2A/PBX1* (5-8). Studies on genetic changes in leukemic cells significantly enhance the precision of diagnosis and allow determining treatment strategy for childhood ALL, especially when specific aberrations are present.

2. Methods

This study was done to detect blast cells taken at early diagnosis from 68 patients with ALL in Children's Medical center. Tehran, Iran. Diagnosis was based on the classification of French American British (FAB) criteria and cyto-

chemistry staining. Informed consent was obtained prior to peripheral blood and bone marrow aspiration.

Immunophenotyping: The leukemic cells were immunophenotyped using monoclonal antibodies to define the lineage and to determine the level of differentiation. The panel included: *CD34*, *CD45*, *HLA-DR*, *CD117*, *CD10*, *CD19*, *CD4*, *CD7*, *CD8*, *CD38*, *Tdt*, *CD2*, *CD3*, *CD20* and *CD22*. Antigen expression was determined by indirect immunofluorescence (*BD*, *FAC S* Calibur) evaluated by flow cytometry (9, 10).

Isolation of mononuclear cells from the collected samples was performed by Ficoll Hipaque density gradient centrifugation (Sigma Diagnostics) and total RNA was isolated from the thawed cells by Trizol method according to manufacturer's instructions. The total RNA was run on a agarose gel containing ethidium bromide to visualize integrity of bands. Thus, reverse-transcription and PCR amplification of *E2A/PBX1*, *MLL/AF4*, *M-BCR* and *m-BCR* fusion genes were carried out according to a standardized protocol by Van Dongen and colleagues (11). Moreover, all cases were compared with positive and negative controls. The specific primers for RT-PCR analysis of these fusion genes are as shown in Tables 1-4.

Table 1. The Primers for *E2A/PBX1*

Primer Code	5' Position (Size)	Sequence 5'-3'
E2A-A	1434 (19)	CACCAGCCTCATGCACAAC
PBX-B	675 (19)	TCGCAGGAGATTCATCACG
E2A-C	1479 (19)	CACCCTCCCTGACCTGTCT
PBX-D	636 (19)	GGCCTGCTCGTATTTCTCC
PBX-E3'	748 (19)	TGAACTTGCGGTGGATGAT

Table 2. The Primers for *MLL/AF4*

Primer Code	5' Position (Size)	Sequence 5'-3'
MLL-A	3916 (17)	CCGCCTCAGCCACCTAC
AF4-B	1714 (20)	TGTCACTGAGCTGAAGGTCG
MLL-C	3936 (18)	AGGACCGCCAAGAAAAGA
AF4-D	1677 (20)	CGTCCTTGCTGAGAATTTG
MLL-E5'	3793 (18)	AAGCCCGTCGAGGAAAAG

3. Results

During the Period of the study between 2009 and 2015, 68 new cases of ALL had been registered in Tehran. Iran, the results of the peripheral blood and bone marrow examinations of all 68 pediatric patients prior to the start of chemotherapy are summarized in Table 5. The table shows

Table 3. The Primers for *M-BCR*

Primer Code	5' Position (Size)	Sequence 5'-3'
BCR-b1-A	3086 (22)	GAAGTGTTTCAGAÅGCTTCTCC
ABL-a3-B	458 (21)	GTTTGGGCTTCACACCATTCC
BCR-b2-C	3126 (21)	CAGATGCTGACCAACTCGTGT
ABL-a3-D	441 (21)	TTCCCCATTGTGATTATAGCCTA
ABL-a3-E3'	505 (23)	TGACTGGCGTGATGTAGTTGCTT

Table 4. The Primers for *m-BCR*

Primer Code	5' Position (Size)	Sequence 5'-3'
BCR-e1-A	1479 (21)	GACTGCAGCTCCAATGAGAAC
ABL-a3-B	458 (21)	GTTTGGGCTTCACACCATTCC
BCR-e1-C	1602 (21)	CAGAACTCGCAACAGTCCTTC
ABL-a3-D	441 (23)	TTCCCCATTGTGATTATAGCCTA
ABL-a3-E3'	505 (23)	TGACTGGCGTGATGTAGTTGCTT

the data for all morphologic, immunologic and genetic studies of cases for diagnosis as well as the outcomes of several years' treatment with control. Among the 68 patients evaluated, 45 (44.1%) were male and 23 (33.9%) were female.

Blood counts with differential and bone marrow aspiration analysis usually confirmed the diagnosis of ALL. The major clinical findings included anemia. hepatomegaly and splenomegaly. The most important laboratory results (Table 6) were white blood cell (WBC) < 5000 (22%), 5000-10000 (23.6%). 10000-50000 (41.2%) and > 50000 (13.2%), and hemoglobin (Hb) < 5 (10.3%), 5 - 10 (67.7%) and > 10 (22%). Patient's age was mainly 1 - 4 and 4 - 10 years. For molecular analysis, we used published experiences to optimize PCR program also by using agarose gel as a powerful separation method based on the detection of presence or absence of the target sequence and length of the fragment; in fact we analyzed DNA fragments generated by RT-PCR following the standard protocols of agarose gel preparation and loading the products to the gel. The final pictures were used to detect the fusion genes and different controls. Finally, *E2A/PBX1* was positive only in patient 14 and negative in the other patients. *MLL/AF4* and *M-BCR* were negative in them, and *m-BCR* was positive in patients 61, 67, 68 and negative in the others. In follow up, 44 patients were in complete remission stage, 6 relapsed and 18 died (Table 5). Based on FAB classification of ALL in our results, 47 individuals were of type L1; 11, L2; 4, L3 and 6 assumed as ALL.

Table 7 shows the relationship between fusion genes and ALL immunophenotypes. In this study early pre-B was the most common in the newly diagnosed patients (27 cases) followed by pre-B (21 cases), and T-ALL (8 cases) types. Other cases included two pro-B ALL, two early pre-B

with CD13 and CD33, two early pre-B along with CD13, two pre B plus CD7, one pre-B with CD33, one with B+T lymphoid cells and two with B-ALL. The prevalence of *E2A/PBX1*, *MLL/AF4*, *BCR/ABL (M-BCR)* and *BCR/ABL (m-BCR)* in childhood ALL were 1.5% (1/68), 0% (0/68), 0% (0/68) and 4.4% (3/68) respectively.

Table 6. FAB Classification and Association with Age, WBC Count, Hemoglobin and Outcome in ALL Patients

Patients	
Age, year	
1 - 10	62
> 10	6
WBC Count, *10³/ml	
< 50	59
50 - 100	4
> 100	5
Hemoglobin, g/dl	
< 5	7
5 - 10	46
> 10	15
Gender	
Male	45
Female	23
Outcome	
C.R	44
Relapse	6
Died	18
French-American British classification	
L1	47
L2	11
L3	4
ALL	6

4. Discussion

Using the gene expression profiling showed a great promise in classification of human hematopoietic malignancies (12-14). Of the four markers studied in the present work, we identified only the *BCR-ABL (m-BCR)* and *E2A/PBX1* rearrangements in the peripheral blood and/or bone marrow samples obtained in 4 (6.15%) out of the 68 cases diagnosed with ALL during the period of study. As we know RT-PCR has become a powerful tool in molecular analysis. As we know RT-PCR has become a powerful tool in

molecular analysis and the procedure of diagnostic process in childhood ALL may determine the prognostic factors, it can be used for risk stratification and selection of treatment as well. The identification of prognostic factors in ALL requires application of immunologic, hematologic and molecular techniques (8, 10, 11, 15, 16).

For leukemic cells, immunophenotype is the main prognostic factor in ALL, determined by lineage specific monoclonal antibodies against various clusters of differentiation markers on human leukocytes. T cell ALL used to be considered as a poor prognostic factor. However, in the study, here were no significant differences between groups of patients with B-lineage or T-lineage ALL, because a few number of patients had a relation with T-ALL. Age, Hb, WBC and subtypes of ALL are the other known clinical and hematological prognostic factors.

Age, is found to have a strong impact on outcome in childhood ALL. In our study there were no significant differences found among patients aged 1 to more than 10 years. On the other hand, there were on significant differences in survival rates between patients with WBC under or over 25x103/μl.

In childhood ALL, a strong negative prognostic factor was shown in MLL gene rearrangement (5, 6, 17). The most common rearrangement of MLL is a balanced translocation t (4;11), associated with the expression of *MLL/AF4* fusion gene, high WBC and pro-B ALL immunophenotype. In our study, there was no *MLL/AF4* fusion gene in the patients, which contrasted to report of Trka J, et al. (18), but it did not contrast with the opinions of Soszynska K et al. (19) and Wu, et al. (20).

In about 5% of children, *E2A/PBX1* is expressed with early pre-B ALL and poor prognosis. In our data, *E2A/PBX1* was present in only one (1.5%) child with early pre-B-ALL who achieved early hematological response with complete remission and after that showed hematological relapse and died. Nevertheless, it is associated usually with poor or a better prognosis when ALL is treated more intensively in this fusion gene (19, 21-23), but the death risk in these patients was 2.5 times higher than in the whole study group (19). In the opinion of Soszynska K et al. (19), *E2A/PBX1* was expressed in about 2.8% of children which is in agreement with our findings. E2A/PBX1 expression was reported by Zuo YX et al. (24) in about 17.5% and by Mesquita DR et al. (25) in 9.7% of children which indicate a significant difference with our study of 68 Iranian ALL patients with Philadelphia chromosome analyzed for lineage involvement, 3 were BCR/ABL positive.

In the Study by Zuo YX et al. (24) the frequency of BCR/ABL positive was 13.7% which contrasted to our report. Cetin Z et al. (26) reported with M-BCR in 1.4% which indicates a significant difference to our data and with m-BCR

Table 7. Fusion Gene Analysis and FAB Classification Compared with Different Immunophenotypes in ALL Ppatients

Immunophenotype	Patients	*E2A/PBX1* Positive	*MLL/AF4* Positive	*BCR/ABL (M-BCR)* Positive	*BCR/ABL (m-BCR)* Positive	L1	L2	L3	ALL
Pro-B	2	0	0	0	0	1			1
Early Pre-B	27	1	0	0	1	21	5	1	1
Early Pre-B with CD13	2	0	0	0	0	1	1		
Early Pre-B with CD13 and CD33	2	0	0	0	0	1	1		
Pre-B	21	0	0	0	2	14	3	1	2
Pre-B with CD7	2	0	0	0	0	1			1
Pre B with CD33	1	0	0	0	0	1			
T Cell	8	0	0	0	0	6	1		1
B Cell	2	0	0	0	0			2	
B+T Cell	1	0	0	0	0	1			

in 3.6% which did not contrast our findings. In the opinion of Qin YZ et al. (27) M-BCR and m-BCR were expressed in 4.8% and 9.1% of children with ALL respectively, indicatinng a significant difference with our results. Moreover, Soszynksa K et al. (19) described 2.9% of children with ALL had BCR/ABL fusion gene which did not contrast to our study.

4.1. Conclusions

Our Study reveals a lower frequency of *E2A/PBX1* and *BCR-ABL (m-BCR)* fusion genes in childhood ALL and absence of *MLL/AF4* and *BCR-ABL (M-BCR)* fusion genes in pediatric ALL Patients. The results were confirmed by RT-PCR for detecting hybrid transcripts. Therefore, fusion transcript levels in untreated acute lymphoid leukemia patients were important to estimate the frequency or prevalence of these fusion genes in Iranian pediatric ALL patients. We can say, 1) these fusion genes are likely to be showing the transient genomic instability and/ or 2) possibly they do not define truly clinically apparent disease but rather malignant progression seems to depend on additional factors like the occurrence of oncogenic secondary changes as well as other agents with their effects on hematopoietic microenvironment. Thus, the presence of fusion genes does not seem to be predictive of increasing malignancy, rather it can challenge the prognostic significance of these rearrangements and lastly, improved strategies are necessary for the treatment of acute leukemia patients.

Supplementary Material

Supplementary material(s) is available here [To read supplementary materials, please refer to the journal website and open PDF/HTML].

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Table 5. The Clinical and Hematological Data of ALL Patients and Gene Analysis Results in Study Subjects

Patient	Age at Diagnosis (yr.mo/sex)	Hb g/dl	WBC (X10³μl)	Type of ALL	Immunopheno-Type	T(1.19) E2A/PBX1	T(4;11) MLL/AF4	T(9;22) M-BCR	T(9;22) m-BCR	Outcome
1	4.10/F	5.3	41280	L2	Pre B ALL	-	-	-	-	Died
2	4.1/F	6.6	8170	L1	Early	-	-	-	-	CR
3	9/F	8	11200	L1	Pre B ALL	-	-	-	-	Died
4	3.10/F	4.9	18400	L1	Pro B ALL	-	-	-	-	CR
5	3.5/M	5.9	35020	L1	Early Pre B ALL	-	-	-	-	CR
6	4/M	9.1	22200	ALL	Pre B ALL	-	-	-	-	Relapse
7	4/M	6.3	22640	L1	Early Pre B ALL along CD13	-	-	-	-	Died
8	6.10/F	8.9	7000	L1	Early Pre B ALL	-	-	-	-	Died
9	7.9/F	7.5	4300	L1	Early Pre B	-	-	-	-	Died
10	3.5/M	6.7	173300	L2	T- ALL	-	-	-	-	CR
11	2/M	7	29330	L1	Early Pre B ALL	-	-	-	-	CR
12	3/F	11.8	8380	L1	Pre B ALL	-	-	-	-	CR
13	4/M	10.8	12170	L2	Early Pre B ALL	-	-	-	-	Died
14	7/M	8.1	29450	L2	Early Pre B ALL	+	-	-	-	Died
15	3/F	4.6	16000	L1	Pre B ALL	-	-	-	-	CR
16	1.7/M	5.9	7620	L1	Early Pre B ALL	-	-	-	-	CR
17	2.5/M	10.8	13490	L1	Early Pre B ALL	-	-	-	-	Died
18	8/M	10.8	24140	L1	T-Cell ALL	-	-	-	-	Relapse
19	3.2/F	10	6320	L3	Early Pre B ALL	-	-	-	-	Died
20	8.2/M	9.5	2680	L1	Early Pre B ALL	-	-	-	-	Died
21	3.7/M	8.1	3600	L2	Early Pre B ALL	-	-	-	-	CR
22	2/M	10.4	1540	L3	Pre B ALL	-	-	-	-	Died
23	11/F	11.9	16600	ALL	Pre B ALL	-	-	-	-	CR
24	3/M	6.2	39700	L1	Early Pre B ALL	-	-	-	-	CR
25	4.5/M	10.1	5100	L1	Pre B ALL	-	-	-	-	CR
26	2/F	7.6	79600	L1	Early Pre B ALL	-	-	-	-	CR
27	1.5/M	7.9	10500	L1	Pre B ALL	-	-	-	-	CR
28	2/M	10.5	7600	L1	Pre B ALL	-	-	-	-	CR
29	8/F	6.2	12700	L1	Early Pre B ALL	-	-	-	-	CR
30	2/F	8.7	3800	L2	Early Pre B ALL	-	-	-	-	CR
31	7/M	8.8	15560	L1	T ALL	-	-	-	-	CR
32	4/M	5.3	1470	L1	Pre B ALL	-	-	-	-	CR
33	2/M	11	2530	L1	Early Pre B ALL	-	-	-	-	CR
34	1/F	13.1	7150	L1	Pre B ALL	-	-	-	-	CR
35	12/F	4.2	14210	ALL	Early Pre B ALL	-	-	-	-	CR
36	2/M	7.9	5790	L1	Early Pre B ALL	-	-	-	-	Relapse
37	6/M	10.8	113180	L1	T ALL	-	-	-	-	CR
38	3/M	7.9	11150	L1	Early Pre B ALL	-	-	-	-	CR
39	5/M	5.6	19710	L2	Pre B ALL	-	-	-	-	CR
40	3/M	10.8	6680	L1	T ALL	-	-	-	-	CR
41	2/M	7.5	3260	L1	Pre B ALL	-	-	-	-	CR
42	11/M	5.2	14300	L1	Pre B ALL	-	-	-	-	Died
43	5/M	7.1	9770	L1	Pre B ALL	-	-	-	-	Died
44	11/M	5.7	3800	L2	Early Pre B ALL	-	-	-	-	Died
45	9/M	10.9	16800	L2	Early Pre B with CD13and CD33	-	-	-	-	CR
46	5/F	3.3	5790	L1	Early Pre B ALL	-	-	-	-	CR
47	4/F	8.1	1700	L1	B+T lymphoid cells	-	-	-	-	CR
48	2/M	10.6	14260	L1	Pre B	-	-	-	-	CR
49	13/M	5.7	231740	ALL	Pre B with CD7	-	-	-	-	CR
50	8/F	9	7720	L1	Pre B with CD33	-	-	-	-	CR
51	10/M	6.4	17450	ALL	T-ALL	-	-	-	-	CR
52	6/M	6.9	84610	L1	Early Pre B	-	-	-	-	CR
53	2/M	10	2450	L1	Early Pre B	-	-	-	-	CR
54	4/F	6.9	3940	L1	Early Pre B With CD13 and CD33	-	-	-	-	CR
55	3/F	5	7730	L3	B-ALL	-	-	-	-	CR

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56	2/F	5.3	940	L1	Pre B ALL	-	-	-	-		CR
57	3/F	4.4	40540	ALL	Pre B ALL	-	-	-	-		CR
58	3/M	8.7	21650	L3	B-ALL	-	-	-	-		Died
59	4/M	8.2	6380	L1	Early Pre B	-	-	-	-		CR
60	4/M	7.1	13510	L1	Early Pre B	-	-	-	-		CR
61	3/F	4.9	52300	L1	Early Pre B	-	-	-	+		Relapse
62	8/M	6.4	6180	L1	T-ALL	-	-		-		Died
63	4/M	5.5	101000	L2	Early Pre B with CD13				-		Relapse
64	14/M	8.4	365000	L1	T-ALL						
(more compatible with early cortical T-cell ALL)	-	-	-	-	CR						
65	12/M	11.3	2007	L1	Pre BALL with CD7	-	-	-	-		Relapse
66	7/M	8.9	6500	L1	Early Pre B	-	-	-	-		Died
67	8/M	10	12000	L2	Pre B	-	-	-	+		CR
68	6/M	4.9	3800	L1	Pre B	-	-	-	+		Died