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

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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 c*himeric 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.

K e y w o r d s: 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-

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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 $\geq$ 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[a] part of commercial kits (manufactured by Cytocell aquarius, Germany).

Real-time PCR was performed in 25 ul 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 ΔΔCT = = (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 polypeptideos 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-ABL1* 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 obseved in pediatric ALL. *E2A* locus is located on chro-

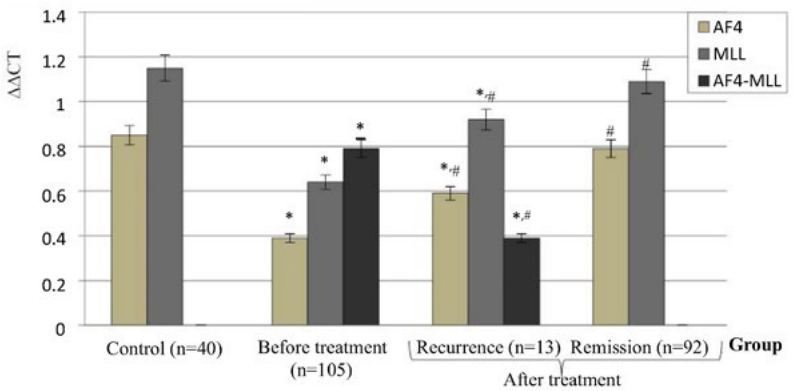

Fig. 1. The expression of *AF4*, *MLL*-genes and the oncogene *AF4-MLL* in bone marrow cells of children with ALL after cytostatic therapy

** — statistically significant difference compared to the indicators of healthy children, $p<0.05$; # — statistically significant difference compared to the indicators observed before treatment, $p<0.05$.*

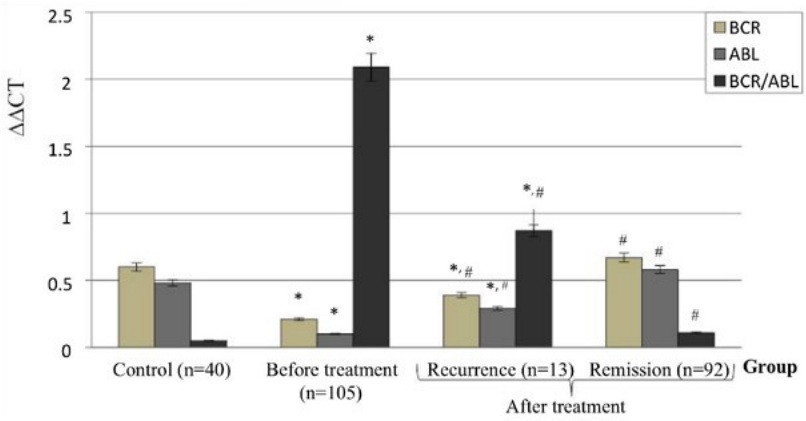

Fig. 2. The expression of *BCR*, *ABL* genes and the oncogene *BCR-ABL* in bone marrow cells of children with ALL after cytostatic therapy

*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.*

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 ΔΔCT = 0.78±0.069 (Fig. 3). The reduced expression of this gene in ALL patients to ΔΔCT = 0.28±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

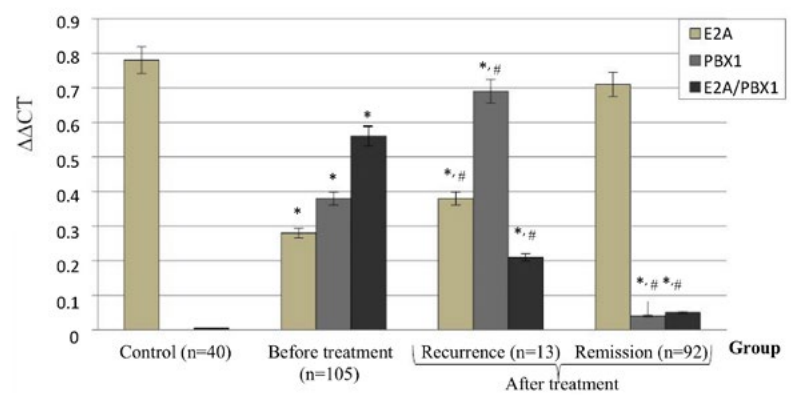

Fig. 3. The expression of *E2A*, *PBX1* genes and the oncogene *E2A-PBX1* in bone marrow cells of children with ALL after cytostatic therapy

*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.*

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 remision 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 c 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-AML* oncogene was 26.7 times higher than that of the control (Fig. 4). Expression of the chimeric *TEL-AML* provokes the accumulation of reactive oxygen species (ROS), which cause DNA damage and, as a

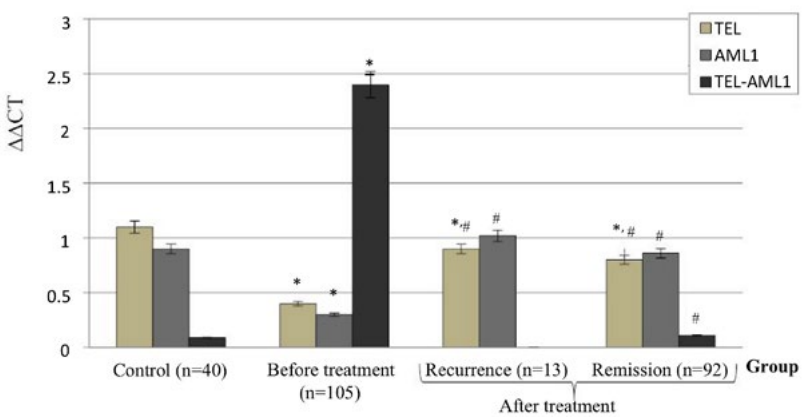

Fig. 4. The expression of *TEL*, *AML1* genes and the oncogene *TEL-AML1* in the bone marrow cells of children with ALL after cytostatic therapy

*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,

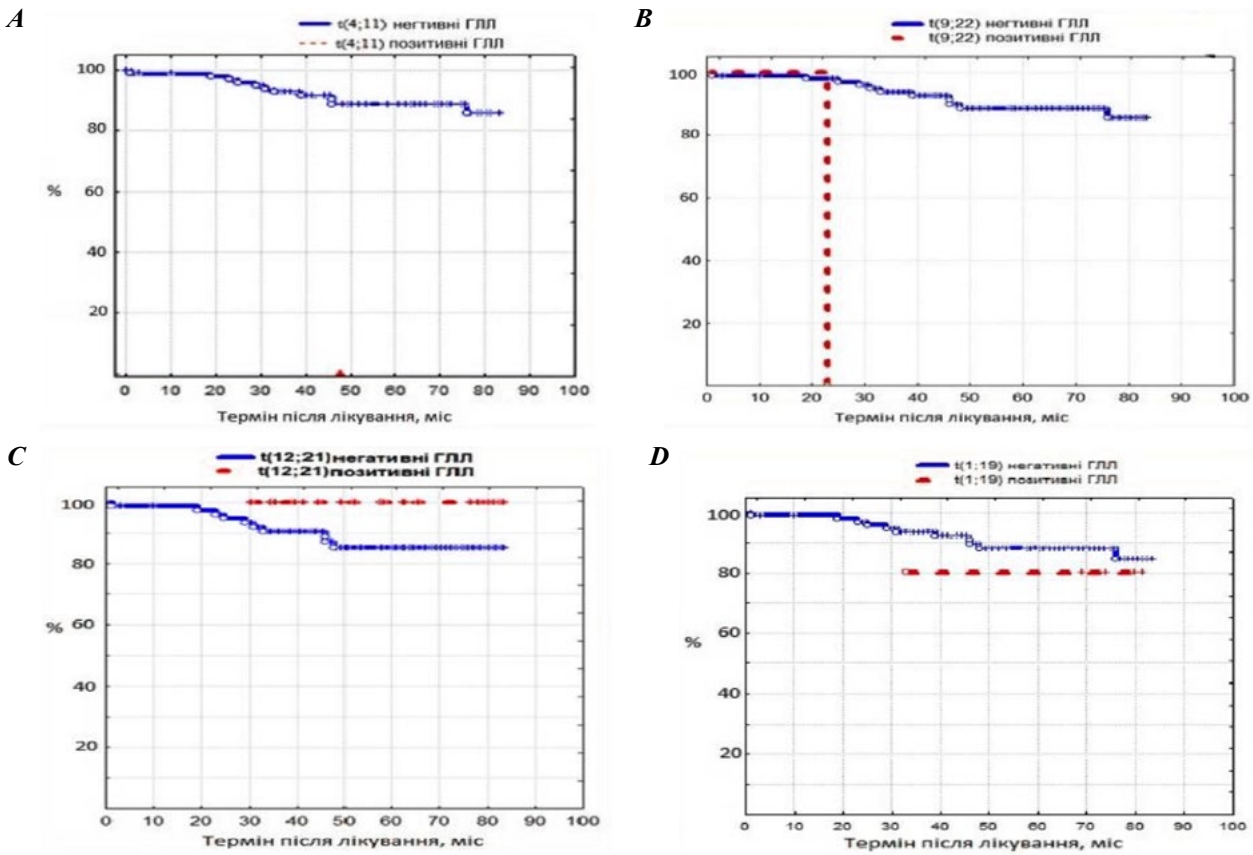

Fig. 5. Relapse-free survival of patients with ALL depending on the expression of oncogenes in the bone marrow

*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 toxicicty. 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

1. *Moorman AV.* New and emerging prognostic and predictive genetic biomarkers in B-cell precursor acute lymphoblastic leukemia. *Haematologica.* 2016; **101**(4):407–16.
2. *Lopes BA, Barbosa TC, Souza BKS, Poubel CP, Pombo-de-Oliveira MS, Emerenciano M.* IKZF1 Gene in childhood B-cell precursor acute lymphoblastic leukemia: interplay between genetic susceptibility and somatic abnormalities. *Cancer Prev Res (Phila).* 2017; **10**(12):738–44.
3. *Kiem HT, Nhu HP, Kim HNT, Van HC.* Causes of death in childhood acute lymphoblastic leukemia at hue central hospital for 10 years (2008-2018). *Glob Pediatr Health.* 2020; **7**:2333794X20901930.
4. *Goto H.* Childhood relapsed acute lymphoblastic leukemia: Biology and recent treatment progress. *Pediatr Int.* 2015; **57**(6):1059–66.
5. *Yokota T, Kanakura Y.* Genetic abnormalities associated with acute lymphoblastic leukemia. *Cancer Sci.* 2016; **107**(6):721–5.
6. *DeAngelo DJ, Jabbour E, Advani A.* Recent advances in managing acute lymphoblastic leukemia. *Am Soc Clin Oncol Educ Book.* 2020; **40**:330–42.
7. *Britten O, Ragusa D, Tosi S, Kamel YM.* MLL-rearranged acute leukemia with t(4;11)(q21;q23)-

current treatment options. *Is there a role for CAR-T cell therapy? Cells*. 2019; **8**(11):1341.
8. *Pi WC, Wang J, Shimada M, Lin JW, Geng H, Lee YL, Lu R, Li D, Wang GG, Roeder RG, Chen WY.* E2A-PBX1 functions as a coactivator for RUNX1 in acute lymphoblastic leukemia. *Blood*. 2020; **136**(1):11–23.
9. *Ajuba IC, Madu AJ, Okocha C, Ibegbulam OG, Okpala I, Nna OE.* Frequency and clinical impact of ETV6/RUNX1, AF4-MLL, and BCR/ABL fusion genes on features of acute lymphoblastic leukemia at presentation. *Niger J Clin Pract.* 2016; **19**(2):237–41.
10. *Lilleyman JS, Hann IM, Stevens RF, Eden OB, Richards SM.* French American British (FAB) morphological classification of childhood lymphoblastic leukaemia and its clinical importance. *J Clin Pathol.* 1986; **39**(9): 998–1002.
11. *Koh CM.* Isolation of genomic DNA from mammalian cells. *Methods Enzymol.* 2013; **529**:161–9.
12. *Livak K.J., Schmittgen T.D.* Analysis of relative gene expression data using realtime quantitative PCR and the 22DDCT method. Methods. 2001; **25**: 402–8.
13. *Takahashi S, Yokoyama A.* The molecular functions of common and atypical MLL fusion protein complexes. *Biochim Biophys Acta Gene Regul Mech.* 2020; **1863**(7):194548.
14. *Tasian SK, Loh ML, Hunger SP.* Philadelphia chromosome-like acute lymphoblastic leukemia. *Blood.* 2017; **130**(19):2064–72.
15. *Vinhas R, Lourenço A, Santos S, Lemos M, Ribeiro P, de Sousa AB, Baptista PV, Fernandes AR.* A novel BCR-ABL1 mutation in a patient with Philadelphia chromosome-positive B-cell acute lymphoblastic leukemia. *Onco Targets Ther.* 2018; **11**:8589–98.
16. *Cuellar S, Vozniak M, Rhodes J, Forcello N, Olszta D.* BCR-ABL1 tyrosine kinase inhibitors for the treatment of chronic myeloid leukemia. *J Oncol Pharm Pract.* 2018; **24**(6):433–52.
17. *Hong Y, Zhao X, Qin Y, Zhou S, Chang Y, Wang Y, Zhang X, Xu L, Huang X.* The prognostic role of E2A-PBX1 expression detected by real-time quantitative reverse transcriptase polymerase chain reaction (RQ-PCR) in B cell acute lymphoblastic leukemia after allogeneic hematopoietic stem cell transplantation. *Ann Hematol.* 2018; **97**(9):1547–54.
18. *Ketsa OV, Marchenko MM.* Free radical oxidation in liver mitochondria of tumor-bearing rats and its correction by essential lipophilic nutrients. *Ukr Biochem J.* 2020; **92**(1):127–34.
19. *Kantner HP, Warsch W, Delogu A, Bauer E, Esterbauer H, Casanova E, Sexl V, Stoiber D.* ETV6/RUNX1 induces reactive oxygen species and drives the accumulation of DNA damage in B cells. *Neoplasia.* 2013; **15**(11):1292–300.
20. *Rose-James A, Shiji R, Kusumakumary P, Nair M, Suraj KG, Sreelekha TT.* Profiling gene mutations, translocations, and multidrug resistance in pediatric acute lymphoblastic leukemia: a step forward to personalizing medicine. *Med Oncol.* 2016; **33**(9):98.

Експресія химерних генів у клітинах кісткового мозку дітей з гострою лімфобластною лейкемією за умов застосування цитостатичної терапії

О. А. Винницька, Л. Я. Дубей

Мета. Дослідити експресію химерних генів у тканинах кісткового мозку як прогностичних факторів перебігу гострої лімфобластної лейкемії (ГЛЛ) у дітей та оцінити їх роль в резистентності пухлин до цитостатичної хіміотерапії. **Методи.** Рівень експресії химерних генів *AF4-MLL, BCR-ABL, E2A-PBX, TEL-AML1* визначали методом полімеразної ланцюгової реакції з використанням комплементарної ДНК, отриманої реакцією зворотної транскрипції з РНК за допомогою AMV транскриптази. **Результати.** Встановлено, що під час ремісії ГЛЛ у дітей не експресувався онкоген *AF4-MLL*, а експресія всіх інших досліджуваних онкогенів знаходилася на низькому рівні. За умов рецидиву захворювання відсутня експресія онкогена *TEL-AML* та виявлена висока експресія химерного гена *BCR-ABL*. **Висновки.** Розвиток, перебіг та виживання пацієнтів з ГЛЛ значною мірою залежать від експресії химерних генів *AF4-MLL, BCR-ABL, E2A-PBX1, TEL-AML* у кістковому мозку, що необхідно враховувати для виявлення ранніх рецидивів у пацієнтів після проведення курсу хіміотерапії.

Ключові слова: гостра лімфобластна лейкемія, онкоген, *AF4-MLL*, *BCR-ABL*, *E2A-PBX*, *TEL-AML*.

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