# RT-PCR ANALYSIS OF E2A-PBX1, TEL-AML1, BCR-ABL AND MLL-AF4 FUSION GENE TRANSCRIPTS IN B-LINEAGE ACUTE LYMPHOBLASTIC LEUKEMIA

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**Keywords:** immunophenotyping, fusion genes, acute lymphoblastic leukemia, reverse transcription-PCR

**Abstract:** Acute lymphoblastic leukemia represents a heterogeneous group of hematological malignancies, defined by clonal proliferation of lymphoid cells. Immunophenotyping by flow cytometry and molecular analysis for the detection of genetic anomalies are clinical standard procedures for diagnosis, sub-classification and post-therapeutic evaluation. Samples from 105 patients diagnosed with acute lymphoblastic leukemia were immunophenotyped at diagnosis and were investigated by molecular analysis in order to identify the occurrence of four fusion genes: MLL-AF4, TEL-AML-1, BCR-ABL-p190, E2A-PBX-1. There were no associations found between the immunophenotype and the presence of any fusion genes evaluated. Both methods in combination remain a prerequisite for an improved subclassification of hematological malignancies, therapeutic decision, and evaluation of treatment response.

## INTRODUCTION

Acute lymphoblastic leukemia (ALL) represents a heterogeneous group of hematological malignancies, defined by clonal proliferation of lymphoid cells. ALL is characterized by excessive proliferation of a malignant clone „blocked” in a certain stage of cell development [Inaba et al., 2013]. The diagnosis approach in acute leukemia involves subsequent issues like morphology, cytochemistry, immunophenotyping, cytogenetic and molecular diagnostic studies. It becomes increasingly clear that data provided by molecular biology represent key information for the diagnosis, prognosis and therapy setting. Since most of clinical features in ALL are consequences of chromosomal abnormalities present in leukemia cells, molecular technologies have become indispensable tools for an accurate detection of such disease-related markers [Harrison, 2011].

For routine diagnostics, reverse transcription-polymerase chain reaction (RT-PCR) and real-time PCR are the most utilized techniques for detection of fusion genes (FG) produced as a result of chromosomal translocations. There are several technical advantages of these methodologies, which are not only highly sensitive and specific but also require smaller number of tumor cells [vanDongen et al., 1999; Liang et al., 2010]. The most frequent abnormalities found in ALL are t(9:22), t(4:11), t(12.21), t(1:19) [Swerdlow et al., 2008]. According to the most recent World Health Organization classification of tumors of hematopoietic and lymphoid tissues [Swerdlow et al., 2008], fusion genes are key elements for classification of patients in risk groups and have major prognostic and therapeutic implications. These genetic defects are also useful as reliable markers for a sensitive detection of residual leukemia cells, when ALL patients are monitored during and after treatment,

## MATERIALS AND METHODS

**Patient samples:** Peripheral blood (PB) and/or bone marrow (BM) samples were obtained at diagnosis, based on written informed consent, from 105 B-ALL patients (71 pediatric and 34 adults), hospitalized between June 2009 and April 2013, in “St. Mary” Hospital for Children and Regional Institute of Oncology, Iasi, Romania. Diagnosis of B-ALL was based on FAB classification [Miller et al., 1981]. Immunophenotyping analyses were carried out on a FacsCanto II machine (BD Biosciences, San Jose, USA), following the European Group for the Immunological Characterization of Leukemia (EGIL) criteria of classification (Table I) [Bene et al., 1995; Hoelzer, 2002].

The presence of 4 fusion genes was investigated at diagnosis: MLL-AF4, TEL-AML-1, E2A-PBX-1, BCR-ABL-p190.

**Isolation of RNA and cDNA synthesis:** Lymphocytes ($2x10^7$ cells) were isolated from PB/BM, washed with 1x PBS and suspended in 0.5 mL of Guanidine-Thiocyanate reagent (EZ-RNA Total RNA Isolation Kit - Biological Industries). Total RNA was isolated, according to manufacturer’s instructions and the pellet was suspended in 12 µL nuclease free water. Two µL of RNA were used for spectrophotometric quantification (NanoDrop 2000- Thermo Scientific) and the rest was diluted to 500 ng/µL. Four µg of diluted RNA and 0.5 µg/reaction random hexamers

(Promega) were heated first at 70°C for 5 minutes and then chilled in ice for 5 minutes. Then 15 µL of the reverse transcription reaction mix containing 4µL GoScript™ 5X Reaction Buffer (Promega), 5µL $MgCl_2$ (25mM), 0.5 mM each dNTP, 20U Recombinant RNasin® Ribonuclease Inhibitor (Promega) and 2 µL GoScript™ Reverse Transcriptase (Promega), was added. The annealing was performed at 25°C for 5 minutes, extension at 42°C for one hour and enzyme inactivation at 70°C for 15 minutes. The cDNA solution was then diluted with 80 µL nuclease free water to a final volume of 100 µL.

**Table I: GEIL/EGIL classification of B-cell ALL**

| | CD19; CD22 s+ic; CD79a ic | CD10 | CD20 | IgM | κ or λ |
|---|---|---|---|---|---|
| **B1** | + | - | - | - | - or - |
| **B2** | | + | - | - | - or - |
| **B3** | | +/- | + | + | - or - |
| **B4** | | +/- | + | +/- | + or + |

B1 = pro-B-ALL, B2 = Common B-ALL, B3 = pre-B-ALL, B4 = mature B-ALL, ic = intracitoplasmatic, s = surface

**RT-PCR analysis of E2A-PBX1, TEL-AML1, mBCR-ABL and MLL-AF4 fusion gene transcripts**

Five µL of cDNA (equivalent of 100ng initial RNA) was PCR amplified for ABL housekeeping gene to check the integrity of cDNA. Then 5 µL of cDNA was used in a 25 µL reaction volume for the detection of fusion genes transcripts. For all genes, PCR reaction was performed using 12.5µl of GoTaq Green Master Mix (Promega) and 10 pmol of forward and reverse primers each. The primer sequences are given as follows:

ABL-f exon 2: 5'-TGTGATTATAGCCTAAGACCCGGAGCTTTT-3'
ABL-r exon 3; 5'-TCAGCGGCCAGTAGCATCTGACTT-3' [Hughes et al., 1991]
BCR-f exon 1: 5'-GACTGCAGCTCCAATGAGAAC-3'
ABL-r exon 3: 5'-GTTTGGGCTTCACACCATTCC-3'
E2A-f exon 13: 5'-CACCAGCCTCATGCACAAC- 3'
PBX1-r exon 2: 5'-TCGCAGGAGATTCATCACG-3'
TEL-f exon 5: 5'-TGCACCCTCTGATCCTGAAC-3'
AML1-r exon 3: 5'-AACGCCTCGCTCATCTTGC-3'
MLL-f exon 8: 5'-CCGCCTCAGCCACCTAC-3'
AF4-r exon 8: 5'-TGTCACTGAGCTGAAGGTCG-3' [van Dongen et al., 1999]

After an initial denaturation at 95˚C for 2 min, 35 cycles of denaturation at 94˚C for 30 s, annealing at 55˚C (for ABL) and 65˚C (for the fusion genes) for 30 s and extension at 72˚C for 1min, followed by a final extension at 72˚C for 10 min, was performed in a Corbett Research Thermal cycler.

Plasmids containing the fusion gene inserts were used as positive controls (Ipsogen). The PCR products were run on a 2% agarose gel to visualize the amplified products of fusion gene transcripts [Mihăşan et al., 2012].

Fusion transcripts variants were estimated based on the length of PCR products. When unusual amplicons were detected, they were sequenced to confirm the presence of breakpoints in the fusion gene and checked for the presence of variants in the fusion region.

## RESULTS AND DISCUSSIONS

Hierarchy of patients is based on clinical criteria, age at diagnosis, number of leukocytes at diagnosis, immunophenotype, presence or absence of translocation, response to corticosteroid therapy and chemotherapy [Pui et al., 1995].

Patient's stratification in risk groups is crucial for therapy initiation and setting of a specific chemotherapy branch. The response to treatment is a very important prognostic factor in acute leukemia and is quantified at day 8 after corticosteroid therapy and day 33, after the induction. The patient is considered cortico-sensitive if there are no blasts presents in peripheral blood or their number has declined and cortico-resistant if the number of blasts is the same as or higher then at diagnosis. The response to chemotherapy is quantified by bone marrow smear, while chemo-sensitivity can be appreciated by the degree of remission. Fusion genes represent important markers for tracking the effectiveness of treatment as well as prognostic markers [Bruggemann et al., 2010].

Under the current study, common ALL (cALL) represented 44.7% (47/105) of the patients: 31/71 (43.6%) representing pediatric cases (0-16 years, median value = 5 years) and 16/34 (47%) adult cases (19-80 years, median value = 57 years). Pre-B cases were registered in 43/105 (40.9%) patients, including 33/71 (46,4%) children and 10/34 (29,4%) adults. The pro-B subset was clonally expanded in 14/105 (13,3%) patients, including 6/71 (8,4%) children and 8/34 (23,5%) adults. Only one pediatric case was found to match the mature-B ALL subtype. The male-to-female ratio in all groups was 1.4: 1 (62 males to 43 females), the report being maintained both in the pediatric subgroup (41 males to 29 females) and in the adults subgroup (20 males to 14 females).

As a reference, cDNA synthesized from all 105 B-ALL samples was amplified for the ABL1 housekeeping gene (amplicon size 200 bp) in order to determine the integrity and amount of amplifiable matrices (figure 1A).

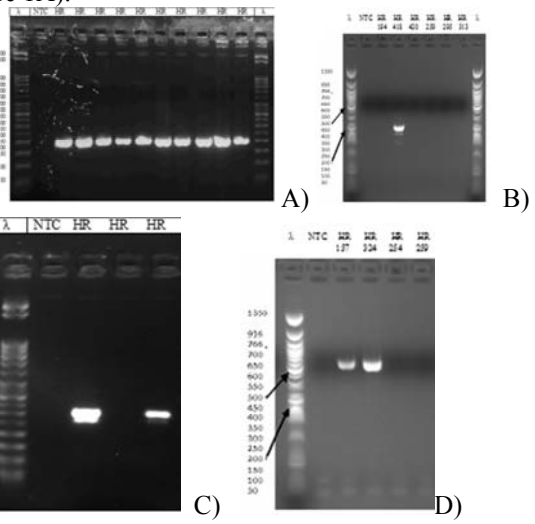



Figure 1: PCR amplification of the ABL1 reference gene – 200bp(A), E2A-PBX1 (B), TEL-AML1 (C) and BCR-ABL1 (D) fusion genes; λ= 50bp ladder; NTC = no template control; HR= ALL patient

Twenty eight of the 105 (26,6%) samples were found to contain one of the fusion gene transcripts detected by RT-PCR. Fusion genes were identified in 18 pediatric and 10 adult patients. Seven patients (6.6%) were found to be positive for E2A-PBX1 fusion gene, eight (7.6%) patients were TEL-AML1 positive, eight patients (7.6%) had mBCR-ABL fusion gene, while MLL-AF4 fusion gene was detected in five patients (4,7%).

E2A-PBX1 translocation is more frequently found in non-Caucasians, representing 5 to 6% of all translocations detected in childhood ALL and 3% of all translocations detected in adult ALL [Crist et al., 1990; Faderl et al., 1998]. In the study presented here, among the 7 patients showing E2A-PBX1 fusion gene, 4 were diagnosed with cALL and 3 patients were assigned to the Pre-B subtype. Estimation of the E2A-PBX1 transcript variant starting from the amplicon dimension revealed a constant junction of exon 13 of E2A gene to the exon 2 of PBX1 (figure 1B). White Blood Cell (WBC) counts in patients with E2A-PBX1 fusion gene transcripts were higher compared to other translocations, or ALL cases negative for any of the fusion genes evaluated.

TEL-AML1 is the most frequent anomaly found in pediatric ALL in the West Europe and the United States, with an incidence of 25% [Liang et al., 2010; Swerdlow et al., 2008]. Most pediatric patients are reported to be between 1-12 years with a peak between 2-5 years [Loh and Rubnitz, 2002]. The presence of TEL-AML1 is not reported in infants, its frequency decreases in older children, while in adults is found very rarely [Swerdlow et al., 2008]. Some studies have reported its association with a favorable prognosis [Romana et al., 1995]. Under the current study, among the 8 patients with TEL-AML1, 6 were cALL and 2 patient were assigned to the Pre-B subtype. The TEL-AML1 fusion transcripts found were generated by joining exon 5 of TEL to the exon 2 of AML1 gene, in all cases (figure 1C). The transcript was not identified in the adults ALL group, confirming its exclusive incidence in pediatric ALL.

The frequency of TEL-AML1 fusion gene is highly variable in different geographic areas. In our study its frequency is 11,2% among pediatric cases, comparable to that found in the Far East (13.4%), but not with that found in the West Europe and the United States (22.8%) [Liang et al., 2010].

BCR-ABL, which is a hallmark for chronic myeloid leukemia (CML), is found in 5% of ALL cases in children and 20-50% of adult ALL cases, the incidence increasing with age [Maurer et al., 1991]. Sazawal et al., in 2004 reported a BCR-ABL frequency of 2.8% and 14.7% among pediatric and adult ALL cases, respectively and of adult positive ALL patients. The CML-associated fusion in the BCR gene intervenes is in "major breackpoint cluster region" (M-BCR), while in ALL a "minor breackpoint cluster region" (m-BCR) is reported almost exclusively. The chimeric mBCR-ABL protein with increased tyrosine kinase activity induces resistance to apoptosis, growth factor interactions and alters cell–cell and cell–matrix interactions [Bedi et al., 1994]. The presence of Philadelphia chromosome in ALL was associated with a poor prognosis and is also an indication in favor of bone marrow transplantation [Oyekunle et al., 2011].

In our study 8 patients were identified with m-BCR, 5 were cALL and 3 patient were included in the Pre-B subtype. Only 4 (3.1%) children were positive for mBCR-ABL. All mBCR-ABL fusion gene transcripts showed exon a2 of ABL gene joined to the first exon of the BCR gene (e1-a2). All patients with the mBCR-ABL fusion gene had a high WBC count (mean WBC count = $275\times10^9$ /L) compared with patients negative for all four fusion genes investigated.

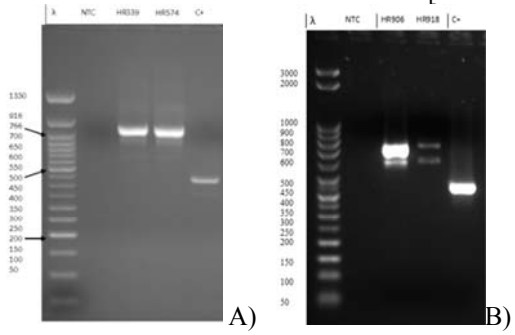
MLL-AF4 is encountered in 50-70% of neonatal acute lymphoblastic leukemia and about 5% of cases of ALL in older children and adults [Swerdlow et al., 2008]. At least 10 breaking points within the gene have been identified in the case of MLL-AF4 [de Braekeleer et al., 2005]. In older children this fusion gene is associated with poor prognostic and represents an indication for bone marrow transplantation [Oyekunle et al., 2011]. Recent studies have demonstrated the fact that some variants of this gene could be found in normal - population, fact which could become the basis for new research projects in the field [Basecke et al., 2002]. Microarray studies have showed that MLL rearranged leukemias represents a unique biological entity characterized by specific gene expression profile [Armstrong et al., 2002].

Under the current study 5 patients with MLL-AF4 were identified, of which 2 were pediatric cases, one, a new-born patient. This two infants were classified one within the pro-B subtype (also showing the aberrant expression of the myeloid marker CD33) and the other within the pre-B subtype. The rest of MLL-AF4 positive cases were adult patients: one with pro-B ALL and two with pre-B ALL. All 5 patients revealed different fusion transcripts: e9-e4, e11-e6, e12-e4, e8-e4, and e10-e4 co-expressed with e11-e4.

The newborn patient with pre-B ALL (27 days old) presented at diagnosis a fusion between exon 12 of MLL gene to exon 4 of AF4 gene (figure 2A), this fusion point being reported for the

first time in a pro-B ALL patient [Ivanov et al., 2013]. The other pediatric patient (80 days old) showed the presence of 2 transcripts in the same time: e10-e4 and e11-e4 (figure 2B). Both patients relapsed with acute myeloid leukaemia after one year and one month, respectively.

One study of 69 B-lineage ALL patients reported mBCR in 2.8% of patients, E2A-PBX1 in 5.7% of cases and absence of TEL-AML1 and MLL-AF4 fusion gene transcripts [Sazawal et al., 2004]. In a similar study by Hill et al. (2005), in 42 B-lineage ALL, only two patients were positive for TEL-AML1 and none for E2A-PBX1 and MLL-AF4[Hill et al. 2005].




A) B)

Figure 2: PCR amplification of the MLL-AF4
A) HR339 (ALL at diagnosis) and HR574 (AML at relapse) = pro-B ALL patient with e12-e4 transcript;
B) HR906 (ALL-diagnosis) and HR918 (AML-relapse) = pre-B ALL patient with co-expression of e10-e4 and e11-e4;
C+ = positive control e9-e4; λ- 50bp ladder, NTC–no template control.

In 2010, Burmeister et al., in a lot of 441 adult cases, reported an incidence of 23 cases with E2A-PBX1 (5.21%) and 10 cases with TEL-AML1 (2.26%)[ Burmeister et al., 2010]. Faiz et al in a study of 103 childhood ALL samples identified 10 cases (9.7%) with TEL-AML1, 14 cases (14%) with MLL-AF4; 2 cases (2%) with E2A-PBX1 and 25 cases (24%) with BCR-ABL1[Faiz et al.,2011].

## CONCLUSIONS

Under the current study evaluating 105 B-lineage ALL patients, the pattern and frequency of the fusion gene transcripts differs from the findings in Western literature. Thus our study reveals a lower frequency of TEL-AML1 (11,2%) fusion gene in childhood ALL. The frequencies of all four investigated fusion genes was lower than those reported in the literature (26 vs ~35%). Immunophenotype and detection of fusion genes are crucially important for diagnosis, prognosis and stratification of patients into groups of therapies. Both methods in combination remain a prerequisite for an improved sub-classification of hematological malignancies, therapeutic decision and evaluation of treatment response.

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**ACKNOWLEDGEMENTS**
This study was possible with financial support from European Social Fund by the POSDRU/107/1.5/S/78702 Project and from research grant UMF Iasi CONTR-24829-22.12.09