

The γ -H2AX foci as an indicator for double-stranded DNA breaks and response to ongoing chemotherapy in breast cancer women: a pilot study

M. AITMAGAMBETOVA¹, G. SMAGULOVA², S. SAKHANOVA³, N. KEREYEVA¹, A. KOISHYBAEV¹, A. AMANZHOLKYZY⁴, A. TULYAEVA¹, D. ZHOLMUKHAMEDOVA¹, G. KANDYGULOVA⁵, N. IMANBAEV¹, A. DONAYEVA⁴, I.A. ABDELAZIM⁶, S. SAPARBAYEV⁴, Z. KHAMIDULLINA⁷, G. GUBASHEVA⁸

¹Department of Oncology, West Kazakhstan Marat Ospanov Medical University, Aktobe, Kazakhstan

²Department of Clinical Pharmacology, West Kazakhstan Marat Ospanov Medical University, Aktobe, Kazakhstan

³Department of the Scientific and Practical Center, West Kazakhstan Marat Ospanov Medical University, Aktobe, Kazakhstan

⁴Department of Normal Physiology, West Kazakhstan Marat Ospanov Medical University, Aktobe, Kazakhstan

⁵Department of Pathological Physiology, West Kazakhstan Marat Ospanov Medical University, Aktobe Kazakhstan

⁶Department of Obstetrics and Gynecology, Faculty of Medicine Ain Shams University, Egypt

⁷Department of Obstetrics and Gynecology №1, Astana Medical University, Astana, Kazakhstan

⁸Department of Obstetrics and Gynecology №2, West Kazakhstan Marat Ospanov Medical University, Aktobe, Kazakhstan

Abstract. – OBJECTIVE: The double-stranded breaks (DSBs) of the DNA can predispose to cancer development. The γ H2AX foci have been proposed for prediction of tumor growth and response to radiotherapy. We aimed to evaluate the γ -H2AX foci as an indicator for DSBs and response to ongoing chemotherapy in breast cancer women.

PATIENTS AND METHODS: Twenty-nine (29) breast cancer women, scheduled for adjuvant chemotherapy were included in this pilot study after obtaining written informed consent and approval of the study by the WKMU ethics committee. Participants received adjuvant chemotherapy, according to the treatment protocol of the Republic of Kazakhstan. A peripheral blood sample was collected from each studied participant for analysis of the γ -H2AX foci in the participants' lymphocytes using the immunofluorescent staining kit. Data of the Fluorescein isothiocyanate (FITC) rupture, and Adenomatous Polyposis Coli (APC) repair channels were analyzed to evaluate the γ -H2AX foci as an indicator for DSBs, and response to ongoing chemotherapy.

RESULTS: About 10.3% (3/29) of the studied breast cancers were luminal A, 72.4% (21/29) were luminal type B, 13.8% (4/29) were basal-like,

and 3.5 (1/29) were HER2 positive. The FITC rupture channel during the ongoing chemotherapy showed significantly decreased foci intensity means ($p=0.0075$), and significantly increased colocalization ($p=0.02$). The APC repair channels during the ongoing chemotherapy showed significantly increased nuclei intensity ($p=0.046$), foci overall ($p=0.0007$), clusters ($p=0.002$), foci mean ($p<0.0001$), and foci mean + clusters ($p=0.0003$). It also showed significantly increased clusters positive cells ($p<0.0001$), foci low-intensity ($p<0.0001$), foci low-intensity + clusters ($p=0.0003$), and clusters of low intensity positive cells ($p=0.002$).

CONCLUSIONS: The γ -H2AX foci, the changes in the FITC rupture, and APC repair channels can serve as real-time indicators for the response to ongoing chemotherapy in breast cancer women. The γ -H2AX foci as an indicator for DSBs/repair pathway, and for the response to ongoing chemotherapy in breast cancer should be evaluated in further studies.

Key Words:

γ -H2AX, Double-stranded breaks, DSBs, DNA, Chemotherapy, Breast cancer.

Introduction

Breast cancer (BC) is one of the most common cancers in women. The incidence of invasive breast cancer in the United States is about 12.5%¹.

In 2020, 35,366 cancer cases were diagnosed in the Republic of Kazakhstan, and 4,390 (12.4%) of them were breast cancers^{2,3}.

The DNA repair pathway is activated upon DNA damage caused by ionizing radiation, hypoxia, and chemicals⁴.

The double-stranded breaks (DSBs) can initiate genomic instability and can predispose to cancer⁴. One of the first events in the DSBs/repair pathway is phosphorylation of histone H2AX at serine 139 residue on each side of the DSBs (referred to as γ -H2AX foci)⁵⁻⁸.

The γ -H2AX foci is a specific indicator/ marker of cellular stress and monitoring of neoplastic diseases⁴. The γ H2AX foci have been proposed for prediction of tumor growth, and response to radiotherapy^{9,10}. The γ H2AX foci can be used to individualize the chemotherapy and/or radiotherapy protocols, increase the treatment efficacy, minimize its side effects, and toxicity¹¹⁻¹⁴.

The γ -H2AX foci is currently the most sensitive method for detecting DSBs¹⁵⁻¹⁶. Immunofluorescent staining of the γ -H2AX foci using the anti- γ H2AX antibody technique provides direct visualization of the γ -H2AX foci and identifies the amount of DSBs¹⁵⁻¹⁶.

The AKLIDES[®] system was previously evaluated and validated for the analysis of the γ -H2AX foci¹⁷⁻²². Therefore, the current pilot study was designed to evaluate the γ -H2AX foci as an indicator for DSBs, and response to ongoing chemotherapy in breast cancer women.

Patients and Methods

Twenty-nine (29) breast cancer women scheduled for adjuvant chemotherapy were included in this pilot study after obtaining written informed consent and approval of the study by the West Kazakhstan Marat Ospanov Medical University (WKMU) Ethics Committee (Approval No. 57, dated 17/01/2020).

Inclusion criteria included breast cancer women (any stage) underwent modified radical mastectomy, or local tumor resection, proper TNM staging, and assessment of tumor receptors [estrogen (ER), progesterone receptors (PR), and hu-

man epidermal growth receptor (HER2)], scheduled for adjuvant chemotherapy, and did not receive radiotherapy, chemotherapy, or molecular targeted therapy.

Women diagnosed with breast cancer, without proper TNM staging or assessment of tumor receptors, received radiotherapy, chemotherapy, or molecular therapies, and/or refused to participate were excluded from this study.

Participants received the adjuvant chemotherapy according to the breast cancer treatment protocol of the Republic of Kazakhstan (No. 56 dated March 01, 2019), and according to the tumor histology in form of cyclophosphamide-based chemotherapy. 1) cyclophosphamide + doxorubicin, or 2) cyclophosphamide + docetaxel²³, or 3) cyclophosphamide + methotrexate + 5-fluorouracil.

Each chemotherapy course was scheduled either weekly or every 3 weeks for 3-6 months according to the participants' clinical condition, and response.

A peripheral blood sample was collected from each studied participant (in EDTA tube) for analysis of the γ -H2AX foci in the participants' lymphocytes using the immunofluorescent staining kit (AKLIDES[®] system; Medipan, Germany) according to the manufacturer' instruction.

The AKLIDES[®] system is a motorized inverse fluorescence microscope combined with various hardware, and software modules to analyze and evaluate the γ -H2AX foci in the participants' lymphocytes/peripheral mononuclear cells.

At least 80-100 lymphocytes were analyzed, and the following parameters were evaluated: (1) Nuclei with increased intensity; (2) Number/percentage of nuclei with foci; (3) Foci diameter, mean intensity for foci, and total number of foci; (4) Number/percentage of nuclei with clusters, nuclei with clusters of low intensity, and total number of clusters; (5) Colocalization.

Colocalization means spatial overlap between ≥ 2 different fluorescent labels. Foci diameter is 0.25-1.2 μ m, and the clusters are foci with >1.2 μ m diameter.

The γ -H2AX foci, and the data of the Fluorescein isothiocyanate (FITC) rupture and Adenomatous Polyposis Coli (APC) repair channels were analyzed to evaluate the γ -H2AX foci as an indicator for DSBs, and response to ongoing chemotherapy in 4 stages.

Stage 1 (before the 1st chemotherapy course), stage 2 (after the 1st chemotherapy course), stage 3 (before the 3rd chemotherapy course), and stage 4 (before the 4th chemotherapy course).

Collected participants' data were analyzed to evaluate the γ -H2AX foci as an indicator for DSBs, and response to ongoing chemotherapy in breast cancer women.

Statistical Analysis

More than 100 lymphocytes were needed for this pilot study to produce a statistically acceptable figure. The mean and standard deviation (\pm SD) were used to present numerical variables. The one-way ANOVA test post-hoc Turkey HSD was used to compare between different studied stages. $p < 0.05$ was considered significant.

Results

Twenty-nine (29) breast cancer women underwent modified radical mastectomy, or local tumor resection, proper TNM staging, and assessment of tumor receptors, and scheduled for adjuvant chemotherapy were included in this pilot study.

The total number of lymphocytes collected from the studied participants using automated AKLIDES[®] system was 113 lymphocytes (>100 lymphocytes were needed to produce a statistically acceptable figure). Data of the FITC rupture, and APC repair channels were analyzed to evaluate the γ -H2AX foci as an indicator for DSBs, and response to ongoing chemotherapy.

The mean age of the studied women was 56.1 ± 12.2 years, 86.2% (25/29) of studied breast can-

cers were T2 (tumor >20 mm and \leq 50 mm), and 13.8% (4/29) were stage T3 (tumor >50 mm)²³.

According to tumor immunohistochemistry, about 10.3% (3/29) of the studied breast cancers were luminal A (ER positive, PR positive, and HER2 negative), 72.4% (21/29) were luminal type B (ER positive, PR negative, and HER2 positive), 13.8% (4/29) were basal-like (triple negative for ER, PR, and HER2)²⁴, and 3.5 (1/29) were only HER2 positive (ER, and PR negative)^{25,26}.

The FITC rupture channel during the ongoing chemotherapy showed significantly decreased foci intensity means from 69.9 ± 17.97 in stage 1, to 73.1 ± 16.3 in stage 2, 64.96 ± 13.6 in stage 3, and 61.7 ± 9.45 in stage 4, ($p=0.0075$), (Table I and Figure 1).

Additionally, there was significantly increased colocalization from 18.6 ± 19.1 in stage 1, to 33.6 ± 28.17 in stage 2, 28.9 ± 24.7 in stage 3, and 22.8 ± 35.19 in stage 4 ($p=0.02$), (Table I and Figure 2).

The APC repair channels during the ongoing chemotherapy showed significantly increased nuclei intensity from 76.8 ± 25.6 in stage I, to 96.0 ± 32.79 in stage 2, 97.3 ± 38.4 in stage 3, and 89.3 ± 53.3 in stage 4 ($p=0.046$), (Table II and Figure 3).

Significantly increased foci overall from 288.1 ± 251.8 in stage 1, to 492.3 ± 428.5 in stage 2, 718.6 ± 541 in stage 3, and 658.8 ± 507.66 in stage 4, ($p=0.0007$) are shown in Table II and Figure 4.

Significantly increased clusters from 49.3 ± 80.94 in stage 1, to 75.5 ± 124.4 in stage 2, 177.03 ± 141.2 in stage 3, and 124.3 ± 113.0 in stage 4 ($p=0.002$) are shown in Table II and Figure 5.

Table I. The fluorescein isothiocyanate (FITC) rupture channel during the ongoing chemotherapy stages.

Variables	Stage 1	Stage 2	Stage 3	Stage 4	p-value
Foci diameter	7.3 ± 0.68	7.68 ± 0.8	7.48 ± 0.69	7.64 ± 0.75	0.47
Nuclei intensity	35.95 ± 10.8	35.3 ± 10.36	34.16 ± 9.9	31.49 ± 8.87	0.91
Nuclei with foci	55.7 ± 37.0	68.9 ± 43.2	68.8 ± 41.1	60.0 ± 53.3	0.49
Foci overall	167.17 ± 219.7	241.7 ± 329.0	200.4 ± 213	162.55 ± 200.66	0.64
Foci intensity means	69.9 ± 17.97	73.1 ± 16.3	64.96 ± 13.6	61.7 ± 9.45	0.0075*
Clusters	0.24 ± 0.79	0.34 ± 1.08	0.14 ± 0.58	0.07 ± 0.26	0.68
Foci mean	1.45 ± 1.58	2.01 ± 1.9	1.78 ± 1.56	1.3 ± 1.25	0.68
Foci mean + clusters	1.46 ± 1.59	2.02 ± 1.9	1.79 ± 1.57	1.3 ± 1.26	0.68
Clusters positive cells	51.13 ± 29.87	62.01 ± 29.5	61.7 ± 29.6	50.17 ± 30.5	0.44
Clusters of low intensity positive cells	2.45 ± 1.81	3.19 ± 2.2	3.1 ± 1.9	2.22 ± 1.64	0.14
Colocalization	18.6 ± 19.1	33.6 ± 28.17	28.9 ± 24.7	22.8 ± 35.19	0.02*

*: Significant difference. Clusters are foci with a diameter > 1.2 μ m. Clusters of low Intensity positive cells: Number/percentage of nuclei with clusters of low intensity. Clusters positive cells: Number/percentage of nuclei with clusters. Colocalization: Spatial overlap between ≥ 2 different fluorescent labels. Data presented as mean \pm SD. Foci intensity means: Mean intensity for foci. Foci mean: Number of foci/cell. Foci overall: Total number of foci. Nuclei intensity: Nuclei with increased immunofluorescence intensity. Nuclei with foci: Number/percentage of nuclei with foci. One-way ANOVA test post-hoc Turkey HSD was used to compare between different studied stages. Stage 1 (before the 1st chemotherapy course). Stage 2 (after the 1st chemotherapy course). Stage 3 (before the 3rd chemotherapy course). Stage 4 (before the 4th chemotherapy course).

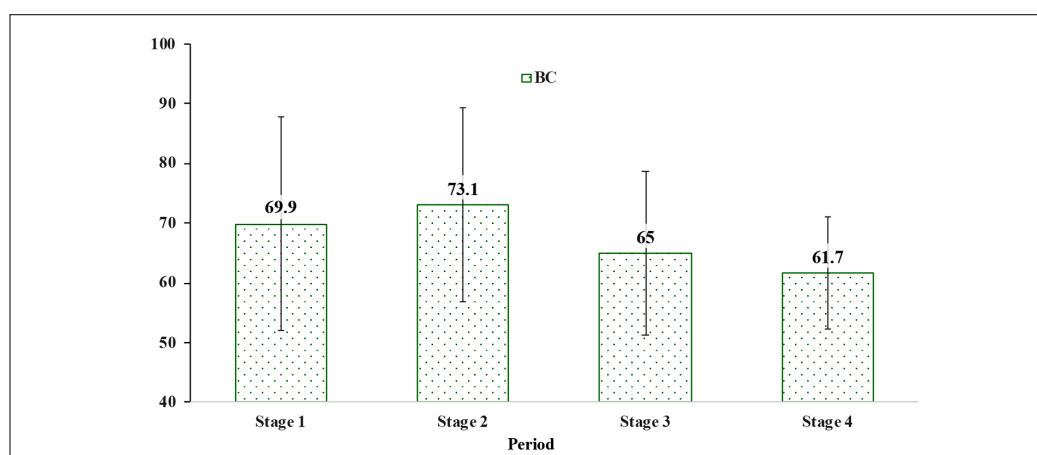


Figure 1. Foci intensity means during the ongoing chemotherapy stages.

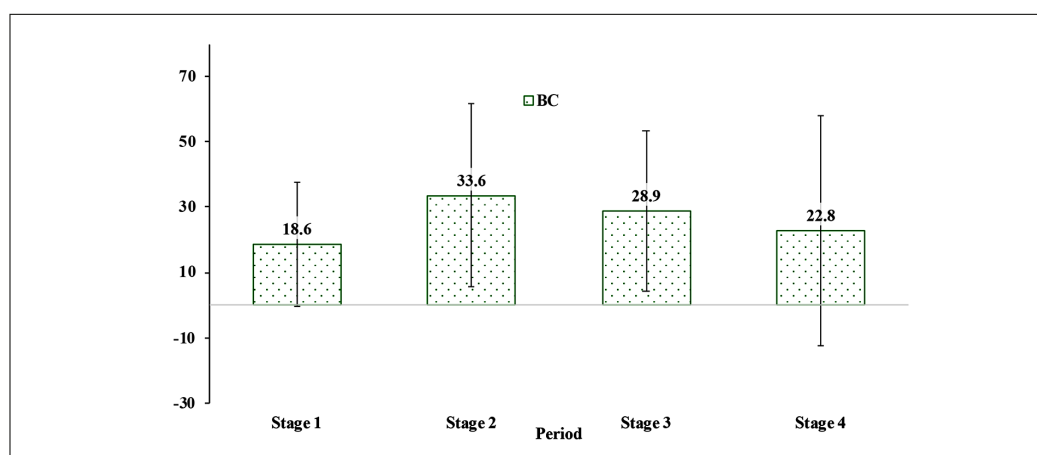


Figure 2. Colocalization during the ongoing chemotherapy stages.

Table II. The Adenomatous Polyposis Coli (APC) repair channels during the ongoing chemotherapy stages.

Variables	Stage 1	Stage 2	Stage 3	Stage 4	p-value
Foci diameter	455.2 ± 286.58	410.97 ± 272.07	371.6 ± 266.57	250.36 ± 135.9	0.078
Nuclei intensity	76.8 ± 25.6	96.0 ± 32.79	97.3 ± 38.4	89.3 ± 53.3	0.046*
Foci overall	288.1 ± 251.8	492.34 ± 428.5	718.6 ± 541	658.79 ± 507.66	0.0007*
Foci intensity means	302.94 ± 81.62	315.5 ± 85.46	278.64 ± 100.8	232.85 ± 84.27	0.066
Clusters	49.3 ± 80.94	75.5 ± 124.4	177.03 ± 141.2	124.3 ± 113.0	0.002*
Foci mean	2.8 ± 2.96	4.3 ± 2.7	6.05 ± 3.4	6.4 ± 3.6	< 0.0001*
Foci mean + clusters	8.05 ± 13.3	10.2 ± 13.13	37.5 ± 59.5	17.5 ± 16.1	0.0003*
Clusters positive cells	70.4 ± 23.7	86.7 ± 12.7	87.2 ± 17.3	84.8 ± 22.8	< 0.0001*
Foci low intensity	3.2 ± 2.8	4.5 ± 2.7	6.3 ± 3.33	6.7 ± 3.56	< 0.0001*
Foci low intensity + clusters	8.4 ± 13.14	10.4 ± 13.0	37.7 ± 59.4	17.8 ± 16.0	0.0003*
Clusters of low intensity positive cells	81.7 ± 16.5	89.9 ± 12.2	89.7 ± 14.8	87.2 ± 23.3	0.002*

*: Significant difference. Clusters are foci with a diameter > 1.2 μ m. Clusters positive cells: Number/percentage of nuclei with clusters. Data presented as mean \pm SD. Foci diameter is 0.25-1.2 μ m. Foci Intensity means: Mean intensity for foci. Foci mean: Number of foci/cell. Foci overall: Total number of foci. Nuclei intensity: Nuclei with increased immunofluorescence intensity. Nuclei with foci: Number/percentage of nuclei with foci. One-way ANOVA test post-hoc Turkey HSD was used to compare between different studied stages. Stage 1 (before the 1st chemotherapy course). Stage 2 (after the 1st chemotherapy course). Stage 3 (before the 3rd chemotherapy course). Stage 4 (before the 4th chemotherapy course).

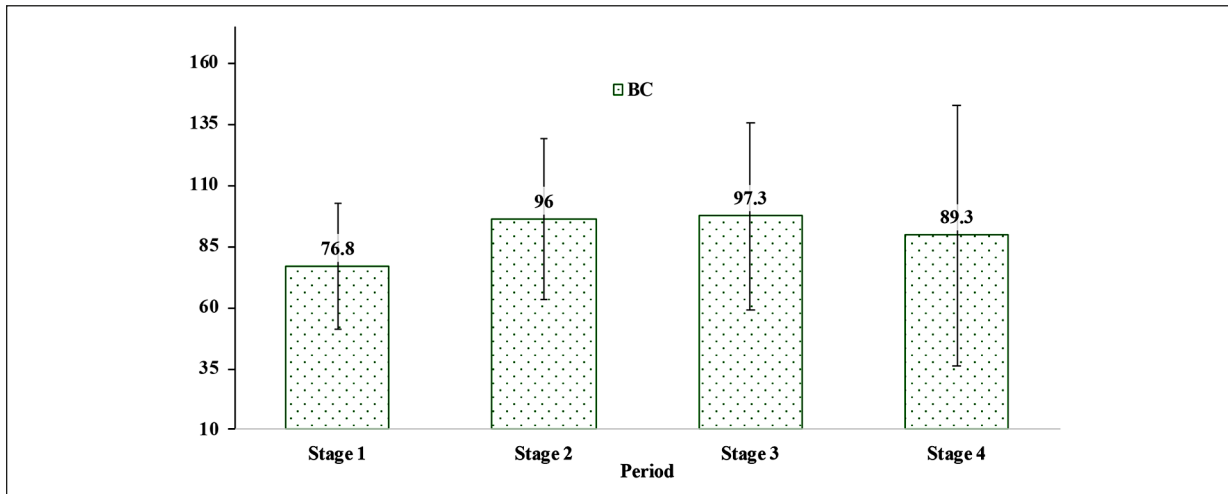


Figure 3. Nuclei intensity during the ongoing chemotherapy stages.

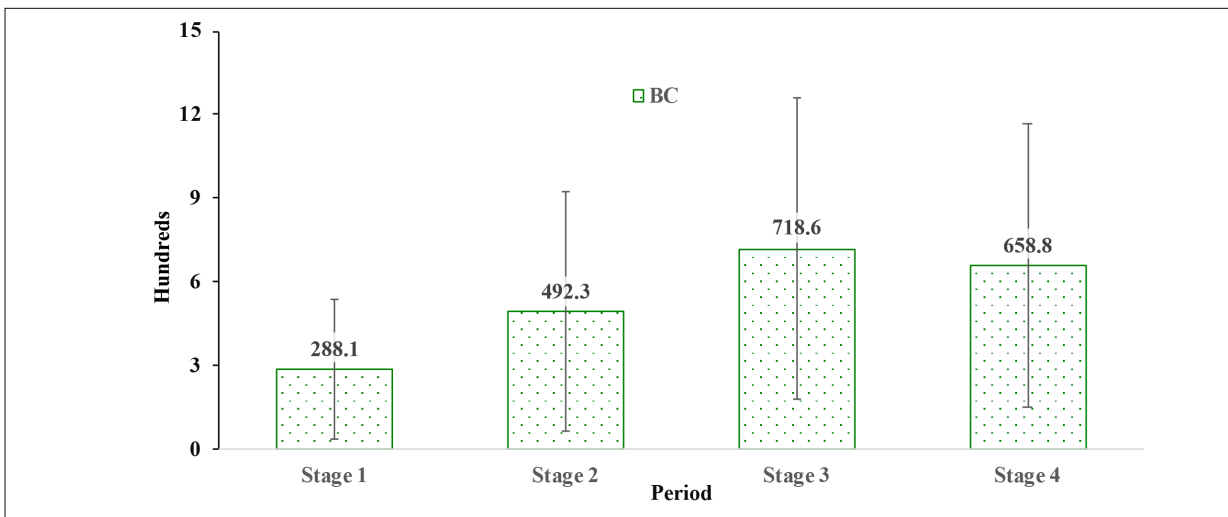


Figure 4. Foci overall during the ongoing chemotherapy stages.

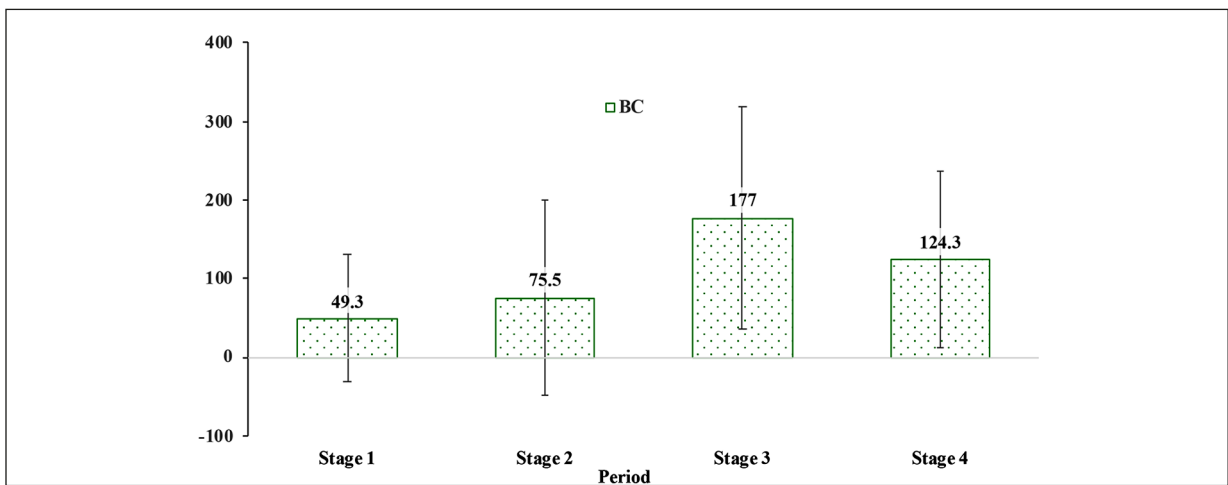


Figure 5. Clusters during the ongoing chemotherapy stages.

Significantly increased foci mean from 2.8 ± 2.96 in stage 1, to 4.3 ± 2.7 in stage 2, 6.05 ± 3.4 in stage 3, and 6.4 ± 3.6 in stage 4 ($p < 0.0001$) are shown in Table II and Figure 6.

Significantly increased foci mean + clusters from 8.05 ± 13.3 in stage 1, to 10.2 ± 13.13 in stage 2, 37.5 ± 59.5 in stage 3, and 17.5 ± 16.1 in stage 4 ($p = 0.0003$) are shown in Table II and Figure 7.

Significantly increased clusters positive cells from 70.4 ± 23.7 in stage 1, to 86.7 ± 12.7 in stage 2, 87.2 ± 17.3 stage 3, and 84.8 ± 22.8 in stage 4 ($p < 0.0001$) are shown in Table II and Figure 8.

Significantly increased foci low intensity from 3.2 ± 2.8 in stage 1, to 4.5 ± 2.7 in stage 2, 6.3 ± 3.33 in stage 3, and 6.7 ± 3.56 in stage 4 ($p < 0.0001$) are shown in Table II and Figure 9.

Significantly increased foci low intensity + clusters from 8.4 ± 13.14 in stage 1, to 10.4 ± 13.0 in stage 2, 37.7 ± 59.4 in stage 3, and 17.8 ± 16.0 in stage 4 ($p = 0.0003$) are shown in Table II and Figure 10.

Significantly increased clusters of low intensity positive cells from 81.7 ± 16.5 in stage 1, to 89.9 ± 12.2 in stage 2, 89.7 ± 14.8 in stage 3, and 87.2 ± 23.3 in stage 4 ($p = 0.002$) are shown in Table II and Figure 11.

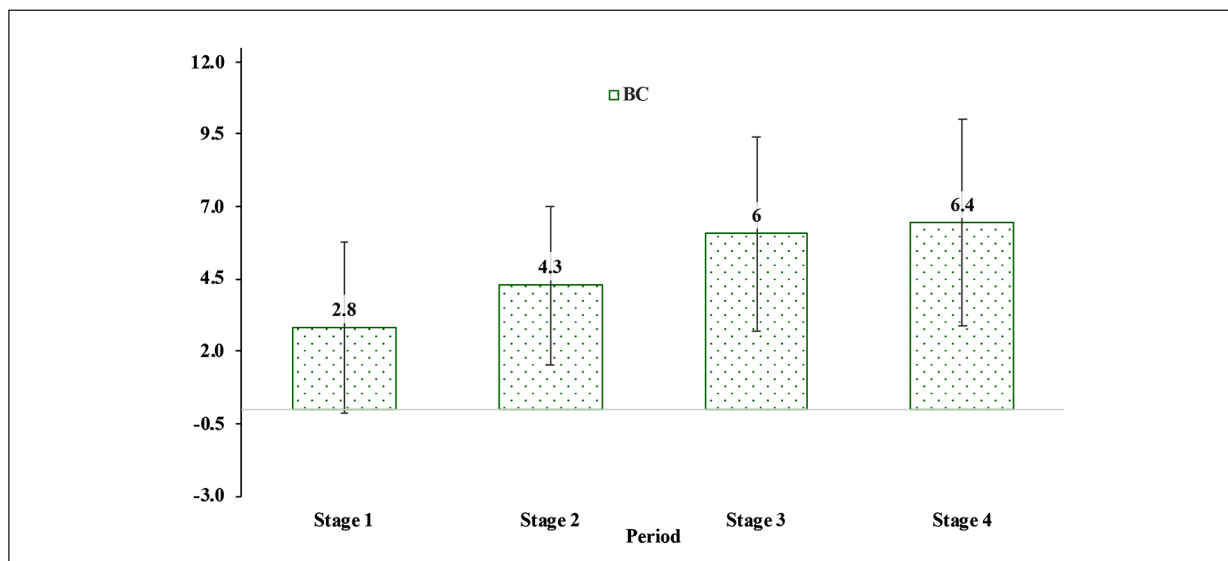


Figure 6. Foci mean during the ongoing chemotherapy stages.

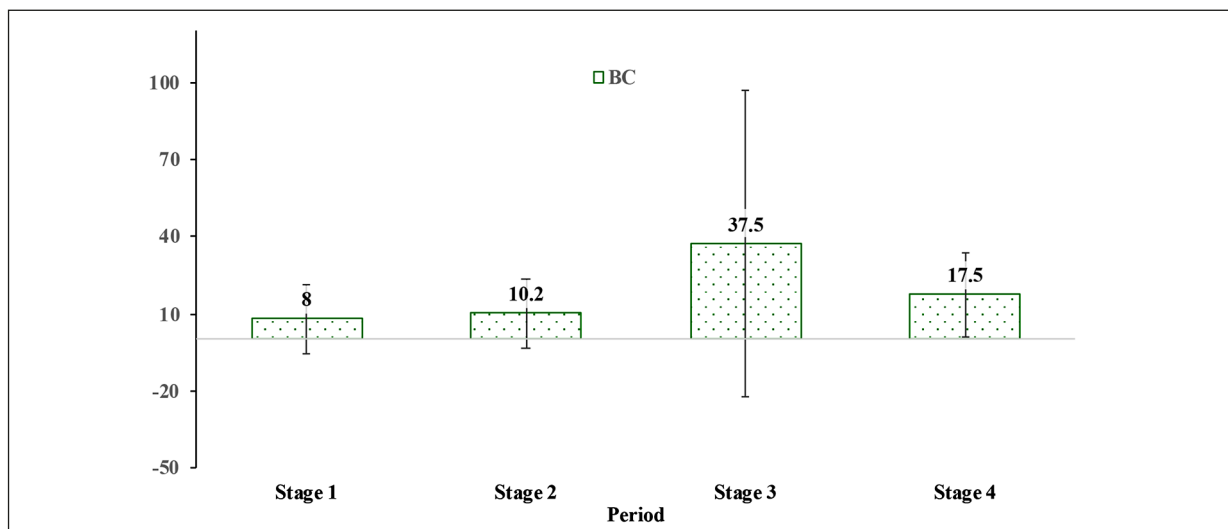


Figure 7. Foci mean + clusters during the ongoing chemotherapy stages.

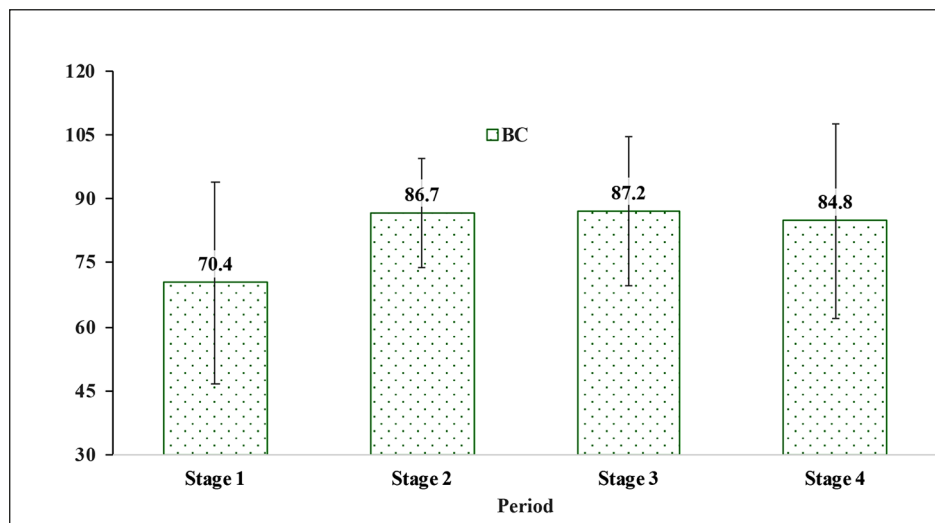


Figure 8. Clusters positive cells during the chemotherapy stages.

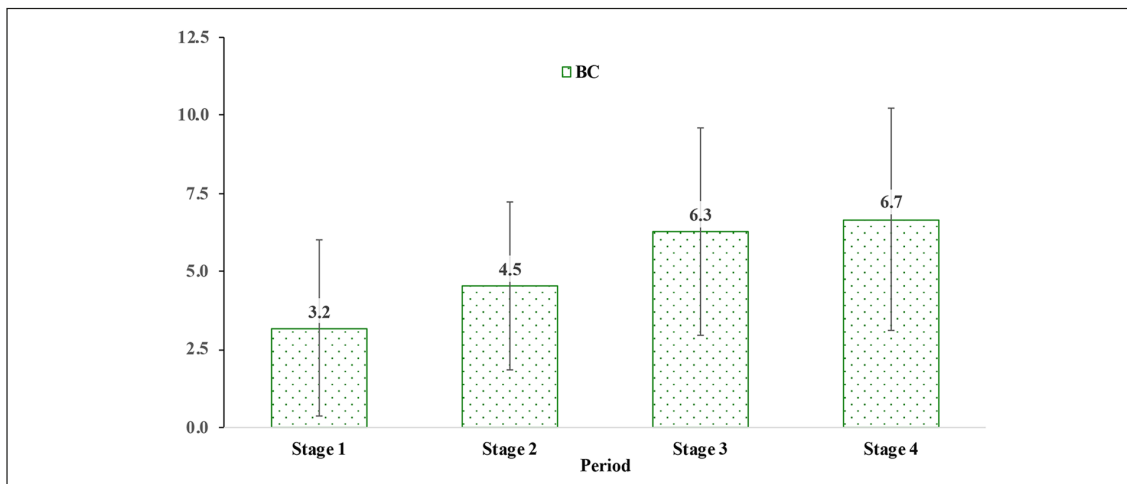


Figure 9. Foci low intensity during the ongoing chemotherapy stages.

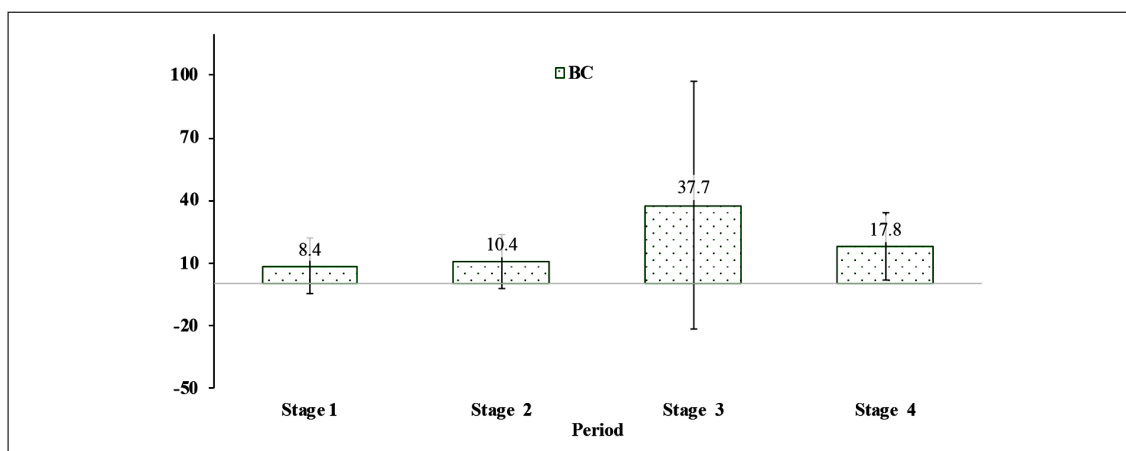


Figure 10. Foci low intensity + clusters during the ongoing chemotherapy stages.

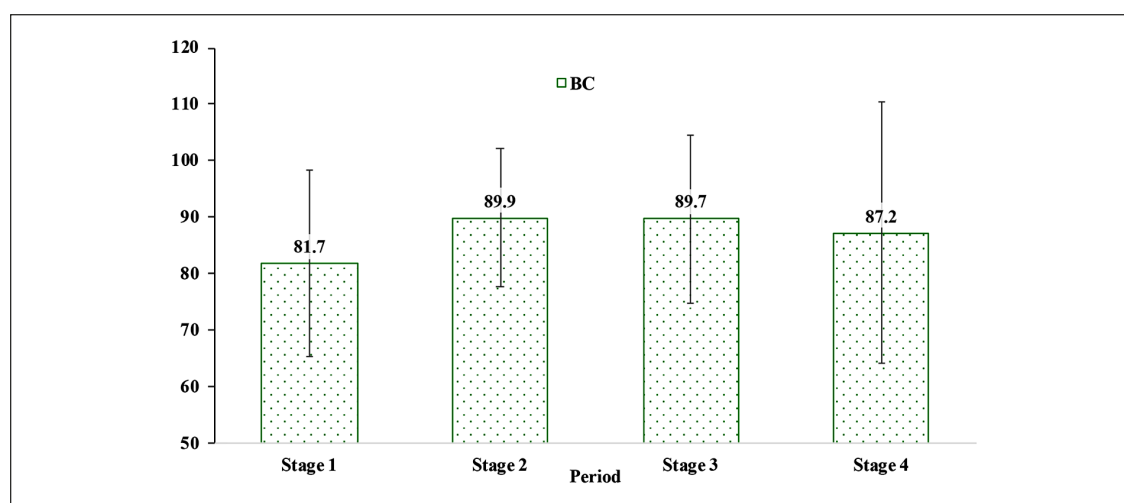


Figure 11. Clusters of low intensity positive cells during the ongoing chemotherapy stages.

Discussion

The DNA repair pathway is activated upon DNA damage⁴. The DSBs can initiate genomic instability and can predispose to cancer⁴.

One of the first events in the DSBs/repair pathway is phosphorylation of histone H2AX at serine 139 residue on each side of the DSBs (referred to as γ -H2AX foci)⁵⁻⁸.

The γ -H2AX foci is a specific indicator/marker of cellular stress, and monitoring of tumor progression, and response to treatment²⁷.

The common methods used for analysis of DSBs foci, and visual assessment of immunofluorescent labeled γ -H2AX foci, are time-consuming, and characterized by high intra- and interobservers variations²².

Unlike the common methods, the AKLIDES[®] system is a motorized inverse fluorescence microscope combined with various hardware, and software modules to analyze and evaluate the γ -H2AX foci in the participants' lymphocytes/peripheral mononuclear cells²⁸.

The authors from the West Kazakhstan Marat Ospanov Medical University (WKMU), Aktobe, Kazakhstan are authorized and trained from the SAMRUNI (Med., Co., Nur-Sultan, Kazakhstan) to use and interpret the results of the automatic AKLIDES[®] system (serial number 2017 AKL 2-42) with FITC rupture (LOT-TT200316-1), and APC (LOT-TT190729-2) repair channels.

The AKLIDES[®] system was previously evaluated and validated for the analysis of the γ -H2AX foci¹⁷⁻²².

The tumor infiltrating cells are known as the tumor micro-environment which play a crucial role in tumor progression.

The activated cytotoxic T lymphocytes exert direct cytotoxic action which related to better survival in women with breast cancer. The regulatory T lymphocytes are responsible for immune self-tolerance and protect against autoimmunity²⁹.

Analysis of 3,771 triple negative breast cancer women undergoing adjuvant chemotherapy showed that 10% in the tumor infiltrating lymphocytes was associated with longer disease-free survival and overall survival (OS)³⁰.

The cancer treatment could be improved and/or modified if the information regarding the individual's response to specific radio and/or chemotherapy protocol are available.

The response to specific radio and/or chemotherapy protocol can be determined by the tumor size reduction, and OS³¹. Nonetheless, the evaluation of the tumor size is time-consuming, and necessitates sophisticated imaging techniques. The γ H2AX is a sensitive marker for DNA damage/repair during chemotherapy courses, and it allows detection of chemotherapy-induced cytotoxicity, and tumor progression³².

Moreover, the γ H2AX as a marker for DSBs allows immediate and long-term evaluation of the patient's response to chemotherapy, and to low dose radiation³³.

Therefore, the real-time response to ongoing chemotherapy was evaluated in this pilot study using the γ -H2AX foci, and the data of the FITC rupture and APC repair channels in 4 stages.

Stage 1 (before the 1st chemotherapy course), stage 2 (after the 1st chemotherapy course), stage 3 (before the 3rd chemotherapy course), and stage 4 (before the 4th chemotherapy course).

The FITC rupture channel during the ongoing chemotherapy showed significantly decreased foci intensity means ($p=0.0075$) and significantly increased colocalization ($p=0.02$).

The APC repair channels during the ongoing chemotherapy showed significantly increased nuclei intensity ($p=0.046$), foci overall ($p=0.0007$), clusters ($p=0.002$), foci mean ($p<0.0001$), and foci mean + clusters ($p=0.0003$). It also showed significantly increased clusters positive cells ($p<0.0001$), foci low intensity ($p<0.0001$), foci low intensity + clusters ($p=0.0003$), and clusters of low intensity positive cells ($p=0.002$).

The most significant fluctuation in the level of foci was observed before the 3rd chemotherapy course (stage 2), which manifested by increased DSBs repair and indicates the participants' response to ongoing chemotherapy.

The changes in the FITC rupture, and APC repair channels can serve as real-time indicators/markers for the response to ongoing chemotherapy in breast cancer women.

Dickey et al⁹, found that the γ -H2AX detection was a powerful tool for monitoring cancer development and progression.

Wang et al⁴, reported high frequency of γ -H2AX in breast cancer cells compared to normal cells in the same women with breast cancer, which could improve the early diagnosis of breast cancer.

Mahmoud et al³⁴, analyzed γ H2AX as a marker of radio-sensitivity in the blood cells of breast cancer women.

Recently, Durdik et al³⁵, suggested the detection of γ H2AX foci in the lymphocytes of women with breast cancer after radiotherapy to assess the tumor radiosensitivity.

This study was the first pilot study conducted in the Republic of Kazakhstan including 29 breast cancer women received adjuvant chemotherapy to evaluate the γ -H2AX foci and the changes in the FITC rupture and APC repair channels as real-time indicators for the response to ongoing chemotherapy.

The small sample size, and the limited data regarding the γ -H2AX as an indicator/marker for the response to ongoing chemotherapy in breast cancer women were the limitations of this study.

The γ -H2AX foci as an indicator for DSBs/repair pathway, and as an indicator for the response

to ongoing chemotherapy in breast cancer should be evaluated in further larger studies.

Conclusions

The γ -H2AX foci, and the changes in the FITC rupture, and APC repair channels can serve as real-time indicators for the response to ongoing chemotherapy in breast cancer women. The γ -H2AX foci as an indicator for DSBs/repair pathway, and for the response to ongoing chemotherapy in breast cancer, should be evaluated in further studies.

Conflict of Interest

The authors declare that they have no conflict of interests.

Acknowledgements

The authors are grateful to the studied participants for giving consent and participation in this pilot study.

Funding

The article processing charges were funded by the authors themselves.

Informed Consent

Informed consent was obtained from all the subjects involved in the study.

Ethics Approval

The study was approved by the West Kazakhstan Marat Ospanov Medical University (WKMU) Ethics Committee (Approval No. 57, dated 17/01/2020).

Availability of Data and Materials

The datasets generated and/or analyzed during the current pilot study are available from the corresponding author upon reasonable request.

Authors' Contribution

MA, GS, and SS are responsible for study concept and design, data collection, statistical analysis, and final revision before submission for publication. NK, AK, and AA are responsible for explaining the aim/objective of the study, interviewed the participants, data collection, and final revision before submission for publication. AT is responsible for statistical analysis, Microsoft editing, and literature re-

view, and final revision before submission for publication. DZ, GK, NI, and AD are responsible data interpretation, literature review, and final revision before publication. IAA is responsible for Microsoft editing, literature review, and final revision before submission for publication. SS, ZK, and GG are responsible for explaining the aim/objective of the study, Microsoft editing, literature review, and final revision before submission for publication. All the authors have read and agreed to the published version of the manuscript.

ORCID ID

Ibrahim A. Abdelazim: <http://orcid.org/0000-0002-7241-2835>; Marzhan Aitmagambetova: <https://orcid.org/0000-0002-0346-5829>; Gaziza Smagulova: <http://orcid.org/0000-0001-7222-620X>; Svetlana Sakhanova: <https://orcid.org/0000-0001-9786-6326>; Nurgul Kereyeva: <https://orcid.org/0000-0001-6205-4029>; Aryp Koishybaev: <http://orcid.org/0000-0002-6164-8009>; Ainur Amanzholkyzy: <https://orcid.org/0000-0002-1980-9032>; Anar Tulyaeva: <https://orcid.org/0000-0001-7149-0121>; Dinara Zholmukhamedova: <https://orcid.org/0000-0002-3899-2856>; Gulnar Kandygulova: <https://orcid.org/0000-0002-1219-9366>; Nauryzbai Imanbaev: <https://orcid.org/0000-0002-8339-9373>; Ainur Donayeva: <https://orcid.org/0000-0002-7363-0789>; Samat Saparbayev: <https://orcid.org/0000-0002-9570-4240>; Zaituna Khamidullina: <https://orcid.org/0000-0002-5324-8486>; Gulnara Gubasheva: <https://orcid.org/0000-0001-9869-3930>.

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