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QUANTIFICATION OF DNA DOUBLE-STRAND BREAKS IN BENIGN AND MALIGNANT BREAST DISEASES

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ABSTRACT

Relevance: *Double-strand DNA breaks are the most dangerous DNA damage. Analysis of foci of phosphorylated histone protein H2AX (γH2AX) is currently the most sensitive method for detecting DNA double-strand breaks. This protein modification can become a biomarker of cellular stress, especially in diagnosing and monitoring neoplastic diseases. In this study, we used novel pattern recognition algorithms on the AKLIDES® platform to automatically analyze immunofluorescent images of γH2AX foci and compare the results with visual scores. The γH2AX foci formation on peripheral blood mononuclear cells of women with breast cancer or benign breast tumors was studied.*

The study aimed to *quantify DNA double-strand breaks in peripheral blood lymphocytes in women with breast cancer and benign breast masses to identify a possible biomarker.*

Methods: *γ-H2AX foci in lymphocytes were analyzed using the automated AKLIDES system in patients with breast cancer (n=29) and benign breast tumors (n=24).*

Results: *When comparing the parameters of the main and control groups in the channel of ruptures "FITC," a statistically significant difference was found in the parameters "Foci diameter" (p=0.0382), "Foci intensity means" (p=0.0166), "Colocalisation" (p=0.0486). In the repair channel "APC," significant differences were found in the parameters "Nuclei intensity" (p=0.0166) and "Foci intensity means" (p=0.0118).*

Conclusion: *The revealed changes of DNA double-strand breaks along the FITC break channels and APC repair between the main and control groups can possibly serve as a breast cancer diagnostic marker.*

Keywords: *DNA double-strand breaks, H2AX histone protein, breast cancer.*

Introduction: Breast cancer is the most commonly diagnosed invasive cancer among women worldwide and the number one cause of female cancer deaths [1].

H2AX histone undergoes phosphorylation in response to DNA double-strand breaks (DSBs), which are part of the oncogenic process. Eukaryotic cells have developed a set of complex signaling networks that detect these DNA damages, organize cell cycle checkpoints, and eventually lead to their repair to prevent the catastrophic consequences of persistent DNA double-strand breaks. Together, these signaling networks constitute a response to DNA damage [2, 3]. Double-strand breaks are one of the first procedures that occur during the formation and progression of cancer due to endogenous and exogenous factors. The H2AX histone variant is phosphorylated on serine 139 due to double-strand breaks, while gamma-H2AX is formatted due to genome instability [4, 5]. There are two main DSB repair pathways, namely non-homologous end joining and homologous recombination; the pathway choice is partially controlled by post-translational histone modifications, including ubiquitination [6, 7]. Thus, activated components of the DNA damage and repair pathway can be used as cancer biomarkers, with H2AX being the most sensitive. Thus, measurements of H2AX levels can help detect precancerous lesions or cancer at an early stage [8-10]. Immunofluorescent staining with anti-γH2AX antibody provides visualization of these nuclear foci that have been found and correlates with the amount of DSB [11, 12].

Fluorescent microscopy allows for a rapid and standardized γH2AX assay and a quick assessment of DNA damage in clinical practice. The platform, called AKLIDES (Medipan, Germany), allows not only fully automated screening evaluation of antinuclear immunofluorescent antibodies [13] but also conducts a computational analysis of γ-H2AX foci, which has now been successfully validated by several independent study groups [14-18].

The study aimed to quantify DNA double-strand breaks in peripheral blood lymphocytes in women with breast cancer and benign breast masses to identify a possible biomarker.

Materials and Methods: This prospective cohort study involved two groups of female patients: the main group of 29 patients with primary verified breast cancer and 24 controls with a histologically verified benign breast tumor. The study was performed at the Medical Center and Scientific-and-Practical Center of West Kazakhstan Marat Ospanov Medical University (Aktobe, the Republic of Kazakhstan). Each participant submitted written informed consent. The project was approved by

the local Commission on Bioethics (Minutes No. 57 of Jan. 17, 2020).

In the main group (29 patients with breast cancer), the average age was 56.10±12.23 years. By disease stage, 25 (86.2%) had stage II disease, and four (13.8%) had stage III. By tumor immune histochemistry, 3 (10.3%) had Luminal type A, 21 (72.4%) had Luminal type B, four (13.8%) had a triple-negative tumor, and one (3.4%) patient had a HER+ cancer.

In the controls, 24 patients had a verified «Mammary Gland Benign Neoplasia» (BI-RADS M2); their average age was 43.08 ± 10.12 years.

The study object was peripheral venous blood in a volume of 10 ml (EDTA tube) containing mononuclear cells. γ-H2AX foci in lymphocytes were analyzed using the γ-H2AX immunofluorescent staining kit (AKLIDES Nuk Human Lymphocyte Complete, Medipan) following the manufacturer's instructions.

The AKLIDES system is based on a motorized inverse fluorescence microscope combined with various hardware and software modules to fully automate image acquisition, analysis, and evaluation. In each sample, we analyzed 80-100 cells at least. DNA double-strand breaks in γ-H2AX were assessed by 12 parameters in the AKLIDES automated system tear channel (FITC) and the repair channel (ARC):

1. Foci diameter;
2. Nuclei intensity;
3. Nuclei with foci;
4. Foci overall;
5. Foci intensity means%
6. Clusters;
7. Foci mean;
8. Foci mean + clusters;
9. Clusters positive cells;
10. Clusters of low intensity;
11. Damaged cells;
12. Colocalization.

Statistical analysis included comparisons of two groups on numerical variables using the nonparametric Mann-Whitney method. The statistical significance of group differences for binary and categorical parameters was determined using Pearson's Chi-square method.

The statistical significance level was fixed at 0.05. Statistical data was processed using the Statistica 10 and SAS JMP 11 application packages.

Results: Analysis of the results of the AKLIDES automated system showed that the average number of cells counted in the main group of patients with breast cancer (113) and in the control group (108) corresponded to the minimum number (100) of cells required for the study.

Tables 1-2 present the results of the analysis of foci of γ-H2AX in the control and main groups (stage 1) according to the rupture channel (FITC) and the repair channel (APC).

Table 1 - Comparison of parameters in the main (breast cancer) and control groups according to the FITC gap channel (Average value ± standard deviation)

Parameter	Group		p-value
	Breast cancer (n=29)	Control (n=24)	
Foci diameter	7.34±0.68	7.00±0.52	0.0382
Nuclei intensity	35.95±10.84	39.13±10.25	0.1921
Nuclei with foci	55.66±37.00	58.25±31.50	0.5918
Foci overall	167.17±219.72	141.67±119.92	0.7342
Foci intensity means	69.88±17.97	81.83±19.28	0.0166
Clusters	0.24±0.79	0.12±0.45	0.5477
Foci mean	1.45±1.58	1.29±1.10	0.9005
Foci mean + clusters	1.46±1.59	1.30±1.10	0.8863
Clusters positive cells	51.13±29.87	53.22±27.89	0.8025
Clusters of low intensity	2.45±1.81	2.10±1.40	0.5554
Damaged cells	72.46±27.92	74.27±23.13	0.8442
Colocalization	18.62±19.12	9.71±13.69	0.0486

It was found that three parameters differed statistically significantly between the two compared groups in the FITC discontinuity channel. A statistical difference was found for the "Foci diameter" parameter, which in the main group was higher than the control group parameter (p=0.0382), and for the "Foci intensity means," this parameter was lower in the main group than in the control group (p=0.0166). The parameter "Colocalization" in the main group was higher than in the control group (p=0.0486) (Figures 1-3).

Two statistically significant parameters were identified when comparing the parameters in the main and control groups on the APC repair channel. Thus, significant differences were found for the parameter "Nuclei intensity," which in the main group was lower than in the control group (p=0.0166), and the parameter "Foci intensity means" in the main group was lower than in the control group (p=0.0118) (Figures 4-5).

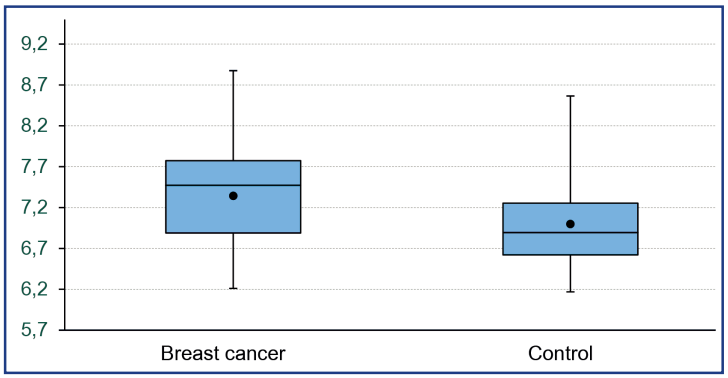

Figure 1 - Comparison of the parameter "Foci diameter" in the main and control groups

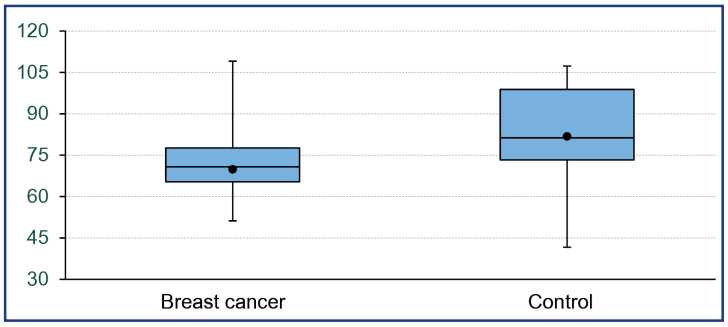

Figure 2 - Comparison of the parameter "Foci intensity means" in the main and control groups

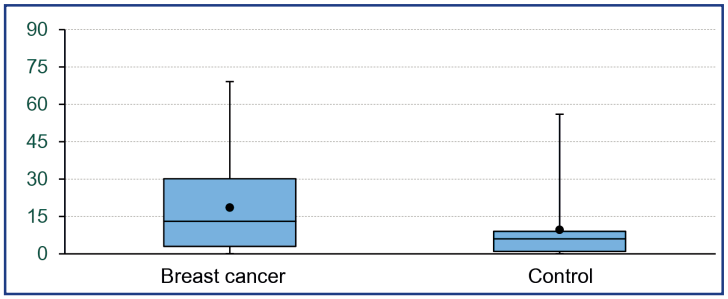

Figure 3 - Comparison of the parameter "Colocalization" in the main and control groups

Table 2 - Comparison of parameters in the main (breast cancer) and control groups on the APC repair channel (Average value ± standard deviation)

Parameter	Group		p-value
	Breast cancer (n=29)	Control (n=24)	
Nuclei intensity	455.23±286.58	738.93±512.67	0.0166
Nuclei with foci	76.79±25.57	76.04±20.51	0.7749
Foci overall	288.10±251.77	217.88±141.48	0.5494
Foci diameter	0.56±0.05	0.54±0.03	0.0830
Foci intensity means	302.94±81.62	369.91±109.65	0.0118
Clusters	49.34±80.94	46.50±100.59	0.9712
Foci mean	2.81±2.96	2.00±1.28	0.7750
Foci mean + clusters	8.05±13.26	6.68±11.43	0.9715
Clusters positive cells	70.38±23.74	69.99±17.44	0.6551
Clusters of low intensity	3.17±2.83	2.28±1.18	0.2918
Damaged cells	81.74±16.52	79.40±10.93	0.1333

Next, we decided to identify significant factors influencing the development of breast cancer. We have obtained the results of statistical one-factor forecasting of the target parameter of breast cancer development "BC (+)" for quantitative and binary factors.

Table 3 presents the TOP-12 list of risk factors for the "BC(+)" parameter.

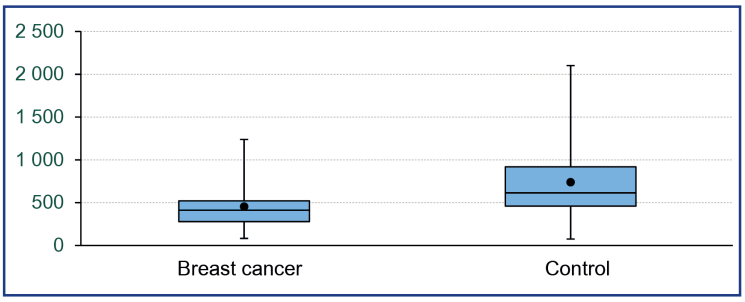

Figure 4 - Comparison of the parameter "Nuclei intensity" in the main and control groups

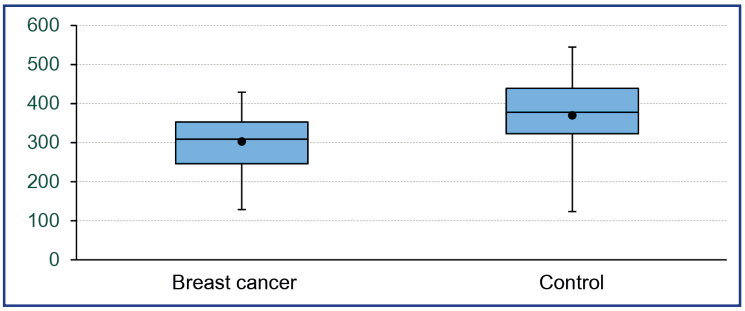

Figure 5 - Comparison of the parameter "Foci intensity means" in the main and control groups

Table 3 - TOP-12 key factors influencing the risk of developing breast cancer

Factor	BC(+): frequency (risk, %)		Risk change, % (95% CI)	Relative risk (95% CI)	p-value
	Factor: No	Factor: Yes			
Age ≥48.0 years old	5 (23.8%)	24 (75.0%)	51.2 (27.6; 74.8)	3.15 (1.43; 6.95)	0.0003
Foci intensity means (Stage 1) <341.6	8 (32.0%)	21 (75.0%)	43.0 (18.7; 67.3)	2.34 (1.27; 4.31)	0.0017
Foci intensity means (Stage 1) <77.8	7 (31.8%)	22 (71.0%)	39.1 (14.0; 64.3)	2.23 (1.16; 4.28)	0.0048
Nuclei intensity (Stage 1) <574.8	6 (30.0%)	23 (69.7%)	39.7 (14.2; 65.2)	2.32 (1.15; 4.71)	0.0049
Foci diameter (Stage 1) ≥0.5	21 (46.7%)	8 (100.0%)	53.3 (38.8; 67.9)	2.14 (1.57; 2.93)	0.0052
Nucleus diameter (Stage 1) ≥7.5	15 (41.7%)	14 (82.4%)	40.7 (16.4; 64.9)	1.98 (1.27; 3.08)	0.0055
Percentage of damaged cells (Stage 1) ≥89.3	19 (45.2%)	10 (90.9%)	45.7 (23.0; 68.4)	2.01 (1.37; 2.94)	0.0068
Colocalization (Stage 1) ≥9.0	11 (39.3%)	18 (72.0%)	32.7 (7.5; 58.0)	1.83 (1.09; 3.09)	0.0169
Foci diameter (Stage 1) ≥0.5	9 (37.5%)	20 (69.0%)	31.5 (5.8; 57.1)	1.84 (1.04; 3.26)	0.0220
Nuclei intensity (Stage 1) <35.6	12 (41.4%)	17 (70.8%)	29.5 (3.9; 55.0)	1.71 (1.03; 2.83)	0.0320
Percentage of nuclei with foci in low-intensity clusters (Stage 1) ≥87.3	15 (44.1%)	14 (73.7%)	29.6 (3.7; 55.5)	1.67 (1.05; 2.66)	0.0381

Based on univariate forecasting, it can be concluded that 12 factors have a statistical significance of influencing the risk of developing "BC +" with a range of risk levels from 69.0% to 100.0%. The leading statistically significant factors for the development of breast cancer with a risk of 75.0% to 81.0% are "Age ≥48.0 years old" and "Foci intensity means (Stage 1) <341.6". At the end of the list of statistically significant factors are "Percentage of nuclei with foci in low-intensity clusters (Stage 1) ≥87.3", "Nuclei intensity (Stage 1) <35.6," and "Foci diameter (Stage 1) ≥0.5", which increase the risk level from 69.0% to 73.7%.

Table 4 and Figure 6 present the results of the analysis performed by POC on the channels of FITC breaks and APC repair of the target parameter "Foci intensity means" to determine the sensitivity and specificity of the technique.

Table 4 – Prognostic parameters for the target parameter "Foci intensity means" for the FITC break channel and the APC repair channel

Parameter	Value	
	through the FITC break channel	through the APC repair channel
Cutoff point	341.6	77.83
Area Under Receiver Operating Characteristic (AuROC)	0.70	0.69
Sensitivity	72.41%	75.86%
Specificity	70.83%	62.50%
Efficiency	71.62%	69.18%

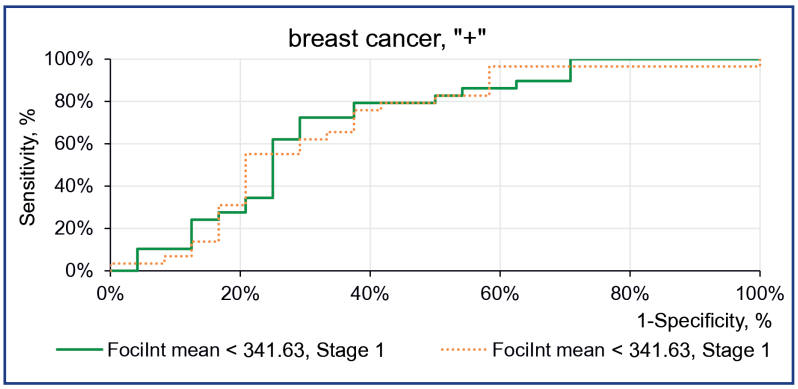

Figure 6 – Prognostic parameters for the target parameter "Foci intensity means" for the channel of FITC breaks and APC repair

During the study, 2 out of 29 main group patients died. In one patient, the cause of death was the progression of the tumor process, and the second died of covid-associated pneumonia. One-year survival was 93%.

In our study, when analyzing foci of γ-H2AX in patients with breast cancer in the channel of FITC ruptures, the parameters " Foci diameter " and " Colocalization " turned out to be higher than in patients with benign tumors, while the parameter " Foci intensity means " was lower than in the control group. In the APC repair channel, the parameters "Nuclei intensity" and "Foci intensity means" in patients with breast cancer were lower than in patients with benign tumors. The revealed changes in the parameters for the FITC rupture channel and the APC repair channel suggest that the main and control groups differ, which may serve as a diagnostic marker for the detection of breast cancer.

Discussion: DNA damage and genomic stability are well-known factors associated with the transition of normal tissues to precancerous and then to malignant states. γ-H2AX, a marker of genomic instability, may be a marker of cancer formation and progression [19].

The most common method for analyzing DNA double-strand break foci, visual assessment of immunofluorescently labeled γ-H2AX foci, is time-consuming. In addition, it is not standardized and is characterized by high intra- and inter-laboratory variability in estimates [18].

We developed a pilot study design to test the possibility of detecting double-strand breaks represented by γ-H2AX foci in human blood using automated fluorescence microscopy and the automated AKLIDES system in patients with breast cancer. We analyzed γH2AX foci on peripheral blood mononuclear cells (lymphocytes) in 29 patients with newly verified breast cancer and 24 control women with a verified benign disease of the mammary glands.

A study by B. Wang et al. reported a high positive frequency of γ-H2AX in tumor cells compared to normal breast tissues in the same patients with breast cancer. The significant difference in tumor and adjacent healthy tissues demonstrates that γ-H2AX can help improve the efficiency of early diagnosis [20].

However, these studies were performed in tissues, and in most cases, the collection of tumor samples is a complex medical procedure, especially when repeated samples are required. Therefore, clinicians often have to turn to safer and less invasive procedures that can be routinely used in the clinic to assess response to therapy, with the potential for reproducible results. In this regard, we used a safer method for detecting foci of γ-H2AX in peripheral blood mononuclear cells in patients with breast cancer using the automated AKLIDES system.

Our analysis of γH2AX foci showed a statistically significant difference in the main and control groups. Thus, in the channel of breaks "FITC," the parameter "Foci diameter" was slightly higher than in the control group (p=0.0382). The parameter "Colocalisation" in the main group was higher than in the control group (p=0.0486). The parameter "Foci intensity means" was lower in the breast cancer group (p=0.0166) (Table 1). In the "APC" repair channel, the parameter "Nuclei intensity" in the main group was lower than in the control group (p = 0.0166). The parameter "Foci intensity means" in the channel of ruptures and repair was lower in the main group than in the control group (p=0.0118) (Table 2).

In the literature we studied, no study was found to quantify DNA double-strand breaks in cancer patients with benign neoplasms.

We found published studies on mobile phones' potential genotoxic radiofrequency effects on human peripheral blood mononuclear cells in vitro measured using the automated AKLIDES system [21].

Studies have also been conducted on the analysis

of foci of γ -H2AX on the automated AKLIDES system in athletes during rest after exercise. The parameters were the analysis of the diameter of γ-H2AX foci and the number of γ-H2AX foci per affected cell [21].

There is experience in using the automated system AKLIDES in Kazakhstan for the diagnosis of systemic autoimmune diseases, where antinuclear antibodies, cytoplasmic antineutrophil antibodies, and perinuclear antineutrophil antibodies were studied in patients with rheumatoid diseases [22].

As a pilot project, our study had some limitations and limits, including:

1) the lack of a standard study methodology;

2) we have not conducted a study of long-term results;

3) the lack of standard reference parameters to interpret the results and make conclusions.

DNA DSBs are a personalized response of the body to certain risk factors so that they can vary individually, and this can create barriers to population-based validation. Like any other biomarker, γH2AX has biological variability, which could be predictable and cyclic [24].

The lack of experimental standardization of the γH2AX assay leads to wide heterogeneity of the results obtained and problems with their interpretation, making it difficult to use γH2AX as a routine biomarker in population studies. Further research is needed to standardize the results, with a strict organization of the research and individual training of personnel [24].

Z. Zhang [26] states, "Laboratory medicine is aimed at providing tests for clinical decision-making." The result of using a predictive biomarker in this pilot study will serve as the basis for a larger study using γH2AX lesions in breast cancer patients to develop methods for the real-time detection of neoplasms.

Conclusion: An increase in the parameters "Foci diameter" (p=0.0382), "Foci intensity means" (p = 0.0166), and "Colocalisation" (p=0.0486) was found in the breast cancer group in the "FITC" channel of ruptures. In the "APC" repair channel, the parameters "Nuclei intensity" (p=0.0166) and "Foci intensity means" (p=0.0118) in the breast cancer group were lower than in the control group.

The detected changes in the parameters of DNA double-strand breaks along the "FITC" break and "APC" repair channels between the main and control groups can possibly serve as a breast cancer diagnostic marker.

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АНДАТПА

СҮТ БЕЗІНІҢ ҚАТЕРСІЗ ЖӘНЕ ҚАТЕРЛІ ІСІК АУРУЛАРЫНДА ДНҚ ҚОС ТІЗБЕКТІ ҮЗІЛІСТЕРІН САНДЫҚ АНЫҚТАУ

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Өзектілігі: *ДНҚ зақымдануының ең қауіпті түрі – ДНҚ қос тізбекті үзілуі. Фосфорланған гистон ақуызының H2AX (γH2AX) ошақтарын талдау қазіргі уақытта ДНҚ қос тізбекті үзілістерін анықтаудың ең сезімтал әдісі болып табылады. Бұл ақуыз модификациясы жасушалық стресстің жеке биомаркеріне айналуы мүмкін, әсіресе ісік ауруларының диагностикасы мен мониторингінде. Бұл зерттеуде біз γH2AX ошақтарының иммунофлуоресцентті кескіндерін автоматты түрде талдау және нәтижелерді көрнекі ұпайлармен салыстыру үшін AKLIDES® платформасында жаңа үлгіні тану алгоритмдерін қолдандық. Сүт безінің қатерлі ісігі бар науқастар мен сүт безінің қатерсіз ісігі бар әйелдердің шеткергі қан мононуклеарлы жасушаларында γH2AX ошақтарының түзілуі зерттелді.*

Зерттеудің мақсаты: *мүмкін болатын биомаркерді анықтау үшін сүт безі қатерлі ісігі және сүт безінің қатерсіз ауруы бар әйелдердегі шеткергі қан лимфоциттеріндегі ДНҚ қос тізбекті үзілістерін сандық түрде анықтау.*

Әдістері: *Сүт безінің қатерлі ісігі (n=29) және сүт безінің қатерсіз ісіктері (n=24) бар науқастарда автоматтандырылған AKLIDES жүйесін қолдану арқылы лимфоциттерде γ-H2AX ошақтарын талдау.*

Нәтижелер: *«FITC» үзілу арнасындағы негізгі және бақылау топтарының көрсеткіштерін салыстыру кезінде «Орташа өзек диаметрі» (p=0,0382), «Барлық ошақтар үшін орташа қарқындылық мәні» (p=0,0166), «Екі арнадағы қабаттасатын ошақтардың саны» (p=0,0486) көрсеткіштерінде статистикалық маңызды айырмашылық анықталды. «APC» жөндеу арнасында «Люминесценция қарқындылығы жоғары ядролар» (p=0,0166) және «Барлық ошақтар үшін орташа қарқындылық мәні» (p=0,0118) көрсеткішкерде айтарлықтай айырмашылықтар анықталды.*

Қорытынды: *Негізгі және бақылау топтары арасындағы FITC үзіліс және «APC» жөндеу арналары бойынша ДНҚ қос тізбекті үзілу жылдамдығының анықталған өзгерістері сүт безі обырын анықтау үшін диагностикалық маркер ретінде қызмет етуі мүмкін.*

Түйінді сөздер: *ДНҚ қос тізбекті үзілуі, H2AX гистон протеині, сүт безі қатерлі ісігі.*

ABSTRACT

КОЛИЧЕСТВЕННАЯ ОЦЕНКА ДВУЦЕПОЧЕЧНЫХ РАЗРЫВОВ ДНК ПРИ ДОБРОКАЧЕСТВЕННЫХ И ЗЛОКАЧЕСТВЕННЫХ ЗАБОЛЕВАНИЯХ МОЛОЧНОЙ ЖЕЛЕЗЫ

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Актуальность: *Наиболее опасным типом повреждений ДНК являются двуцепочечные разрывы ДНК. Анализ очагов фосфорилированного гистонового белка H2AX (γH2AX) в настоящее время является наиболее чувствительным методом обнаружения двуцепочечных разрывов ДНК (ДЦР). Эта модификация белка может стать индивидуальным биомаркером клеточного стресса, особенно при диагностике и мониторинге неопластических заболеваний. В этом исследовании нами были использованы новые алгоритмы распознавания образов на платформе AKLIDES® для автоматического анализа иммунофлуоресцентных изображений фокусов γH2AX и сравнения результатов с визуальными оценками. Изучено формирование очагов γH2AX на мононуклеарных клетках периферической крови женщин с раком молочной железы (РМЖ) и доброкаче-ственными образованиями молочных желез.*

Цель исследования *– провести количественную оценку двуцепочечных разрывов ДНК в лимфоцитах периферической крови у женщин с раком молочной железы и доброкачественными образованиями молочных желез для определения возможного биомаркера.*

Методы: *Проведение анализа очагов γ-H2AX в лимфоцитах на автоматизированной системе AKLIDES у женщин с РМЖ (n=29) и доброкачественными образованиями молочных желез (n=24).*

Результаты: *При сравнении показателей основной и контрольной групп в канале разрывов «FITC» обнаружена статистически значимая разница показателей «Средний диаметр ядра» (p=0,0382), «Среднее значение интенсивности для всех очагов» (p=0,0166), «Количество перекрывающихся очагов в двух каналах» (p=0,0486). В канале репарации «APC» выявлены достоверные различия показателей «Ядра с повышенной интенсивностью свечения» (p=0,0166) и «Среднее значение интенсивности для всех очагов» (p=0,0118).*

Заключение: *Выявленные изменения показателей двуцепочечных разрывов ДНК по каналам разрывов FITC и репарации APC между основной и контрольной группами, возможно, могут служить биомаркером для выявления РМЖ.*

Ключевые слова: *двуцепочечные разрывы ДНК, гистоновый белок H2AX, рак молочной железы (РМЖ).*

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