Prevalence of ETV6/RUNX1 Fusion Gene in Pediatric Patients with Acute Lymphoblastic Leukemia in Iran

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Abstract

Objective: ETV6/RUNX1 (also known as TEL/AML1) is the most frequent gene fusion in childhood acute lymphoblastic leukemia (ALL). Sixty-three patients were enrolled in this study to explore the distribution of this gene in Iranian population.

Methods: This study used 63 peripheral blood and bone marrow (PB/BM) samples from children with ALL. Immunophenotyping of PB and BM samples were performed using flow cytometry to illustrate the lineage. Moreover, reverse transcriptase polymerase chain reaction (RT-PCR) technique was used to amplify transcripts of leukemia-specific chromosome fusion gene ETV6/RUNX1 and to monitor the expression levels of the ETV6/RUNX1 in patients according to Van Dongen et al protocol.

Findings: On the basis of French-American-British (FAB) classification, 47 individuals had ALL-L1. The incidence of ETV6/RUNX1 fusion gene in this study was 34.9%. The laboratory and clinical features of twenty two ETV6/RUNX1 positive ALL cases were similar to those of other studies. The most positive cases of ETV6/RUNX1 fusion gene had the early pre B ALL and pre B ALL immunophenotypes.

Conclusion: The ETV6/RUNX1 fusion gene is a common genetic anomaly in Iranian childhood ALL patients and the prevalence of the ETV6/RUNX1 fusion gene is similar to that of ALL patients in other countries. However early pre B cells were the most common type in studied patients.

Key Words: Acute Lymphoblastic Leukemia; Reverse Transcriptase; ETV6/RUNX1 Fusion; Polymerase Chain Reaction

Introduction

Acute lymphoblastic leukemia (ALL) is the most common malignancy in children. The precise diagnosis and classification of ALL is based on morphology, cytochemistry, immunophenotype, and molecular analyses of bone marrow cells. In pediatric B-lineage ALL, the t(12;21) (p13;q22) chromosomal translocation is very common and usually found in about 25% of all cases. The t(12;21) (p13;q22) was first described in 1994[1] and is not detectable by conventional cytogenetic methods. It leads to the fusion of two genes, RUNX1 (AML1) on chromosome 21 and ETV6 (TEL) on chromosome12[2,3]. The RUNX1 belongs to the core binding factor family of transcription
Prevalence of ETV6/RUNX fusion gene in ALL

Subjects and Methods

The initial diagnosis of ALL was established by morphological, cytochemical and immune phenotypic assessments. The French-American-British (FAB) classification is based on morphology and cytochemical stains[7].

Immunophenotyping was determined by flow cytometry using a panel of monoclonal antibodies to define the lineage and to determine the level of differentiation[8]. The default panel established included: CD34, CD 45, HLA-DR, CD117, CD10, CD19, CD4, CD7, CD8, CD38, TdT, CD2, CD3, CD20 and CD22.

Molecular analysis: mononuclear cells were isolated from PB/BM samples by Ficoll-Hypaque density centrifugation and the target genes amplified using the specific primers as follows:

| Primer code | 5' Position (size) | Sequence (5'-3') |
|-------------|-------------------|-----------------|
| TEL-A       | 845 (20)          | TGCACCCCTCCTGATCCTGAAC |
| AML1-B      | 611 (19)          | AACGCTTGCATCTTTCGC |
| TEL-C       | 928 (22)          | AACGCCATCAACCTCTCTATC |
| AML1-D      | 577 (18)          | TGAAAGCAGGGGTAGAAC |
| TEL-ES'     | 692 (20)          | CGCACCCAGGAAACAACCCAC |

For the reverse transcriptase-polymerase chain reaction (RT-PCR) assay, total RNA was extracted by a single-step method with Trizol (Invitrogen). To quantify ETV6/RUNX1 fusion gene the RT-PCR was performed according to a standardized protocol by Van Dongen and colleagues[9]. Moreover, all cases were analyzed and reevaluated using positive and negative controls.

Findings

The correlation of the hematological and clinical prognostic factors with the outcome of the disease was analyzed. Among the sixty three patients evaluated, 39 (62%) were boys and 24 (38%) girls and their age at the time of diagnosis varied between 1 year and 13 years. The results of hematological, immunological and molecular analysis are presented in Table 1.

Of 63 patients, 56 children (88.9%) developed leukemia from B-lineage and seven (11.1%) from T-lineage. The immunophenotyping of B-lineage analysis permitted the characterization of 28 cases (44.4%) as early pre B ALL, 22 (34.9%) as pre B ALL and 4 (6.3%) pro B ALL. The co-expression of lymphoid and myeloid antigens shown in Table 2 was confirmed as follows: one (1.6%) with early pre B ALL associated with CD7 co-expression and one (1.6%) was the early pre B along with aberrant expression of CD13.

In follow up it was found that 59 patients were at complete remission stage and 4 died. Based on FAB classification of ALL in our results, 47 individuals were of type L1; in which immunologic classification was as follows: 21 early pre B, 17 pre B, 3 pro B and 6 T-ALL. The immunophenotypes of ALL patients with TEL/AML1 fusion transcripts were early pre B, pro B, pro B and T-ALL types. No ETV6/RUNX1 fusion transcripts were detected in early pre B, with CD2 but detected in early pre B along with aberrant expression of CD13. The ETV6/RUNX1 fusion gene was identified through RT-PCR among 22 (34.9%) patients in which ten had early pre B, 10 pre B ALL, one pro B and one T-ALL. The prevalence of ETV6/RUNX1 was 37.5% (21/56) in childhood B-lineage ALL. The ETV6/RUNX1+ patients were studied with regard
Table 1: Hematological, immunological data of ALL patients and RT-PCR fusion gene amplification

| Patient | Age of diagnosis (yr,mon/sex) | Hgb g/dL | WBC x10^3 mL | Type of ALL | Immunophenotype | t(12;21) ETV6/RUNX1 | Outcome |
|---------|--------------------------------|----------|---------------|-------------|----------------|---------------------|---------|
| 1       | 7/9/F                           | 7.5      | 4300          | L1          | Early pre B ALL | -                   | CR      |
| 2       | 2.10/M                          | 8.9      | 9840          | L1          | Pre B ALL      | -                   | CR      |
| 3       | 3.5/M                           | 6.7      | 173300        | L2          | T-ALL          | -                   | CR      |
| 4       | 2/M                             | 7        | 29330         | L1          | Early pre BALL | -                   | CR      |
| 5       | 3/F                             | 11.8     | 8380          | L1          | Pre B ALL      | -                   | CR      |
| 6       | 8.5/M                           | 10.8     | 16560         | L1          | Pre B ALL      | -                   | CR      |
| 7       | 4/M                             | 10.5     | 12730         | L2          | Early pre B ALL| -                   | CR      |
| 8       | 7/M                             | 10.4     | 10400         | L1          | Pro B ALL      | -                   | CR      |
| 9       | 1.5/F                           | 10.2     | 8600          | L2          | Pre B ALL      | +                   | CR      |
| 10      | 7/M                             | 8.1      | 29450         | L2          | Early pre B ALL| -                   | CR      |
| 11      | 3/F                             | 4.6      | 16000         | L2          | Pre B ALL      | +                   | Died    |
| 12      | 3.8/F                           | 7.4      | 2130          | L1          | T ALL          | +                   | CR      |
| 13      | 9/F                             | 7.6      | 5720          | L2          | Early pre B ALL| -                   | CR      |
| 14      | 1.7/M                           | 5.9      | 7620          | L1          | Early pre B ALL| -                   | CR      |
| 15      | 10/M                            | 9.8      | 2470          | L1          | Early pre B ALL| +                   | CR      |
| 16      | 2.5/M                           | 10.8     | 13490         | L1          | Early pre B ALL| -                   | CR      |
| 17      | 8/M                             | 10.8     | 24140         | L1          | T ALL          | -                   | CR      |
| 18      | 12.5/F                          | 6.7      | 10600         | L1          | Pre B ALL      | -                   | Died    |
| 19      | 4/F                             | 6.7      | 77980         | L2          | Early pre B ALL| +                   | CR      |
| 20      | 3.2/F                           | 10       | 6320          | L3          | Early pre B ALL| +                   | CR      |
| 21      | 4.10/F                          | 5.3      | 41280         | L2          | Pre B ALL      | +                   | CR      |
| 22      | 4.1/F                           | 6.6      | 8170          | L1          | Early pre B ALL| +                   | CR      |
| 23      | 9/F                             | 8        | 11200         | L1          | Pre B ALL      | -                   | CR      |
| 24      | 3.10/F                          | 4.9      | 18400         | L1          | Pro B ALL      | -                   | CR      |
| 25      | 3.5/M                           | 5.9      | 35020         | L1          | Early pre B ALL| +                   | CR      |
| 26      | 8/F                             | 6.6      | 14000         | L1          | Early pre B ALL along with aberrant expression CD2 | - | CR |
| 27      | 5/M                             | 7.1      | 9770          | L1          | Pre B ALL      | +                   | CR      |
| 28      | 4/M                             | 9.1      | 22200         | ALL         | Pre B ALL      | +                   | CR      |
| 29      | 4/M                             | 6.3      | 22640         | L1          | Early pre B ALL along with aberrant expression of CD13 | + | CR |
| 30      | 6.10/F                          | 8.9      | 70000         | L1          | Pre B ALL      | +                   | CR      |
| 31      | 8.2/M                           | 9.5      | 2680          | L1          | Early pre B ALL| -                   | CR      |
| 32      | 3.7/M                           | 8.1      | 3600          | L2          | Early pre B ALL| -                   | CR      |
| 33      | 2/M                             | 10.4     | 1540          | L3          | Early pre B ALL| +                   | Died    |
| 34      | 3.9/M                           | 6.6      | 26400         | L3          | Early pre B ALL| +                   | CR      |
| 35      | 11/F                            | 11.9     | 16600         | L1          | Pre B ALL      | +                   | CR      |
| 36      | 12/M                            | 8.3      | 803370        | L1          | T-ALL          | -                   | Died    |
| 37      | 7/F                             | 9.3      | 5700          | L1          | Pre B ALL      | -                   | CR      |
| 38      | 3/M                             | 6.2      | 39700         | L1          | Early pre B ALL| +                   | CR      |
| 39      | 11/M                            | 7.9      | 2100          | L1          | Pre B ALL      | -                   | CR      |
| 40      | 13/M                            | 4.6      | 11970         | L1          | Pro B ALL      | -                   | CR      |
| 41      | 1.8/F                           | -        | 5470          | L1          | Pre B ALL      | -                   | CR      |
| 42      | 8.3/M                           | 5.2      | 14300         | L1          | Pre B ALL      | -                   | CR      |
| 43      | 4.5/M                           | 10.1     | 5100          | L1          | Early pre B ALL| -                   | CR      |
| 44      | 9/M                             | 9.9      | 3900          | L1          | Pro B ALL      | +                   | CR      |
| 45      | 12/M                            | 5.1      | 16930         | L1          | Early pre B ALL| -                   | CR      |
| 46      | 2/F                             | 7.6      | 79600         | L1          | Early pre B ALL| +                   | CR      |
| 47      | 5/M                             | 6.4      | 20700         | L1          | Early pre B ALL| -                   | CR      |
| 48      | 1.5/M                           | 7.9      | 10500         | L1          | Early pre B ALL| -                   | CR      |
| 49      | 4.5/F                           | 3.2      | 12500         | L2          | Pre B ALL      | -                   | CR      |
| 50      | 2/M                             | 10.5     | 11510         | L1          | Pre B ALL      | +                   | CR      |
| 51      | 8/F                             | 6.2      | 27820         | L1          | Early pre B ALL| -                   | CR      |
| 52      | 2/F                             | 8.7      | 15750         | L1          | Early pre B ALL| -                   | CR      |
| 53      | 7/M                             | 8.8      | 15560         | L1          | T-ALL          | -                   | CR      |
| 54      | 4/M                             | 5.3      | 1470          | L1          | Pre B ALL      | -                   | CR      |
| 55      | 2/M                             | 11       | 2530          | L1          | Early pre B ALL| -                   | CR      |
| 56      | 3/M                             | 13.1     | 7150          | L1          | Pre B ALL      | +                   | CR      |
| 57      | 12/F                            | 4.2      | 14210         | ALL         | Early pre B ALL| +                   | CR      |
| 58      | 2/M                             | 7.9      | 5790          | L1          | Early pre B ALL| -                   | CR      |
| 59      | 6/M                             | 10.8     | 11380         | L1          | T-ALL          | -                   | CR      |
| 60      | 3/M                             | 7.9      | 11150         | L1          | Early pre B ALL| -                   | CR      |
| 61      | 5/M                             | 5.6      | 19710         | L1          | Pre B ALL      | +                   | CR      |
| 62      | 3/M                             | 10.8     | 6680          | L1          | T-ALL          | -                   | CR      |
| 63      | 2/M                             | 7.5      | 3260          | L1          | Pre B ALL      | -                   | CR      |

CR: Complete Remission; ALL: Acute Lymphoblastic Leukemia; WBC: White Blood cell; Hgb: Hemoglobin; M: Male; F: Female
Table 2: Fusion gene analysis as well as French-American-British classification and comparison with different immunophenotypes in ALL patients

| Immunophenotype         | Patients | TEL/AML1 positive | L₁ | L₂ | L₃ | ALL |
|-------------------------|----------|------------------|----|----|----|-----|
| Pro-B                   | 4        | 1                | 3  | 5  | 3  | 1   |
| Early pre B             | 28       | 9                | 19 | 5  | 3  | 1   |
| Early pre B with CD13   | 1        | 1                | 1  |    |    |     |
| Early pre B with CD2    | 1        | 1                |    |    |    |     |
| Pre B                   | 22       | 10               | 17 | 4  | 1  |     |
| T cell                  | 7        | 1                | 6  | 1  |    |     |
| **Total**               | **63**   | **22**           | **47** | **10** | **3** | **3** |

ALL: Acute Lymphoblastic Leukemia

to their gender and it revealed that 12 were females and 10 males (Table 3). Based on FAB classification it must be stated that 13 individuals were type L₁; 4 were L₂; 2, L₃ and 3 were assumed as ALL.

In the present study among the 58 patients with WBC count ≤50×10³/µL, 20 were TEL/AML1 positive. The patients with WBC count between 50×10³/µL and 100×10³/µL, 2 were ETV6/RUNX1 positive. However, none of the three patients whose WBC counts were greater than 100×10³/µL was ETV6/RUNX1 positive. The immunologic markers in our cases with regard to ETV6/RUNX1 were as follows: 10 children had early pre B, 10 pre B, 1 pro B and 1 T-ALL.

Discussion

The ETV6/RUNX1 fusion gene is thought to be the most common leukemia-specific fusion gene in children with ALL. The frequency of 34.9% referring to the ETV6/RUNX1 rearrangement, is the upper 25% average reported in the literature[16,17]. It is worth noting that the lower frequency of this fusion gene has also been observed in countries such as India (6%)[10], Mexico (9.6%)[11], Argentina (11.6%)[12], Thailand (12%)[13], China (17.9%)[14] and Taiwan (19%)[15] which indicates a significant difference among them but this difference was not significant in other studies[16-18].

Table 3: Fusion gene analyses and associations with age, white blood cell count and hemoglobin in ALL patients

| Variable                  | TEL/AML1 Positive | TEL/AML1 Negative | Total |
|---------------------------|------------------|------------------|-------|
| **Age (yr)**              |                  |                  |       |
| 1-10                      | 20               | 35               | 55    |
| >10                       | 2                | 6                | 8     |
| **WBC count (×10³/µL)**   |                  |                  |       |
| <50                       | 20               | 38               | 58    |
| 50-100                    | 2                | 0                | 2     |
| >100                      | 0                | 3                | 3     |
| **Hemoglobin (g/dL)**     |                  |                  |       |
| 6-10                      | 13               | 24               | 37    |
| >10                       | 4                | 10               | 14    |
| **Gender**                |                  |                  |       |
| Male                      | 10               | 29               | 39    |
| Female                    | 12               | 12               | 24    |
| **French-American-British classification** | | | |
| L₁                        | 13               | 34               | 47    |
| L₂                        | 4                | 6                | 10    |
| L₃                        | 2                | 1                | 3     |
| **ALL**                   | 3                | 3                |       |

ALL: Acute Lymphoblastic Leukemia; WBC: White Blood Cell
The improvement of medical assessment in Iran has resulted in a significant decrease in infant mortality rates caused by ALL. In relation to the immunophenotypes of ALL patients with ETV6/RUNX1 fusion transcripts, P. Tiensiwakul[24] found in 35 ALL patients, an incidence of 8.6% of ETV6/RUNX1 translocation (12% of B-lineage ALL), which is lower than that reported in Caucasians but is similar to that reported in Japanese and Koreans[25], which indicates a significant difference with our study. In the report of Zuo YX et al, FAB-L2 morphology was commonly observed, but t(12;21) was often absent in those children[26] which indicates a significant difference with our results.

Moreover, for the newly diagnosed B-ALL cases with ETV6/RUNX1 rearrangement, several studies pointed a favorable prognosis[27-28] and some authors have suggested more comprehensive assessment whereas other studies did not identify any significant difference between the prognosis of patients with or without ETV6/RUNX1 rearrangement[22,23]. Other known clinical and hematological prognostic factors including age, WBC, and the presence of early hematological response, play an important role in ALL. In a study, the patients were grouped according to their WBC count at the time of diagnosis: the groups consisted of 21 patients with, <50×10^3/µL one patient with 50-100×10^3/µL, and three patients with >100×10^3/µL. Among the 21 patients with WBC count <50×10^3/µL seven were ETV6/RUNX1 positive. The patient with WBC count between 50 and 100×10^3/µL was also ETV6/RUNX1 positive. However, none of the three patients whose WBC count was greater than 100×10^3/µL was ETV6/RUNX1 positive[18]. So our findings are consistent with those reported in previous literature[18-21]. Among the ETV6/RUNX1 fusion gene positive patients 90.9% (20/22) had WBC count ≤50×10^3/µL, anemia 90.9% (20/22), 95.4% (21/22) with B-lineage immunophenotype, died 4.5% (1/22) and most (20/22) patients were between 1 and 10 years old. In our study, patients aged 1 to 10 years had a better outcome and were similar to the findings in other studies[17,18]. More cases will be required for future research to confirm the efficacy of our quantization method using ETV6/RUNX1 fusion transcripts as the target gene for the estimation of disease progression.

**Conclusion**

The molecular analysis by RT-PCR was shown to be an ideal tool for detecting hybrid transcripts. So, molecular analysis was carried out in every sample, including those that were unsuitable for cytogenetic analysis, the cryopreserved ones and those with little cellularity. Furthermore, molecular analysis is more sensitive and more specific than cytogenetic as it identifies the presence of genetic rearrangements in samples where the cytogenetic result was negative, as well as the absence of important genetic rearrangements in patients with cytogenetically identical translocations. It is known that comprehensive diagnosis of childhood malignancies using molecular assessment is now achievable in Iran. Thus, application of complementary methods to detect clinically relevant specific abnormalities (e.g., ALL with fused gene) is of fundamental importance.

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**Conflict of Interest:** None

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