Expression of chimeric genes in bone marrow cells of children with acute lymphoblastic leukemia upon cytostatic therapy

O. A. Vynnytska, L. Ya Dubey
Danylo Halytsky Lviv National Medical University
69, Pekarska Str., Lviv, Ukraine, 79010
ovinnitska@gmail.com

Aim. To study the expression of chimeric genes in bone marrow cells as prognostic factors of the course of acute lymphoblastic leukemia (ALL) in children and to evaluate their role in resistance to cytostatic chemotherapy. Methods. The AF4-MLL, BCR-ABL, E2A-RVX, TEL-AML1 chimeric gene expression level was determined by RT-qPCR. Results. The AF4-MLL oncogene was not expressed in children during remission, and the expression of all other studied oncogenes was at a low level. In conditions of relapse, there was no expression of the TEL-AML oncogene and a high expression of the BCR-ABL. Conclusions. The development, course, and survival of ALL patients largely depend on the expression of AF4-MLL, BCR-ABL, E2A-PBX1, TEL-AML chimeric genes in the bone marrow; this should be taken into account when detecting early relapses in patients after chemotherapy.

Keywords: acute lymphoblastic leukemia, oncogene, AF4-MLL, BCR-ABL, E2A-PBX, TEL-AML.

Introduction

Acute lymphoblastic leukemia (ALL) is the most common malignant pediatric neoplasm; the ALL development is connected with various somatic gene defects [1], which lead to the increased proliferation and disruption of the differentiation program of lymphopoiesis progenitors [2].

Despite progress in the treatment of this pathology, ALL is among the pediatric pathologies with the highest risk of mortality. One of the causes of high mortality is cytogenetic and molecular diversity of ALL [3]. Additionally, the gene rearrangements in patients with ALL may be an unfavorable prognostic factor in the course of the disease and be associated with a high risk of treatment failure and relapse [4]. ALL is often associated with chromosomal translocations that lead to the formation of chimeric genes and the expression of chimeric proteins with onco-
genic properties. The products of Chimeric gene expression can increase the resistance of tumor clone cells and reduce the effectiveness of cytostatic chemotherapy [5].

The products of chromosome translocations are involved in the development of ALL [6]. The most common chromosome translocations are: AF4-MLL t(4;11)(q23;p23), BCR-ABL t(9;22)(q34;q11), E2A-PBX1 t(1;19) (q23;p13), TEL-AML t(12;21)(q13;q22) [7]. These gene products can be transcription factors and regulators of the mitotic cell cycle that initiate cell transformation and proliferation. Thus, the translocation between chromosomes 1 and 19 leads to the formation of the oncogene E2A-PBX1, which causes 25% of pre-B-cell ALL in children [8]. The AF4/MLL and BCR/ABL oncogenes play an important role in the development of ALL; their expression leads to the dysregulation of hematopoiesis, and as a result to the development of leukemias. TEL/AML1 is generated by t(12;21) (p13;q22); this is the most common gene fusion in malignant neoplasms and occurs in 25% of ALL cases in children [9]. The chimeric gene occurs mainly during fetal hematopoiesis at a rate significantly higher than the apparent clinical course of ALL, which indicates the need for additional secondary and postnatal genetic events [6].

Determination of the expression of chimeric genes in ALL makes it possible both an estimation of the degree of malignant transformation and early identification of an unfavourable prognosis, which can potentially help us in choosing right treatment approach [6].

Here we aimed to study the expression of chimeric genes in bone marrow cells as prognostic factors of the course of ALL in children and to evaluate their role in the neoplasm resistance to the cytostatic chemotherapy.

**Materials and Methods**

We examined 105 children with ALL, aged 12 months to 16 years, who were undergoing inpatient treatment in the Hematology Department. Among the respondents there were 62 boys (59.0%) and 43 girls (41.0%) (n=105). When performing the work, the rules of patient safety were observed.

ALL diagnosis was established according to the FAB classification and blast cell immunophenotype [10] using the ultrasound, chest X-ray, Computed Tomography scan (CT scan), or magnetic resonance imaging (MRI) of the head, organs of the chest, abdomen. To confirm the diagnosis of ALL, the data from the clinical picture, biochemical analysis of peripheral blood, cytological examination of bone marrow (BM) aspirate (percentage of blasts ≥ 25.0% of all non-erythroid cells) were used. Lymphoid origin of the neoplasm was detected by morphological, cytochemical and immunophenotypic studies of bone marrow and blood blasts. The hemogram analysis was performed to determine the absolute number of white blood cells, platelets, percentage of blast cells, and hemoglobin level. The percentage of blast cells in the bone marrow was estimated.

The expression level of the AF4-MLL, BCR-ABL, E2A-RVX, TEL-AML1 chimeric genes in patients was determined by RT-PCR, a polymerase chain reaction method (PCR). PCR was performed using complementary DNA (cDNA) obtained by reverse transcription reaction (RT) with RNA using AMV transcriptase (Promega, USA).
Extraction of genomic DNA from bone marrow cells was performed by the standard phenolic method [11], which consists in denaturation and extraction of proteins with organic solvents (phenol, chloroform) and the transition of DNA to an aqueous solution. From aqueous solutions, DNA was precipitated by adding ethanol. Denaturation of double-stranded DNA was performed at a temperature of 92–95 °C. The primers for gene amplification AF4-MLL, BCR-ABL, E2A-RVX, TEL-AML1 were part of commercial kits (manufactured by Cytocell aquarius, Germany).

Real-time PCR was performed in 25 μl of the reaction mixture containing the appropriate primers and 20-30 ng of genomic DNA. The cycle conditions were as follows: pre-incubation for 10 min at 95 °C, resulting in denaturation of the target DNA and activation of the hot start iTaqTM DNA polymerase. 20 cycles of 20 s at 95 °C and 50 s at 60 °C were conducted. The change in the expression of the target gene was recorded in relation to the internal control gene at the time of the study. The samples were analyzed by real-time quantitative analysis. The expression of the target gene was determined by changing the number of copies of transcripts at each time ∆∆CT = (CTtest – Ccontrol) Time x – (CTtest – Ccontrol) Time 0 [12].

To assess the features of the clinical course of the disease depending on the expression of the chimeric gene, the response of patients to polychemotherapy under the ALLIC-BFM 2009 program was analyzed on the 33rd day after the start of treatment. According to this, the patients were divided into two groups: Group I — patients with complete remission of the disease (n=92); Group II — patients who were found to relapse after a course of polychemotherapy (n=13). The comparison (control) group consisted of healthy people (n=30).

To quantify the results of the study, the values of the arithmetic mean (M) and the error of the arithmetic mean (m) were calculated — M±m. Statistical processing of the obtained results was performed using the licensed statistical analysis packages of Microsoft Excel and Statistica 5.5. To assess the reliability of the difference in average indicators between the groups, the Student’s t-test was used. The difference was considered significant with a probability factor of p<0.05.

Results and Discussion
Genetic disorders and additional chromosomal abnormalities that accompany the development of ALL may help to identify the patients with an unfavorable prognosis. Accurate diagnosis and selection of an effective treatment regimen for ALL require the understanding of various genetic abnormalities, their impact on the course of the disease, and the knowledge how these chimeric genes are expressed to predict the development of relapses after [the] treatment.

The MLL gene rearrangements are associated with acute leukemias. MLL is the gene, which is localized on 11q23; it encodes for lysine(K)-specific methyltransferase 2A (KMT2A). MLL polypeptides processed by threonine aspartase, with the formation of MLL-N and MLL-C domains, involved in the formation of a protein complex. The MLL t activates the transcription of target genes [13].

We observed a 1.8-fold decrease in the MLL gene expression in ALL patients before cytostatic therapy compared to the control (p<0.05).
(Fig. 1), this may be associated with the chromosomal rearrangements and the formation of chimeric genes. One of the chimeric genes involving MLL is AF4-MLL, formed by the fusion of fragments of two genes: AF4 (4q21) and MLL (11q23) [7]. ALL patients had an increased expression of AF4-MLL, which is not detected in the control (Fig. 1). MLL leads to the synthesis of chimeric proteins that bind to nuclear factors (for example, MEN1, LEDGF). Additionally, AF4-MLL promotes mutations of the RAS gene [7].

It was found that the expression of the AF4-MLL chimeric gene after the ALLIC BFM 2009 chemotherapy remained detectable in the relapsed patients 0.39±0.045 copies of mRNA (Fig. 1). In patients with a remission, AF4-MLL was not expressed, at the same time the expression of MLL (11q23) and AF4 (4q21) genes was increased (Fig. 1).

Thus, the chimeric gene AF4-MLL expression may be associated with the ALL relapse. Therefore, targeted inhibition of its activity can improve cytostatic therapy and prevent the relapse.

Another important marker of ALL is an unusual short chromosome (the Philadelphia chromosome (Ph)), which is a result of t(9;22) that leads to the formation of a fusion gene between a non-receptor tyrosine kinase ABL1, on chromosome 9, and the BCR gene on chromosome 22 [14].

ALL patients had reduced values of BCR and ABL gene expressions by 2.85 times and 4.8 times, respectively, compared to the control (p<0.05) and a significantly increased expression of BCR-ABL with ΔΔCT of 2.09 ±1.98 copies (Fig. 2). After a ALLIC BFM 2009 cytostatic therapy, a decrease in the BCR-ABL expression by 19 times was observed in patients with the complete remission and by 2.4 times in patients with the relapse (Fig. 2). At the same time, cytostatic chemotherapy resulted in an increased expression of BCR and ABL genes to control values while their expression was increased by 1.85 and 2.9 times in the relapsed patients (p<0.05) (Fig. 2).

These results show that BCR-ABL1 is an indicator of an unfavorable prognosis for pediatric ALL patients. Presumably, tyrosine kinase inhibitors can significantly increase a patient’s chances of recovery and survival [16].

The E2A-PBX1 oncogene is also observed in pediatric ALL. E2A locus is located on chromosome 21. E2A-PBX1 is encoded by the E2A gene on chromosome 21 and is a transcription factor that plays a key role in the development of hematopoietic cells. The E2A-PBX1 oncogene is involved in the development of acute lymphoblastic leukemia (ALL) and other hematological malignancies. The E2A-PBX1 oncoprotein is a member of the E2A-PBX family of transcription factors, which are involved in the regulation of gene expression and the development of hematopoietic cells. The E2A-PBX1 oncoprotein is able to bind to specific DNA sequences and activate the transcription of target genes, including the genes that are involved in the development of hematopoietic cells. Therefore, the E2A-PBX1 oncoprotein is a key player in the development of hematopoietic malignancies, and its inhibition is a potential therapeutic target.

**Fig. 1.** The expression of AF4, MLL, and the oncogene AF4-MLL in bone marrow cells of children with ALL after cytostatic therapy.

* — statistically significant difference compared to the indicators of healthy children, p<0.05; # — statistically significant difference compared to the indicators observed before treatment, p<0.05.
mosome 19 and gives rise to two genetic products — E12 and E47, which is a result of alternative splicing of the original transcript. The synthesis products E12 and E47 contain DNA binding sites in the regions of promoters/enhancers of immunoglobulin (Ig) genes, thereby provoking Ig synthesis [17]. E2A has been shown to play an important role in the development, maturation, and differentiation of B-lymphocytes, also through direct involvement in the Ig chain rearranging, which is key to B-cell maturation. Additionally, E2A is actively involved in the formation of T-lymphocytes [17]. In healthy children, E2A expression was $\Delta\Delta CT = 0.78 \pm 0.069$ (Fig. 3). The reduced expression of this gene in ALL patients to $\Delta\Delta CT = 0.28 \pm 0.03$ (2.8 times compared to the control) (Fig. 3) contributed to the disruption of the immune system.

The PBX1 gene produced a homeodomain protein that can bind to HOX and MEIS proteins, forming heterodimeric complexes, and is involved in the development of a number of malignant diseases [18] including melanoma and breast cancer [8]. In normal BM cells, the
*PBX1* gene was not expressed, similarly to the chimeric gene (Fig. 4).

Expression of both *E2A* and *PBX1* genes, as well as of *E2A-PBX1*, was observed in patients with ALL (Fig. 3). The *E2A-PBX1* fusion gene can produce two isoforms: E2A-PBX1a and E2A-PBX1b, which are the products of alternative PBX1 splicing and have the same ability to transform cells. Detection of this chimeric transcript is a marker of an unfavorable prognosis. Translocation *E2A-PBX1* entails the development of other genetic changes that increase ALL progression. Among them the *PAX5* deletion accelerates the division of progenitors and enhances the effect of blocking of B-cell differentiation. The involvement of Pax5 in signaling pathways is associated with apoptosis and cell cycle regulation [8].

During remission after the $ALLIC$ BFM 2009 chemotherapy, the *E2A-PBX1* expression decreased 11-fold as compared to the initial values (p<0.05) (Fig. 3). At the same time, in the patients with relapse, the *E2A-PBX1* expression decreased only 2.7-fold (p<0.05) (Fig. 3). Presumably, the *E2A-PBX1* protein interaction with chromatin remodelling factors SWI/SNF and histone acetyltransferase SVR/p300, leads to a strong transcriptional activation of the *HOXA* genes, whose expression products have powerful carcinogenic properties [8].

Therefore, the detection of t(1;19) may be an important indicator in ALL differential diagnosis and choice of the treatment regimen. The detection of *E2A-PBX1* in the children with ALL using RT-qPCR can be considered as the most promising method. Another common translocation found in ALL patients is t(12; 21) (p13; q22), which leads to the fusion of the *TEL* and *AML1* genes. It occurs in the prenatal period and causes a predisposition to the disease, whereas leukomogenesis is provoked by the appearance of additional gene mutations.

Before ALL treatment, the expression of *TEL* and *AML1* was decreased in the patients compared to the control whereas the expression of the *TEL-AML1* oncogene was 26.7 times higher than that of the control (Fig. 4). Expression of the chimeric *TEL-AML1* provokes the accumulation of reactive oxygen species (ROS), which cause DNA damage and, as a

![Fig. 4. The expression of TEL, AML1 genes and the oncogene TEL-AML1 in the bone marrow cells of children with ALL after cytostatic therapy](image)

Note: * — statistically significant difference compared to the indicators of healthy children, p<0.05; # — statistically significant difference compared to the indicators observed before treatment, p<0.05.
result, the formation of driver genetic mutations and aberrations [19].

A minor expression of TEL-AML (ΔΔCT = 0.11±0.013) was detected in patients with remission after ALLIC BFM 2009 chemotherapy whereas in the patients with relapse this oncogene was not identified at all (Fig. 4).

Since this oncogene is not identified after chemotherapy in patients with relapse, it cannot be considered [as] a valuable marker for the diagnosis and prediction of ALL relapse in children. At the same time, the detection of TEL-AML in patients with ALL may indicate a favorable prognosis, sensitivity to drug therapy, and an increased chance of complete remission. Therefore, this chimeric transcript is an important target for the diagnosis, prognosis, and even possible treatment of acute leukemia. Additionally, t(12; 21)(q13; q22) occurs at the early stages of B-cell progenitor formation in the embryonic period; therefore chimeric TEL-AML is detected in healthy newborns,

Fig. 5. Relapse-free survival of patients with ALL depending on the expression of oncogenes in the bone marrow

Note: a — patients with AF4-MLL expression; b — patients with BCR–ABL expression; c — patients with E2A/PBX1 expression; d — patients with TEL/AML expression. * — statistically significant difference compared with patients who were positive for the presence of the corresponding gene, p<0.05.
which may indicate the establishment of a pre-leukaemia. This may explain the fact that this oncogene is identified in children with complete remission [20].

To determine the highest risk group in the patients with chimeric gene expression, we investigated the relapse-free survival of patients. The highest risk group was linked to the AF4-MLL and BCR-ABL expression Fig. 5). Thus, the lowest relapse-free survival (highest mortality) was observed in patients with expression BCR-ABL, since 2 years after chemotherapy, the mortality of patients was recorded (Fig. 5B).

The patients with the AF4-MLL oncogene were in the second place with the lowest survival rate, where mortality was recorded at the 5th year of life, after a course of chemotherapy (Fig. 5a); possibly due to its toxicity. The highest, 100% rate of relapse-free survival was observed in patients with the TEL-AML translocation (Fig. 5c) and in patients with the E2A-PBX1, chimeric gene where the relapse-free survival rate was at the level of 80% (Fig. 5d).

Thus, relapse-free survival is one of the main criteria for evaluating the results of complex treatment in ALL, which depend on the presence or absence of chromosomal aberrations in patients.

Conclusions. The development, course, and survival of the ALL patients largely depend on the presence and expression of the AF4-MLL, BCR-ABL, E2A-PBX1, TEL-AML chimeric genes. After the ALLIC BFM 2009 cytostatic therapy, the detected changes in the expression of chimeric genes can be associated with the development of relapses or the onset of complete remission. During ALL remission in children, the AF4-MLL oncogene was not expressed, and the expression of all other oncogenes studied was at a low level. In conditions of the disease relapse, there was no the TEL-AML oncogene expression whereas a high expression of the chimeric gene BCR-ABL was detected, which could be considered as an approach to detect early relapses in patients aftertreatment. Our study will allow us to better determine the degree of malignancy of the process and to identify the risk groups for the course of the disease. It may also prove useful for further modification of the treatment program, which will increase the life expectancy of patients.

REFERENCES
7. Britten O, Ragusa D, Tosi S, Kamel YM. MLL-rearranged acute leukemia with t(4;11)(q21;q23)-