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DNA DOUBLE-STRANDED BREAKS AND REPAIRS IN ACUTE LEUKEMIA

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ABSTRACT

Relevance: *Errors in the damage repair system, such as double-stranded DNA breaks, can lead to mutations that will be passed on to subsequent generations of cells, and some of these mutations may have oncogenic potential.*

The study aimed to *evaluate the number of double-stranded breaks and DNA repairs of peripheral blood lymphocytes in a group of conditionally healthy children and in patients diagnosed with acute leukemia (AL) to develop a method for predicting the outcome of the disease and determining the effectiveness of therapy.*

Methods: *Peripheral blood lymphocytes were studied: a) 38 conditionally healthy children (control group); b) 100 patients diagnosed with acute leukemia (AL); c) 14 children with relapse of the disease. We examine double-stranded DNA breaks/repairs using the Aklides system (MEDIPAN, Germany), consisting of a fluorescent analyzer and the AKLIDES Nuk software.*

Results: *In patients with T-lymphoblastic leukemia, both at admission and the end of Day 7 at the hospital, the number of 53BPI repair foci was, on average, three times higher than the number of DNA damages. In most cases, the ratio of breaks/repairs indicators during treatment did not change among patients with B-line leukemia. Double-stranded DNA breaks prevailed over repairs, with the newly established disease on the 7th, 15th Day, and 3rd month of treatment.*

Conclusion: *The level of lymphocyte DNA damage in patients with B-ALL was higher than expected. In addition, the ratio of double-strand breaks to repairs remained unchanged at all stages of therapy in patients with B-ALL. The changes we suggest in these patients can be observed during and/or after maintenance therapy. Monitoring double-strand breaks/reparations was the initial step in developing a method of predicting the disease outcome and determining the therapy efficacy. The results obtained are of direct interest and require further research.*

Keywords: *double-stranded breaks, DNA repair, acute leukemia, lymphocytes, immunofluorescence.*

Introduction: Among the main types of deoxyribonucleic acid (DNA) damage, double-stranded breaks (DSB) are the most severe form. The double-stranded breaks (DSB) are formed either due to a direct rupture of two complementary sites - so-called "direct" DSB, or formed from other lesions, from single-stranded breaks, as a result of repair disorders during the work of relevant repair enzymes.

Reparation is the body's response to DNA damage. Two following mechanisms can restore the double-stranded breaks:

a) Non-homologous reunion of the DNA ends, in which the damaged chain ends are connected directly;

b) Homologous recombination, in the presence of the DNA fragment identically intact in the nucleotide sequence [1].

However, DNA repair may not be fully effective, and in addition, in some cases, DNA damage repair leads to errors and, as a result, to the occurrence of mutations.

The p53 transcription factor, also known as the "genome guardian," plays a crucial role in ensuring the stability of the genetic apparatus [2]. The p53 protein marks the sites of double-stranded breaks and activates the transcription of genes responsible for repair mechanisms. In turn, to start the DNA repair, the p53 protein binds with the phosphorylated form of H2AX histone (denoted as – γH2AX). A separate γH2AX focus represents each double-stranded break. The presence of standard physiological repair mechanisms leads to successful repair and a decrease in the γH2AX foci [3, 4].

The large amounts of double-stranded breaks and other types of DNA damage initiate an apoptosis program. However, programmed cell death is an exceptional case of maintaining homeostasis (the balance between newly formed and dying cells) since the triggering of apoptosis is possible only if the DNA damage in the nucleus is irreversible and cannot be corrected by the repair system [5].

Thus, the main pathway of the DNA repair system can be presented as the DNA damage → p53 expression → DNA repair/apoptosis (in a condition that threatens the cell life) [6]. The quantitative determination of the DNA breaks and repairs are carried out using flow cytometry, confocal microscopy, and indirect immunofluorescence analysis (IIF). We used the method of indirect immunofluorescence in the current study. The IIF method is based on the detection of a) the H2AX protein, which is formed when double-stranded DNA breaks appear in phosphorylated form; b) the p53-binding protein 1, also known as 53BP1, involved in signal transmission to repair the DNA double-stranded break.

The study aimed to evaluate the number of double-stranded breaks and DNA repairs of peripheral blood lymphocytes in a group of conditionally healthy children

and in patients diagnosed with acute leukemia (AL) to develop a method for predicting the outcome of the disease and determining the effectiveness of therapy.

Materials and methods: The peripheral blood lymphocytes were the material for the study. The following groups of children have been studied:

Group 1 (n=38, median age – 9.7 years) – control (conditionally healthy children);

Group 2 (n=100, median age – 8 years) – patients with AL: acute lymphoblastic leukemia (ALL) = 82, acute myeloid leukemia (AML) = 17, AL of mixed linearity = 1.

Group 3 – (n=14, median age 9.7 years) – children with AL relapse.

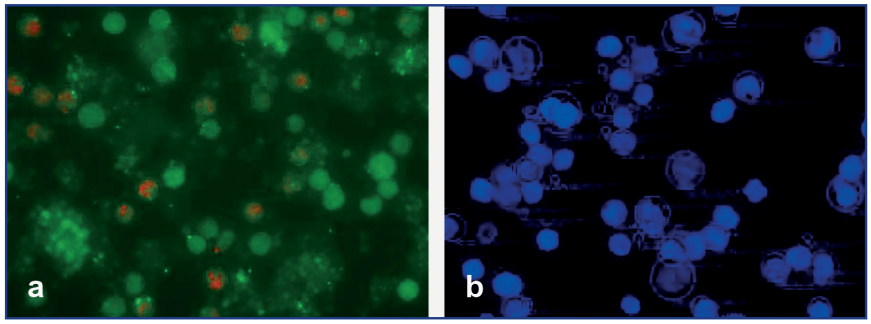
Inclusion criteria: children aged 0 to 18 with primary AL and recurrent AL who were diagnosed and treated at the Scientific Center for Pediatrics and Pediatric Surgery (Almaty, Kazakhstan).

Exclusion criteria: persons over 18 years old; children with severe cytopenia.

The data collection period was from January 2022 to March 2023. The inpatient stay was eight months with ALL and 4-5 months with AML.

The study of double-stranded breaks/DNA repairs was carried out with the application of the Aklides system (MEDIPAN, Germany), consisting of a fluorescence analyzer and AKLIDES Nuk software. The quantitative determination of the double-stranded DNA breaks and repair of lymphocytes in patients with acute leukemia was performed for the first time in Kazakhstan. The commercial AKLIDES Nuk Human Lymphocyte Complete Combi kits were used within the study frames. The method is based on binding specific antibodies with the γH2AX protein component. The second stage of the analysis was to bind the 53BPI-specific antibodies with the initially formed complex. A fluorescence signal displayed the focus of rupture and repair. A green glow in the FITC channel was observed at the rupture focus, and a red glow in the APC channel was observed at the repair center (Figure 1a). The DAPI channel based on autofocus and counterstaining was used to detect the cell nuclei (Figure 1b). The Aklides system has selected cells of the same morphology and typical rounded shape. The calculation of double-stranded breaks/repairs was carried out per 100 lymphocytes. The final result was displayed as reports (Single Report). As stated, the Aklides digital system has been fully standardized and supported the analysis of objects of only specific shapes and sizes. However, the tumor cells are morphologically different when compared with normal cells. In this regard, it is advisable to introduce changes to the sample parameters for subsequent studies of leukemic cells.

The statistical analysis was performed using SPSS Statistics software, version 23.0, for Windows.



a) Viewing the image in simultaneous FITC and APC modes
b) Automatic focus on cells in the DAPI channel
Figure 1 – An example of indirect immunofluorescence assay for the quantitative determination of phosphorylated γH2AX and 5ZBP1 protein in lymphocytes

Results: Table 1 presents the quantification of double-stranded breaks and DNA repair in the control group. The children in the control group's age corresponded to the studied patients' age.

Based on data from Table 1, in the group of children aged 0 to 5 years old, the number of cells with breaks prevailed over the number of cells with repairs, while the total number of repairs was detected. The total number of ruptures and reparations was approximately equal in the age group of 5-10 years old. More double-stranded breaks than repairs were found for the third group of children over ten years old. The elevation of DNA damage with age may be due to the influence of external environmental factors and various biochemical processes of the body.

Table 1 – Indicators of the ratios of breaks/repairs of lymphocyte DNA (LP) in patients of the control group (arithmetic mean M±m)

Age, years	Cells with ruptures and repairs of LP (FITC/APC)	Total number of ruptures and reparations of LP (FITC/APC)	Number of ruptures and reparations per 1 cell
0-5 years old	43±5/38±4*	122±6/131±8*	1/1
5-10 years old	51±4/35±6 (p≤0,05)	109±10/104±9*	1/1
Over 10 years old	48±5/34±6*	114±11/96±8*	1/1

*Note: * - the result is unreliable*

Diagram 1 shows the distribution of the number of patients depending on the AL immunological variant.

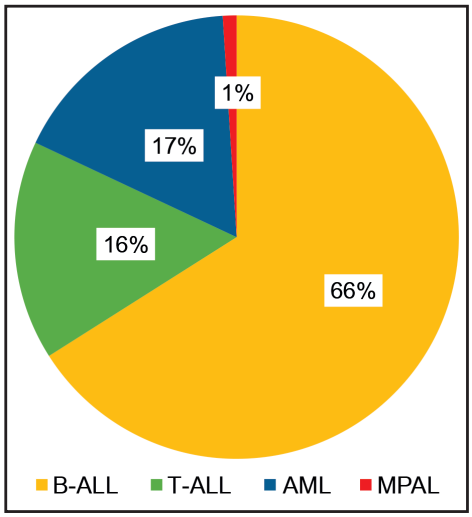


Diagram 1 – Number of patients by AL variants

The distribution of AL variants was as follows: "Acute B-lymphoblastic leukemia" (B-ALL) was identified in 66 patients, comprising 66% of the total number of patients. Among patients with B-ALL, the pro-B1 variant was detected in 9 children (14%), B2-38 (58%), B3-16 (24%), and B4-3 (4%). The diagnosis of *"acute myeloid leukemia"* was established in 17 patients (17%), including 11 (65%) - with AML M1-M2, 3 (18%) - M3, 1 (6%) - M4-M5 and 2 (11%) - M7. Besides, 16 people (16%) were admitted with acute T-lymphoblastic leukemia (T-ALL), ten children (62%) with the T3-cortical immunological variant, and 6 (38%) with T-non-cortical leukemia. Besides, one case of mixed linearity AL (B+myelo) classified as mixed-phenotype acute leukemia (MPAL) has been revealed.

The distribution of patients by age and linear identity of primary AL is presented in Table 2.

According to Table 2, B-ALL was more often detected in children aged 5-10 years old, 45% (n=30). Among children over 10 years old, AML was diagnosed in 42% (n=7).

Table 2 – Distribution of patients with AL by age and immunological variant of the disease

AL variant	Number of patients, abs., (%)		
	0-5 years old	5-10 years old	Over 10 years old
Acute lymphoblastic leukemia	19 (29%)	30 (45%)	17 (26%)
T-acute lymphoblastic leukemia	4 (25%)	7 (44%)	5 (31%)
Acute myeloid leukemia	5 (29%)	5 (29%)	7 (42%)
Acute leukemia of mixed phenotype	1 (100%)	-	-

The number of breaks and DNA repairs of peripheral blood lymphocytes among patients with newly diagnosed AL is presented in Table 3.

Table 3 – Indicators of double-stranded breaks/repairs in patients with primary AL (arithmetic mean M±m)

AL variant	Cells with ruptures and repairs (FITC/APC)	Total number of ruptures and reparations of LP (FITC/APC)	Number of ruptures and reparations per 1 cell
ALL B2	41±3/31±4 (p≤0,05)	95±8/109±7*	1/1
ALL B1	73±5/38±3 (p≤0,01)	186±9/209±8 (p≤0,05)	2/2
ALL B3	29±4/22±5*	52±5/38±4* (p≤0,05)	1/1
ALL B4	20±3/17±3*	59±4/14±3 (p≤0,01)	1/1
ALL T3-cortical	24±4/41±5 (p≤0,05)	53±5/143±9 (p≤0,01)	1/1
ALL T-non-cortical	25±2/44±4 (p≤0,01)	55±7/194±11 (p≤0,01)	1/2
AML M1-M2	34±5/24±4*	62±5/60±7*	1/1
AML M3	60±5/52±4*	153±6/117±8 (p≤0,01)	1/1
AML M4-M5	67±6/2±1 (p≤0,001)	165±10/2±1 (p≤0,001)	2/1
AML M7	34±5/38±3*	76±4/91±6 (p≤0,05)	1/1
MPAL (B+myelo)	84±7/12±3 (p≤0,01)	239±11/42±7 (p≤0,001)	2/1

*Note: * - the result is unreliable*

According to Table 3, in the patient with biphenotypic acute leukemia, the repair has declined by 5.7 times. The prognosis for biphenotypic leukemia is worse than acute lymphoblastic or myeloid leukemia. However, the patient tolerated the polychemotherapy relatively well, and following the therapy, the child's condition stabilized. The average number of ruptures in cortical (n=53) and non-cortical (n=55) variants is approximately equal for the group of patients with T-ALL. At the same time, the number of repairs in patients with T3-cortical ALL (n=143) and T-non-cortical ALL (n=194) was significantly more significant than the number of ruptures themselves. Among patients with AML in M1-M2, the minimum damage has been detected – 62 ruptures, and the maximum in M4-M5 – 165 per 100 lymphocytes. For the latter, it is worth highlighting that the reparation has been reduced by 82 times. For the group of patients with AML, the average number of reparations in M1-M2 composed 60; in M3, it made a total of 117. Among AML, only in M7 were more reparations (n=91) than ruptures (n=76). The prognosis for AML M7 is highly unfavorable.

In patients with B-linear AL, the average number of double-stranded breaks in pro-B1 was the maximum of -186, the minimum in pre-B3 - 52. The average number of cell repair foci in patients with B-ALL has been distributed in B1 – 209, B2 – 109, and B3 – 38. The level of repairs for B4-ALL was the lowest compared to other B-ALL variants. At the same time, the clinically mature cell B4-ALL was characterized by a high proliferative index, which in turn was reflected in a poor outcome.

The number of breaks and DNA repairs of peripheral blood lymphocytes among patients with recurrent AL is presented in Table 4. In the "relapses" group, three patients had a recurrence of AL less than a year after the start of treatment.

Table 4 – Indicators of double-stranded breaks/reparations in patients with recurrence of AL (arithmetic mean M±m)

AL variant	Number of cells with ruptures and repairs (FITC/APC)	Total number of ruptures and reparations of LP (FITC/APC)	Number of ruptures and reparations per 1 cell
Recurrence of ALL B2	36±5/20±5 (p≤0,05)	74±6/60±3 (p≤0,05	1/1
Recurrence of ALL B4-mature cell type	45±7/0 (p≤0,001)	45±4/0 (p≤0,001)	1/0
Relapse of AML M1-M2	29±4/20±3*	50±5/30±3 (p≤0,01)	1/1

*Note: * - the result is unreliable*

As seen from Table 4, among patients with recurrence of the disease, regardless of the variant of AL, the double-stranded breaks prevailed over repairs. Most patients who relapsed the disease were with B2 standard (N = 11). Recurrence of M1-M2 AML was detected in two children, and B4-mature cell ALL was revealed in one child. The repair was utterly absent in B4-ALL, and despite the therapy, the patient died.

The results of counting the number of breaks and DNA repairs of peripheral blood lymphocytes during treatment are presented in Table 5. In patients with ALL, by the treatment protocol at the induction stage, the first seven days included taking glucocorticoid prednisolone. At the same time, for AML, the course introduced the cytotoxic drugs group. The following points for calculating the breaks/repairs were Day 15 and 3rd Month.

In some patients, starting from Day 15th, severe cytopenia was noted due to chemotherapy. In blood samples with a leukocyte concentration of less than 2×10^9/L, the Aklides system did not count the breaks/repairs due to a considerable distance between the cells recorded on the carrier's slot. In addition, the morphological changes in cells, shape, and size have been revealed against the background of taking cytotoxic drugs. It could stem from the developed tumor lysis syndrome, primarily observed in children with hyperleukocytosis. We assume that when cells were isolated from peripheral blood in a specific density gradient, the blasts could be subsequently analyzed in addition to lymphocytes.

According to Table 5, among patients with B-lymphoblastic leukemia, in most cases, the ratio of breaks/repairs rates did not change during treatment. The double-stranded DNA breaks prevailed over repairs at the newly detected AL and on Day 7, 15th Day, and 3rd month of treatment. According to the observations of specialists [7], the B-cell ALL is considered a more favorable option than T-ALL in terms of prognosis. A curious fact is that a positive response to chemotherapy accompanied the reduced repair of lymphocytes in patients with B-ALL. No changes have been registered in patients with T-ALL on Day 7 of prophase. Also, more repairs (n=7) have been detected than ruptures (n=4). However, on the 15th Day of polychemotherapy (n=6) and the 3rd Month (n=3), more patients with ruptures were recorded than with repairs, n=3 and n=2, respectively. Among patients with AML, on Day 7 of therapy - the double-stranded breaks (n=8) prevailed, while on the 15th Day (n=5) and on the 3rd month – the repairs were increased (n=4).

Table 5 – Dynamics of double-stranded breaks/repair ratios during therapy

Day of therapy	Number of patients (n)		
	AL variant	b>r*	r>b*
Day 7	B-ALL	22	15
	T-ALL	4	7
	AML	8	4
Day 15	B-ALL	18	12
	T-ALL	6	3
	AML	4	5
3rd month	B-ALL	22	13
	T-ALL	3	2
	AML	2	4

Note: b – double-stranded breaks; r – repair

Discussion: For many cases of leukemia, lymphoma, or sarcoma, the first events of carcinogenesis are most often the translocations that activate or form the oncogene [8]. Unrepaired damage increases due to inherited mutations in DNA repair genes in replicating somatic cells [9]. The patient with M4-M5 AML had 169 ruptures and two repairs. FISH study revealed a rearrangement of the mixed-lineage leukemia (MLL) gene. These results coincide with published data. The lymphocyte repair in patients with T-ALL of pro-T1, pre-T2, and mature-T4 variants was 1.3 times higher than in patients with cortical T3-ALL. The doctors reported no significant clinical differences; however, patients with T-noncortical ALL variant initially had a worse response to chemotherapy than patients with T-cortical ALL.

Overall, patients with T-lymphoblastic leukemia had fewer double-stranded breaks. At the same time, in almost all patients with this immunological AL variant, both at the time of admission and at the end of Day 7 of prophase, the number of repair foci was, on average, three times higher than the DNA damage. Thus, prednisolone did not play a significant role in changing the nature of ratios of the double-stranded breaks and DNA repair. The obtained results corresponded with clinical signs of the relevant AL variant. By the end of Day 7 of prophase, in most cases, no regression of hyperplastic syndrome was noted, and a high number of blasts in the complete blood count remained. At the same time, cytotoxic drugs increased double-stranded DNA damage and improved the disease's clinical picture. The results of the reparative ability of lymphocytes in patients with T-ALL are debatable. High repair rates could be one of the reasons for poor response to chemotherapy. The repair shall correct the DNA damage, reducing the likelihood of mutations and, therefore, the emergence of the tumor substrate [10, 11]. The results are consistent with the data published by Trenner A. et al., who consider the repair mechanism one of the main resistance factors to chemotherapy [12].

Only a few studies consider double-stranded breaks and DNA repair in patients with acute leukemia. In the available literature, the double-stranded DNA damage and repair were investigated under "in vitro" conditions [3, 13]. In our study, we monitored ruptures/repairs in patients with AL before and after exposure to several anticancer drugs.

Conclusion: The level of lymphocyte DNA damage in patients with B-ALL was higher than expected. In addition, the ratio of double-strand breaks to repairs remained unchanged at all stages of therapy in patients with B-ALL. The changes we suggest in these patients can be observed during and/or after maintenance therapy. Monitoring double-strand breaks/reparations was the initial step in developing a method of predicting the disease outcome and determining the therapy efficacy. The results obtained are of direct interest and require further research.

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АНДАТПА

ЖЕДЕЛ ЛЕЙКОЗ КЕЗІНДЕГІ ДНҚ ҚОСТІЗБЕКТІ ҮЗІЛІСТЕРІ МЕН РЕПАРАЦИЯСЫ

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Өзектілігі: *ДНҚ-ның қос тізбекті үзілістері сияқты зақымдануды қалпына келтіру жүйесіндегі қателер жасушалардың кейінгі ұрпақтарына берілетін мутацияларға әкелуі мүмкін және мұндай мутациялардың кейбіреулері онкогендік потенциалға ие болуы мүмкін.*

Зерттеудің мақсаты *– шартты түрде сау балалар тобындағы және "жедел лейкоз" (ЖЛ) диагнозы қойылған пациенттердің перифериялық қан лимфоциттерінің қос тізбекті үзілістері және ДНҚ репарациясының санын салыстырмалы талдау және оны аурудың нәтижесін болжау мен емнің тиімділігін анықтау әдісін әзірлеу үшін қолдану.*

Әдістері: *тікелей емес иммунофлуоресцентті талдау әдісі флуоресцентті анализатор мен Aklides nuk бағдарламалық қамтамасыз етуден тұратын Aklides (Medipan, Германия) жүйесінде өткізілді. Әдіс арқылы перифериялық қан лимфоциттері зерт-*

телді: а)шартты түрде сау 38 бала (бақылау тобы); б) жедел лейкоз (ЖЛ) диагнозы қойылған 100 науқас; в) аурудың қайталануы бар 14 науқас.

Нәтижелері: *Т-лимфобластикалық лейкозбен ауыратын науқастарда емнің басында және ауруханаға жатқызудың 7-ші күні 53BP1 репарация ошақтарының саны ДНҚ зақымдану санынан орта есеппен 3 есе жоғары болды. В-бағытты лейкозбен ауыратын науқастар арасында көп жағдайда емдеу кезінде ДНҚ қостізбекті үзілісі/репарациясы көрсеткіштерінің арақатынасы өзгерген жоқ. ДНҚ-ның қостізбекті үзілістері ауру алғаш анықталған сәтте, емдеудің 7-ші, 15-ші күні, 3-ші айында да репарациядан басым болды.*

Қорытынды: *B-ALL бар емделушілерде лимфоциттердің ДНҚ зақымдану деңгейі күтілгеннен жоғары болды. Сонымен қатар, екі тізбекті үзілістердің жөндеуге қатынасы B-ALL бар емделушілерде терапияның барлық кезеңдерінде өзгеріссіз қалды. Бұл емделушілерде біз ұсынып отырған өзгерістер демеуші терапия сатысында және/немесе оны аяқтағаннан кейін байқалуы мүмкін. Екі қатарлы үзілістердің/репарациялардың мониторингі аурудың нәтижесін болжау және терапияның тиімділігін анықтау әдісін әзірлеудің бастапқы қадамы болды. Алынған нәтижелер тікелей қызығушылық тудырады және қосымша зерттеулерді қажет етеді.*

Түйінді сөздер: *ДНҚ қостізбекті үзілістері, ДНҚ репарациясы, жедел лейкоз, лимфоциттер, иммунофлуоресценция.*

АННОТАЦИЯ

ДВУЦЕПОЧЕЧНЫЕ РАЗРЫВЫ И РЕПАРАЦИИ ДНК ПРИ ОСТРЫХ ЛЕЙКОЗАХ

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Актуальность: *Ошибки в системе репарации повреждений, таких как двуцепочечные разрывы ДНК, могут привести к возникновению мутаций, которые передадутся последующим поколениям клеток. Некоторые из таких мутаций могут об-ладать онкогенным потенциалом.*

Цель исследования *– анализ количества двуцепочечных разрывов и репараций ДНК лимфоцитов периферической крови в группе условно здоровых детей и у пациентов с диагнозом «острый лейкоз» (ОЛ) для оценки степени повреждения ДНК.*

Методы: *Лимфоциты периферической крови были взяты у: а) 38 условно здоровых детей (контрольная группа); б)100 пациентов с диагнозом ОЛ; в) 14 детей с рецидивом заболевания были исследованы методом непрямого иммунофлуорес-центного анализа (НИФ) и исследованы с помощью системы Aklides (MEDIPAN, Германия), состоящей из флюоресцентно-го анализатора и программного обеспечения AKLIDES Nuk.*

Результаты: *У пациентов с Т-лимфобластным лейкозом как на момент поступления, так и на конец 7-го дня госпита-лизации количество репарационных очагов 53BP1 в среднем в 3 раза превышало количество повреждений ДНК. Среди паци-ентов с В-линейными лейкозами в большинстве случаев отношения показателей разрывов/репараций в ходе лечения не из-менились. Двуцепочечные разрывы ДНК преобладали над репарациями, как при впервые установленном заболевании, так и на 7-й, 15-й день, 3-й месяц лечения.*

Заключение: *Уровень повреждений ДНК лимфоцитов у пациентов с В-ОЛЛ оказался выше ожидаемого. Кроме того, на всех этапах терапии больных В-ОЛЛ отношение двуцепочечных разрывов к репарациям сохранилось. Предполагаемые нами изменения у данных пациентов могут наблюдаться на этапе поддерживающей терапии и/или после ее окончания. Монито-ринг двуцепочечных разрывов/репараций являлся первоначальным этапом для разработки метода прогнозирования исхода заболевания и определения эффективности терапии. Полученные результаты вызывают непосредственный интерес, и требует дальнейших исследований.*

Ключевые слова: *двуцепочечные разрывы, репарация ДНК, острый лейкоз (ОЛ), лимфоциты, иммунофлюоресценция.*

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