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

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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, CD17, CD10, CD19, CD4, CD7, CD8, CD38, Tdt, CD2, CD3, CD20 and CD22. Antigen expression was determined by indirect immunofluorescence (BD, FACSCalibur) 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.

During the Period of the study between 2009 and 2015, 68 new cases of ALL had been registered in Tehran, Iran. Blood counts with differential and bone marrow aspiration 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. Primers for E2A/PBX1

| Primer Code | 5' Position (Size) | Sequence 5'-3' |
|-------------|--------------------|----------------|
| E2A-A       | 1434 (19)          | CACCCAGCTCCAGCTCCAC |
| PBX-B       | 675 (19)           | TGGGTCCAACCTCCAC |
| E2A-C       | 1479 (19)          | CACCCAGCTCCAGCTCCAC |
| PBX-D       | 636 (19)           | TGGGTCCAACCTCCAC |
| PBX-E            | 748 (19)           | CACCCAGCTCCAGCTCCAC |

### Table 3. Primers for M-BCR

| Primer Code | 5' Position (Size) | Sequence 5'-3' |
|-------------|--------------------|----------------|
| M-BCR-a1    | 1479 (21)          | GCTCCACCTCCACCTCCAC |
| ABL-a3-B    | 505 (23)           | TGGGTCCAACCTCCAC |
| BCR-e1-A    | 1474 (21)          | GCTCCACCTCCACCTCCAC |
| ABL-a3-D    | 441 (21)           | TGGGTCCAACCTCCAC |
| ABL-a3-E    | 505 (23)           | TGGGTCCAACCTCCAC |

### Table 4. Primers for m-BCR

| Primer Code | 5' Position (Size) | Sequence 5'-3' |
|-------------|--------------------|----------------|
| BCR-a1      | 1474 (21)          | GCTCCACCTCCACCTCCAC |
| ABL-a3-B    | 505 (23)           | TGGGTCCAACCTCCAC |
| BCR-e1-C    | 1602 (21)          | GCTCCACCTCCACCTCCAC |
| ABL-a3-D    | 441 (21)           | TGGGTCCAACCTCCAC |
| ABL-a3-E    | 505 (23)           | TGGGTCCAACCTCCAC |

### 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 the data for all morphologic, immunologic and genetic studies of cases to 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, L2, L3, and L4 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.

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-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 those aged 1 to more than 10 years. On the other hand, there were on significant differences in survival rate between patients with WBC under or over 25x10³/µl.

In childhood ALL, a strong negative prognostic factor was shown as 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 et al. (18), but it did not contrast with the opinions of Soszynska 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 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 et al. (24) in about 17.5% and by Mesquita 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 et al. (24) the frequency of BCR/ABL positive was 13.7% which contrasted to our report. Cetin et al. (26) reported with M-BCR in 1.4% which indicates a significant difference to our data and with m-BCR in 3.6%
which did not contrast our findings. In the opinion of Qin et al. (27) M-BCR and m-BCR were expressed in 4.8% and 9.1% of children with ALL respectively, indicating a significant difference with our results. Moreover, Soszynska 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 lymphoblastic 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 show the transient genomic instability and 2) possibly they do not define truly clinically apparent disease but other malignant progression seems to depend on additional factors like the occurrence of genetic secondary changes as well as other agents with effects on hematopoietic microenvironment. Thus, the presence of fusion genes does not seem to be predictive of increasing malignancy, rather it can challenge the pragmatic significance of these rearrangements and therefore improved strategies are necessary for the treatment of acute leukemia patients.

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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 (X10³/µl) | Type of ALL | Immunopheno-Type | T (1;19) E2A/ PBX1 | T (4;11) MLL/ AF4 | T (9;22) M-BCR | (9;22) m-BCR | Outcome         |
|---------|-------------------------------|---------|---------------|-------------|------------------|---------------------|-------------------|----------------|----------------|----------------|
| 1       | 4.10/F                        | 5.3     | 40280         | L2 Pre B ALL | -                | -                   | -                 | -              | -              | Died           |
| 2       | 4.1/F                         | 6.6     | 8770          | L1 Early Pre B ALL | -         | -                   | -                 | -              | -              | CR             |
| 3       | 9/F                           | 8       | 11200         | L1 Pre B ALL | -                | -                   | -                 | -              | -              | Died           |
| 4       | 3.10/F                        | 4.9     | 18400         | L1 Pre 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 ALL | -        | -                   | -                 | -              | -              | CR             |
| 10      | 3.5/M                         | 6.7     | 173100        | L2 T- ALL    | -                | -                   | -                 | -              | CR             |
| 11      | 2/M                           | 7       | 29330         | L1 Early Pre B ALL | -         | -                   | -                 | -              | -              | CR             |
| 12      | 3/F                           | 11.8    | 13170         | L2 Pre B ALL | -                | -                   | -                 | -              | CR             |
| 13      | 4/M                           | 10.8    | 16000         | 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      | 2.3/M                         | 10.8    | 13490         | L1 Early Pre B ALL | -       | -                   | -                 | -              | CR             |
| 18      | 8/M                           | 10.8    | 24440         | L1 Early Pre B ALL | -       | -                   | -                 | -              | Died           |
| 19      | 3.2/F                         | 10      | 5320          | L3 Early Pre B ALL | -       | -                   | -                 | -              | Died           |
| 20      | 8.2/M                         | 9.5     | 2680          | L1 Pre B ALL | -                | -                   | -                 | -              | Died           |
| 21      | 3.7/M                         | 8.1     | 3600          | L1 Early Pre B ALL | -       | -                   | -                 | -              | CR             |
| 22      | 2/M                           | 10.4    | 1540          | L1 Pre B ALL | -                | -                   | -                 | -              | CR             |
| 23      | 11/F                          | 11.9    | 1710          | L1 Pre B ALL | -                | -                   | -                 | -              | CR             |
| 24      | 3/M                           | 6.2     | 3940          | L1 Early Pre B ALL | -       | -                   | -                 | -              | CR             |
| 25      | 4.5/M                         | 10.1    | 16000         | L1 Pre B ALL | -                | -                   | -                 | -              | CR             |
| 26      | 2/F                           | 7.6     | 79600         | L1 Early Pre B ALL | -       | -                   | -                 | -              | CR             |
| 27      | 1.5/M                         | 7.9     | 16500         | L1 Pre B ALL | -                | -                   | -                 | -              | CR             |
| 28      | 2/M                           | 10.5    | 16000         | L1 Pre B ALL | -                | -                   | -                 | -              | CR             |
| 29      | 8/F                           | 7.5     | 35020         | L1 Early Pre B ALL | -       | -                   | -                 | -              | CR             |
| 30      | 2/F                           | 8.7     | 18000         | L2 Early Pre B ALL | -       | -                   | -                 | -              | CR             |
| 31      | 3/F                           | 8.8     | 15560         | L1 T- ALL    | -                | -                   | -                 | -              | CR             |
| 32      | 4/F                           | 11      | 2530          | L1 Early Pre B ALL | -       | -                   | -                 | -              | CR             |
| 33      | 11/F                          | 11.1    | 7150          | L1 Pre B ALL | -                | -                   | -                 | -              | CR             |
| 34      | 2/F                           | 11      | 5790          | L1 Early Pre B ALL | -       | -                   | -                 | -              | CR             |
| 35      | 11/F                          | 4.2     | 14200         | L1 Early Pre B ALL | -       | -                   | -                 | -              | CR             |
| 36      | 2/F                           | 7.9     | 5790          | L1 Early Pre B ALL | -       | -                   | -                 | -              | CR             |
| 37      | 6/M                           | 10.8    | 13180         | L1 T- ALL    | -                | -                   | -                 | -              | Relapse        |
| 38      | 3/M                           | 7.9     | 11550         | L1 Early Pre B ALL | -       | -                   | -                 | -              | CR             |
| 39      | 5/M                           | 5.6     | 19710         | L2 Pre B ALL | -                | -                   | -                 | -              | CR             |
| 40      | 3/M                           | 10.8    | 6680          | L2 Pre B ALL | -                | -                   | -                 | -              | CR             |
| 41      | 2/M                           | 7.5     | 3260          | L2 Pre B ALL | -                | -                   | -                 | -              | Died           |
| 42      | 11/M                          | 5.2     | 14300         | L2 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 CD13 and CD33 | -   | -                   | -                 | -              | CR             |
| 46      | 5/F                           | 3.3     | 5790          | L1 Early Pre B ALL | -       | -                   | -                 | -              | CR             |
| No. | Sex | Age | WBC | Stage | Primary Diagnosis | Secondary Markers | Treatment | Status |
|-----|------|-----|-----|-------|------------------|-------------------|-----------|--------|
| 47  | F    | 8.1 | 1700 | L1    | B+T lymphoid cells | -                 | -         | CR     |
| 48  | M    | 10.6| 14260| L1    | Pre B            | -                 | -         | CR     |
| 49  | M    | 5.7 | 231740| ALL   | Pre B with CD7   | -                 | -         | CR     |
| 50  | F    | 9   | 7720 | L1    | Pre B with CD33  | -                 | -         | CR     |
| 51  | M    | 6.4 | 17450| T-ALL | -                | -                 | -         | CR     |
| 52  | M    | 6.9 | 84610| L1    | Early Pre B      | -                 | -         | CR     |
| 53  | M    | 10  | 2450 | L1    | Early Pre B      | -                 | -         | CR     |
| 54  | F    | 6.9 | 3940 | L1    | Early Pre B with CD13 and CD33 | - | - | CR |
| 55  | F    | 5   | 7730 | L3    | B-ALL            | -                 | -         | CR     |
| 56  | F    | 5.3 | 940  | L1    | Pre B ALL        | -                 | -         | CR     |
| 57  | F    | 4.4 | 40540| ALL   | Pre B ALL        | -                 | -         | CR     |
| 58  | M    | 8.7 | 21650| L3    | B-ALL            | -                 | -         | Died   |
| 59  | M    | 8.2 | 6380 | L1    | Early Pre B      | -                 | -         | CR     |
| 60  | M    | 7.1 | 13520| L1    | Early Pre B      | -                 | -         | CR     |
| 61  | F    | 4.9 | 52300| L1    | Early Pre B      | -                 | -         | Relapse|
| 62  | M    | 6.4 | 6180 | L1    | T-ALL            | -                 | -         | Died   |
| 63  | M    | 5.5 | 100000| L2   | Early Pre B with CD13 | - | - | Relapse |
| 64  | M    | 8.4 | 365000| L1   | T-ALL            | -                 | -         | Relapse|
| 65  | M    | 11.3| 2007 | L1    | Pre B ALL        | -                 | -         | Relapse|
| 66  | M    | 8.9 | 6500 | L1    | Early Pre B      | -                 | -         | Died   |
| 67  | M    | 10  | 120000| L2   | Pre B            | -                 | -         | + CR   |
| 68  | M    | 4.9 | 3800 | L1    | Pre B            | -                 | -         | + Died |