J Appl Biotechnol Rep. 2023 June;10(2):1009-1017

Journal of
Applied Biotechnology Reports

doi 10.30491/JABR.2023.366752.1570

Original Article

The Effect of Radiation on the Expression Level of *DANCR* and *TUG1* LncRNAs in Breast Cancer Cell Lines

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Received October 22, 2022; **Accepted** February 19, 2023; **Online Published** June 18, 2023

Abstract

Introduction: Radiotherapy is a standard and effective modality for breast cancer treatment, through induction of DNA damages notably DNA double-strand breaks which are involved in radiation-induced cell death. All radiation-induced DNA damages are subjected to various repair processes, therefore, interference in the DNA repair pathways might result in radio-resistance. Non-coding RNAs are a diverse group of functional RNA molecules that are not translated into proteins. Recent studies have shown that radiation can cause expression changes in non-coding RNAs.

Materials and Methods: MCF-7 and MDA-MB-231 cell lines were grown in a DMEM culture medium supplemented with fetal bovine serum and antibiotics. At exponential growth, cells were exposed to various doses of megavoltage X-rays. 24 and 48 h after irradiation cells were harvested, RNA was extracted and cDNA was synthesized. The expression level of lncRNAs was measured using quantitative real-time PCR.

Results: This study showed that radiation could increase *DANCR* and *TUG1* lncRNAs expression in breast cancer cell lines 24 and 48 h after receiving radiation. Also, the results suggested that after radiation, the expression of *DANCR* in the radioresistant cell line was higher than the radiosensitive one; in the case of *TUG1*, it's unlike *DANCR*.

Conclusions: The radiation increased the expression of *DANCR* and *TUG1* lncRNAs in breast cancer cell lines because the expression of *DANCR* in the MDA-MB- 231 was higher than in the MCF-7. In contrast, the expression level of *TUG1* in MCF-7 was higher than the MDA-MB-231. Therefore, lncRNAs, *DNACR*, and *TUG1* might play a role in the radioresistance and radiosensitivity of breast cancer, respectively.

Keywords: DNA Repair, *DANCR*, *TUG1*, Breast Cancer, Radiosensitivity, Radioresistance

Citation: Nejadtaghi M, Mozdarani H. The Effect of Radiation on the Expression Level of *DANCR* and *TUG1* LncRNAs in Breast Cancer Cell Lines. J Appl Biotechnol Rep. 2023;10(2):1009-17. doi:10.30491/JABR.2023.366752.1570

Introduction

Breast cancer is the most common malignancy in women and the second leading cause of cancer deaths, disturbing 2.1 million women every year.[1,2] Its prevalence is growing in Iran, which is known as the fifth leading cause of death, and the average age of onset in Iranian women is 45 years, while this figure is at least ten years higher in other countries.[3,4]

Radiotherapy is a standard, and effective treatment modality for breast cancer.[5,6] The most important target of ionizing radiation in cells is the structure of DNA, so the DNA repair pathways are one of the major targets of radiotherapy. Homologous recombination and non-homologous end joining (NHEJ) are the main DNA repair pathways for repairing DNA strand breaks.[7,8] Non-coding RNAs are a diverse group of functional RNA molecules that are not translated into proteins, which can be divided into small (<200 nt) ncRNAs, including microRNAs (miRNAs), and long (>200 nt) ncRNAs (lncRNAs).[9-11]

The length of lncRNAs varies from 200 nucleotides to less than 100 kb[12,13] and important biological functions have been defined for them. For example, lncRNAs are involved in tumorigenesis, invasion, metastasis, and angiogenesis.[14,15] In recent years, several lncRNAs have been shown to participate in DNA repair pathways, such as the *ATM*, *ATR*, and *p53* pathways, with both oncogenic function and tumor suppressor function as regulators.[15,16]

DANCR or *ANCR* is an 855 nucleotide lncRNA located on chromosome 4.[17-19] *DANCR* can be involved in DNA repair because of its effect on the EZH2 factor, which is a part of the PRC2 complex[20,21] and is a regulator of key double-stranded DNA repair proteins, including RAD51 and p53, and is associated with altered responses to DNA damage. This lncRNA interacts with *EZH2*, reducing its stability and protein levels in breast cancer cell lines, and causing its degradation.[18,22,23]

TUG1 is a long non-coding RNA of 7.1 kb, located on chromosome 22q12[24] and identified among 39 lncRNAs induced as p53-dependent after DNA damage[25] and observed to be transcriptionally activated after DNA damage[26] and is effective in Multiple cancers acting as an oncogene or tumor suppressor.[26, 27]

Based on their important functions in the DNA repair pathway and in regulating radiosensitivity or resistance in

different cancers, in this study expression level of *DANCR* and *TUG1* lncRNAs. was evaluated in MCF-7 and MDA-MB- 231 cell lines before and after irradiation with different doses of X-rays.

Materials and Methods

Cell Culture

MCF7 and MDA-MB- 231 breast cancer cell lines were obtained from the Pasteur Institute of Iran (Tehran, Iran). Cells were cultured in DMEM (Gibco) medium supplemented with 1% antibiotics, including penicillin (100 IU/ml) plus streptomycin (100 µg/ml) (PAN-Biotech GmbH) and 10% fetal bovine serum (FBS) (Bio-Idea, Iran), under the condition of 5% CO_2 atmosphere, 95% of humidity, and temperature of 37 °C. All the experiments were done when the cells reached the logarithmic growth state.

Irradiation

For irradiation, (3×10^6) cells were plated in 75 cm^2 culture flasks. When cells reached to approximately 70% confluence, the medium was renewed, and cells were irradiated using a six megaelectron volts linear accelerator (LINAC) (Elekta, Sweden) with a dose of 2, 4, and 8 Gy. Also, there was a sham control flask kept at the same condition without being irradiated. For each dose of radiation and control one, we had two flasks; one group of flasks was incubated for the next 24 h, and one group was incubated for the next 48 h. Upon incubation completion, cells were trypsinized and harvested. This procedure was repeated three times independently.

Total RNA Extraction and Quality Control

Total RNA from the cell lines was isolated using TRIzol (Geneall, South Korea) reagent according to the manufacturer's instructions. Nanodrop and agarose gel electrophoresis were utilized to check the purity, concentration, and integrity of RNA. The RNA solution was stored at -80 °C for further use.

DNase Treatment

To remove any probable genomic DNA contamination before cDNA synthesis, the extracted RNAs were treated with a DNase1 enzyme kit (Yektatajhiz, Tehran, Iran), according to the manufacturer's instructions.

RT-PCR and Primer Designing

cDNA synthesis was performed with a cDNA synthesis kit (Beta Bayern, Germany) according to the manufacturer's instructions. For cDNA synthesis, random hexamer primers were used. Also, for the qRT-PCR experiment, specific forward and reverse primers were designed with primer 3, oligo 7, and oligo analyzer software. Then primers were blasted with NCBI primer blast. The primer sequences used in this study are shown in the Table 1.

Table 1. The Sequences of the Specific Primers

Primer Sequences	Forward	Reverse
GAPDH	TGGATGCCACTGGCGTCTTC	TTGCTGATGATGATCTTGAGGCTGT
DANCR	TGACGCGCCACTATGTAGC	ACTTCCGCAGACGTAAGAGAC
TUG1	GCTCTCTTTACTGAGGGTGCTT	GGATCTGTCAAGTCTCAATGTTGG

Evaluation of LncRNA Levels Using Quantitative Real-Time RT-PCR

The qPCR was performed on Applied Biosystems step one plus Real-Time PCR System with SYBR green method using high Rox amplicon master mix by adopting the $2^{-\Delta\Delta ct}$ method. Glyceraldehyde phosphate dehydrogenase (*GAPDH*) was used as an endogenous reference. The lncRNA expression levels were detected by quantitative real-time PCR with the primes, as shown in Table 1. Each Real-Time PCR experiment was repeated two times for each lncRNA.

Statistical Analysis

All statistical analyses and figure creation were performed using GraphPad Prism 8 software. The one-way ANOVA test was used to compare differences in *DANCR* and *TUG1* expression after receiving different doses of radiation compared to the control sample. The independent t-test was used to compare between MCF-7 and MDA-MB-231 cell lines for each dose of radiation.

Results

DANCR LncRNA Expression Changes in Response to Radiation in the MCF-7 Cell Line

As shown in Figure 1 (A, B), irradiation increased the expression of *DANCR* lncRNA in the MCF-7 cell line after 24 h compared to the control sample; this upregulation peaked at 2 Gy irradiation. The increase after 2 Gy irradiation was more significant than the observed increase after 4 Gy. Also, receiving 8 Gy irradiation decreased the expression compared to 2 and 4 Gy.

Measuring this lncRNA's expression level after 48 h showed a dose dependent decrease in its expression after receiving 2, 4, and 8 Gy irradiation. This decrease was more pronounced at 8 Gy. The most significant decrease in expression levels was observed for doses 4 and 8 Gy. Also, this decrease between 2 and 4 Gy and 2 and 8 Gy was significant ($p<0.05$).

DANCR LncRNA Expression Changes in Response to

Radiation in the MDA-MB-231 Cell Line

As shown in Figure 1 (C, D), expression changes of *DANCR* in the MDA-MB-231 cell line, indicates similar expression pattern with MCF-7 cells after 24 h. It means there is a significant increase after receiving 2 and 4 Gy irradiation compared to the control without receiving any radiation. This increase peaked at 2 Gy irradiation and was more significant than the observed increase at 4 Gy. Also, the expression level of *DANCR* decreased after 8 Gy compared to 2 and 4 Gy. There was a significant upregulation after 4 Gy irradiation and at 48 h incubation time compared to those recieved 2 and 8 Gy radiation.

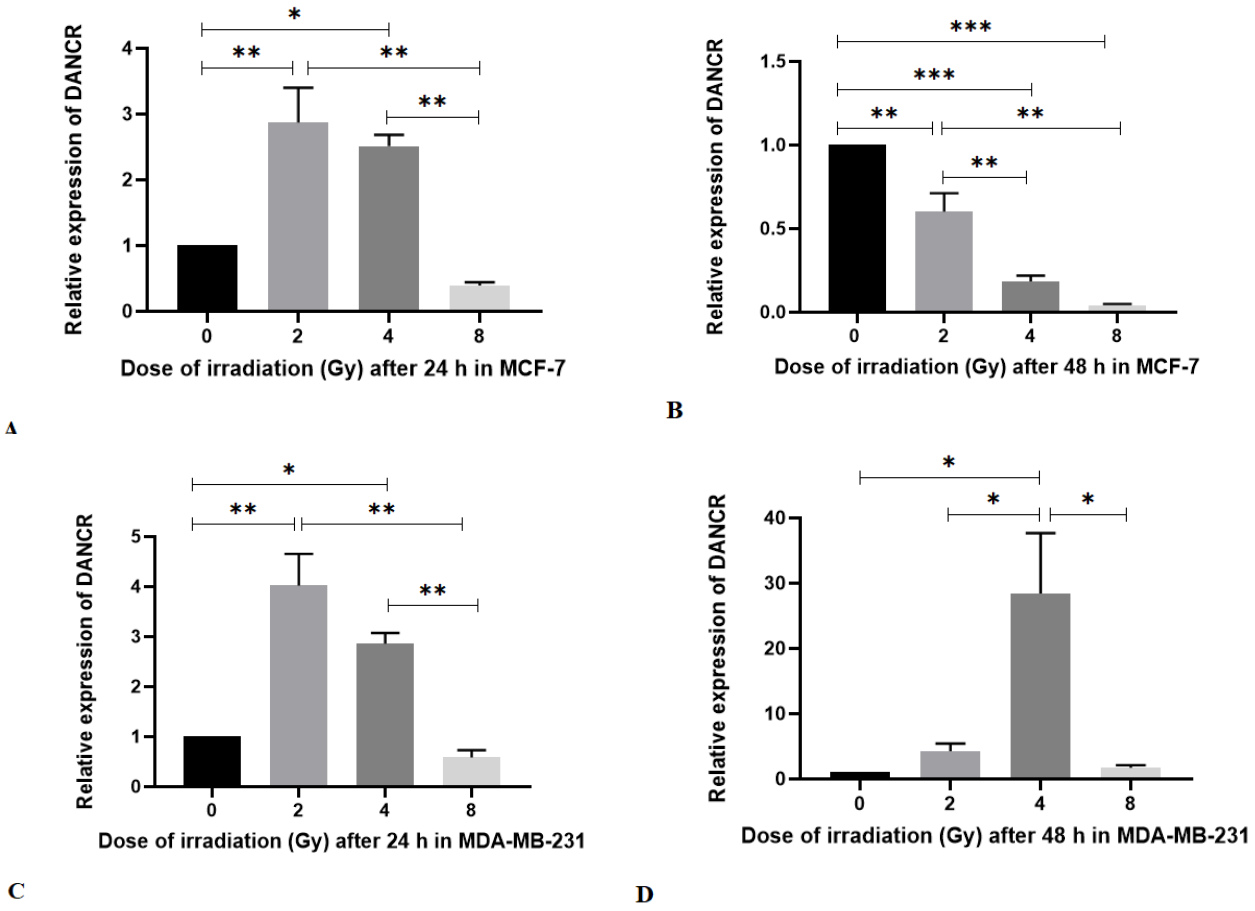

Figure 1. The Expression Changes of *DANCR* LncRNA in Response to Different Doses of Radiation in the MCF-7 Cell Line after 24 h **(A)** and 48 h **(B)**. And in the MDA-MB-231 Cell Line after 24 h **(C)** and 48 h **(D)**. *p<0.05; **p<0.01, ***p<0.001.

A Comparison Between the Relative Expression of DANCR LncRNA in MCF-7 and MDA-MB-231 Cell Lines

As demonstrated in Figure 2, the relative expression of *DANCR* lncRNA in the MDA-MB-231 compared with MCF-7 cell lines was significantly higher for both sampling times (24 and 48 h) for all doses of radiation. However, the expression level of *DANCR* was more pronounced at 48 h.

LncRNA TUG1 Expression Changes in Response to Radiation in the MCF-7 Cell Line

As shown in Figure 3 (A, B), the expression level of *TUG1* in the MCF-7 cells receiving 2, 4, and 8 Gy compared to the control without receiving any irradiation was measured 24 and 48 h after irradiation. The results suggested that 24 h after irradiation, expression of this lncRNA was increased for doses of 4 and 8 Gy in MCF-7 cell lines. Also, there was a significant decrease in its expression after receiving 4 Gy compared to 2 Gy irradiation.

LncRNA TUG1 Expression Changes in Response to Radiation in the MDA-MB-231 Cell Line

Figure 3 (C, D) shows the expression level of *TUG1* in the MDA-MB-231 cell line following receiving radiation doses of 2, 4, and 8 Gy compared to the control at 24 and 48 h post-irradiation incubation. The results demonstrated that 24 h after irradiation, the expression of this lncRNA in 4 Gy in the MDA-MB-231 cell line was increased. Also, the expression in 4 Gy compared to 2 Gy was more significant, and then there was a significant decrease in 8 Gy compared to 4 Gy. The expression level *TUG1* 48 h after irradiation, increased significantly following 8 Gy compared to the control, 2 and 4 Gy irradiated cell.

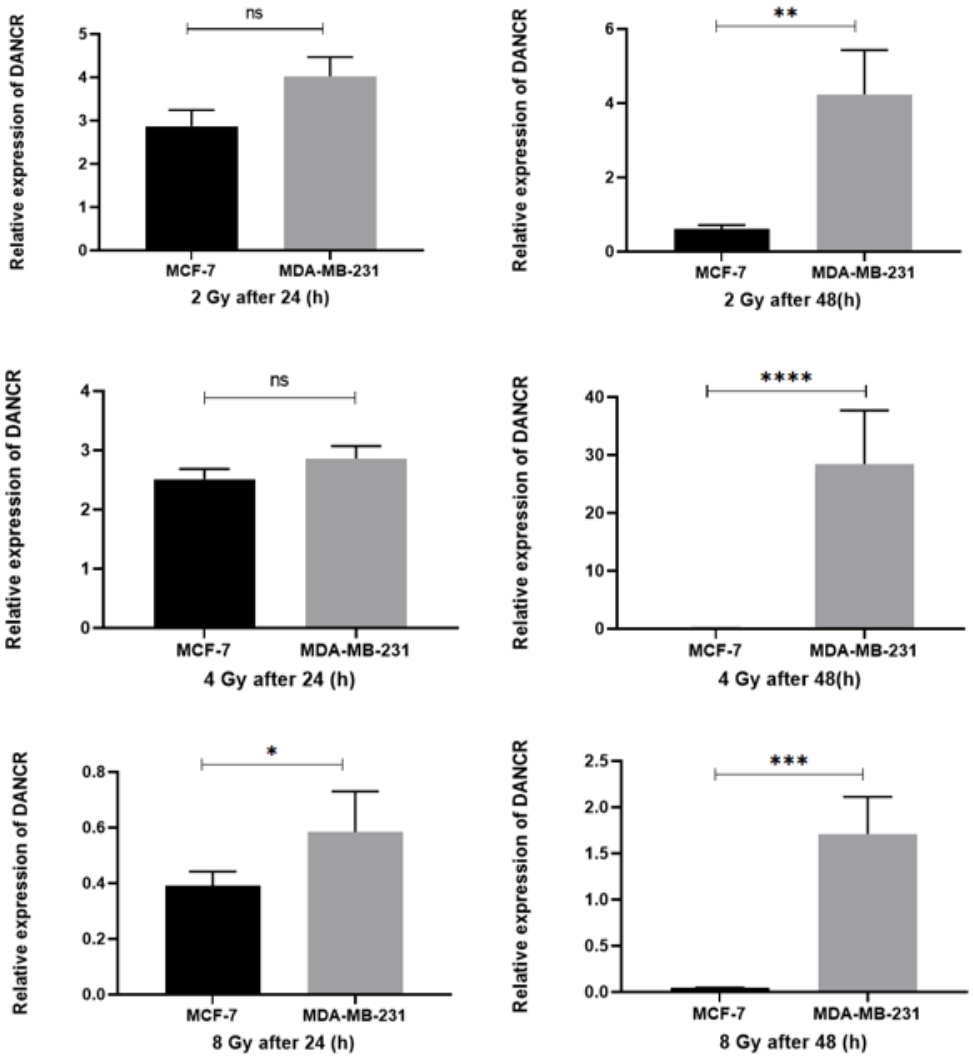

Figure 2. A Comparison of the Relative Expressions of *DANCR* LncRNA between MCF-7 and MDA-MB-231 Cell Lines in each Radiation Dose after 24 h and 48 h. *p<0.05; **p<0.01, ***p<0.001.

A Comparison between the Relative Expressions of TUG1 LncRNA in the MCF-7 and the MDA-MB-231 Cell Lines

As demonstrated in Figure 4, 24 and 48 h after irradiation with different doses of X-rays, the expression of TUG1 in MCF-7 cell line increased significantly compared to MDA-231 cell line. After 48 h post-irradiation with 8 Gy, its expression in the MDA-MB-231 cell line increased significantly compared to the MCF-7 cell line. 24 h after receiving radiation, the most differences between MCF-7 and MDA-231 cell lines were observed at dose of 2 Gy. 48 h after irradiation, the most differences between the two cell lines were observed at 8 Gy. Also, it is shown that after 24 h, there are more differences in the expression level of *TUG1* between MCF-7 and MDA-MB-231 cell lines when receiving different doses of radiation.

Discussion

Cancer is known as one of the principal causes of death worldwide.[28,29] Although radiotherapy is one of the major methods for cancer therapy, normal tissue toxicity and tumor recurrence are main limitations for proper treatment. Therefore, to solve this problem, the radiosensitivity of patients should be optimized.[7,30,31] On the other hand, radiotherapy, in addition to destroying tumor cells, also affects healthy cells around the tumor and causes treatment-related complications that can cause a different response in each patient.[32,33]

The most important target for ionizing radiation in cells is DNA molecule which results in DNA damages, especially DNA double-strand breaks (DSBs).[34,35] DSBs are thought to be the leading cause of cell death, and large-scale genomic changes result from DSB repair defects. So, they can create chromosomal instability directly associated with cancer progression.[36] The two main pathways for DSBs repair are homologous recombination (HR) and non-homologous end junction (NHEJ).[37] The studies suggested that response to

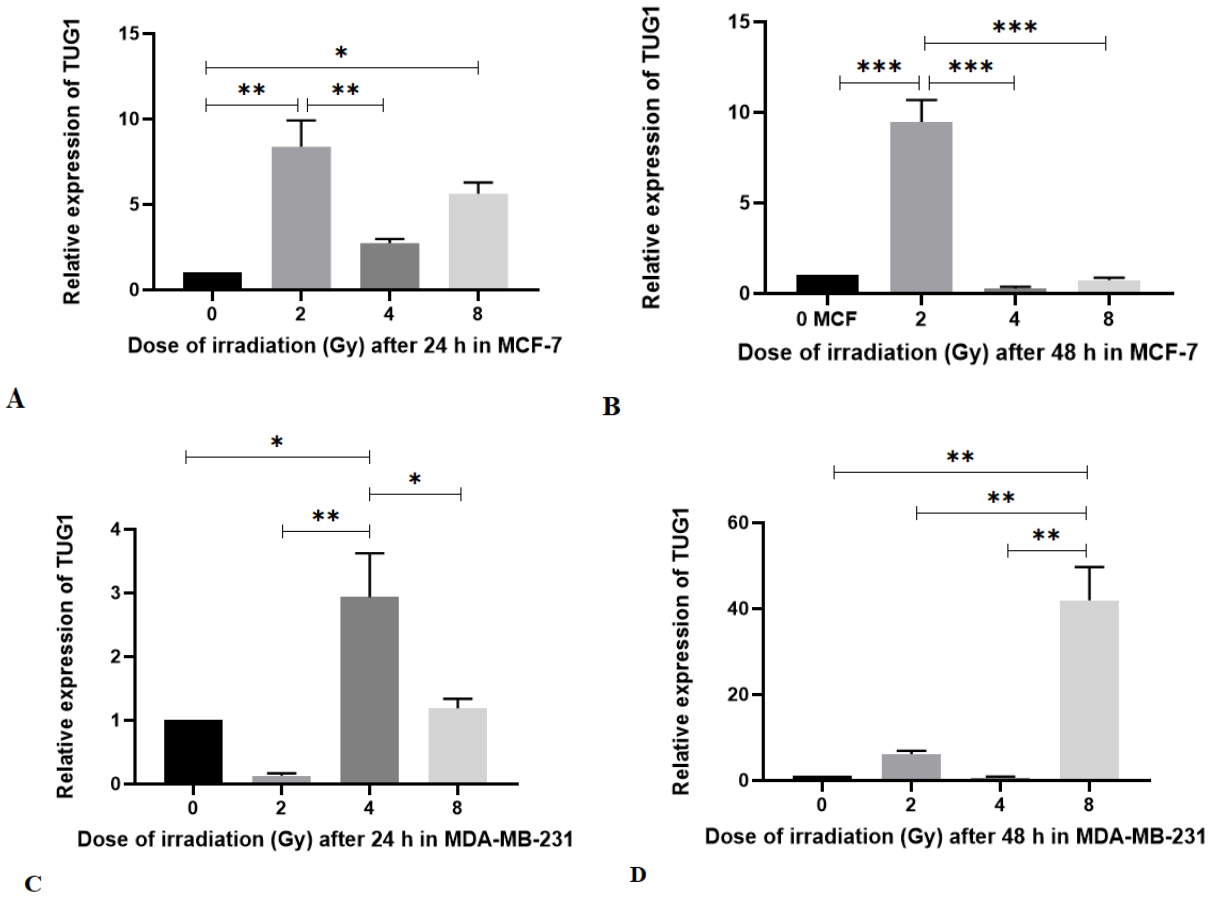

Figure 3. The Expression Changes of *TUG1* LncRNA in Response to Different Doses of Radiation in the MCF-7 Cell Line after 24 h **(A)** and 48 h **(B)**. And in the MDA-MB-231 cell line after 24 h **(C)** and 48 h **(D)**. *p<0.05; **p<0.01, ***p<0.001.

radiation treatment is modulated by cellular processes such as DNA damage repair. Thus, identifying markers playing a role in radioresistance and radiosensitivity is critical for detecting responsive patients and improving the outcome of radiotherapy.[38-41]

Recently, it has been shown that ionizing radiation could change the expression pattern of non-coding RNAs (ncRNAs), including microRNAs and lncRNAs, whose expression levels can be determined by RT-PCR, NGS, and microarray.[42,43] Recent studies showed that lncRNAs were involved in DNA damage repair (DDR) machinery by sensing DNA damage, signal transduction, repairing damaged DNA, activating cell-cycle checkpoints, and inducing apoptosis.[44,45]

It was demonstrated that *DANCR* was overexpressed in many cancers, playing an oncogenic role. For breast cancer, both overexpression and down regulation were shown for *DANCR*.[46-49] To date, there is no report regarding expression changes of *DANCR* in breast cancer cell lines in response to radiation. But in triple negative breast cancer, the upregulation of *DANCR* was demonstrated in cancerous tissues.[47]

In the present study, it was shown that irradiation could increase the expression level of *DANCR* lncRNA in MCF-7 and MDA-MB-231 cell lines after 24 and 48 h post-irradiation. The most effective dose causing upregulation for *DANCR* was 2 and 4 Gy in both cell lines. Also, it was shown that the difference between these two cell lines after 48 h was more evident compared to after 24 h from receiving radiation.

Comparing *DANCR* expression changes between MCF-7 and MDA-MB-231 cell lines suggest that 24 and 48 h after irradiation the expression level of *DANCR* in radioresistant MDA-MB-231 cell line was more significant than radiosensitive MCF-7 cell line. This result suggests the probable role of *DNACR* in breast cancer radioresistance. The pathways that this lncRNA play a role in the DNA repair system in response to radiation might be different. *DANCR* may cause repression of *EZH2*,[50] a key regulator of double-stranded DNA repair proteins, including RAD51 and p53. Also, *EZH2* is associated with altered responses to DNA damage.[51] In addition, in a study with nasopharyngeal carcinoma cells, it is shown that *DANCR* causes repression of *PTEN*,[52] which plays an important role in the promotion of the DNA repair system.[53] Moreover, the Ras/phosphoinositide 3-kinase *(PI3K)/PTEN* pathway is one of the activated pathways in breast cancer that cause radioresistance[54] and *DANCR* plays a role in activating the *PI3K/AKT* pathway.[49] In another

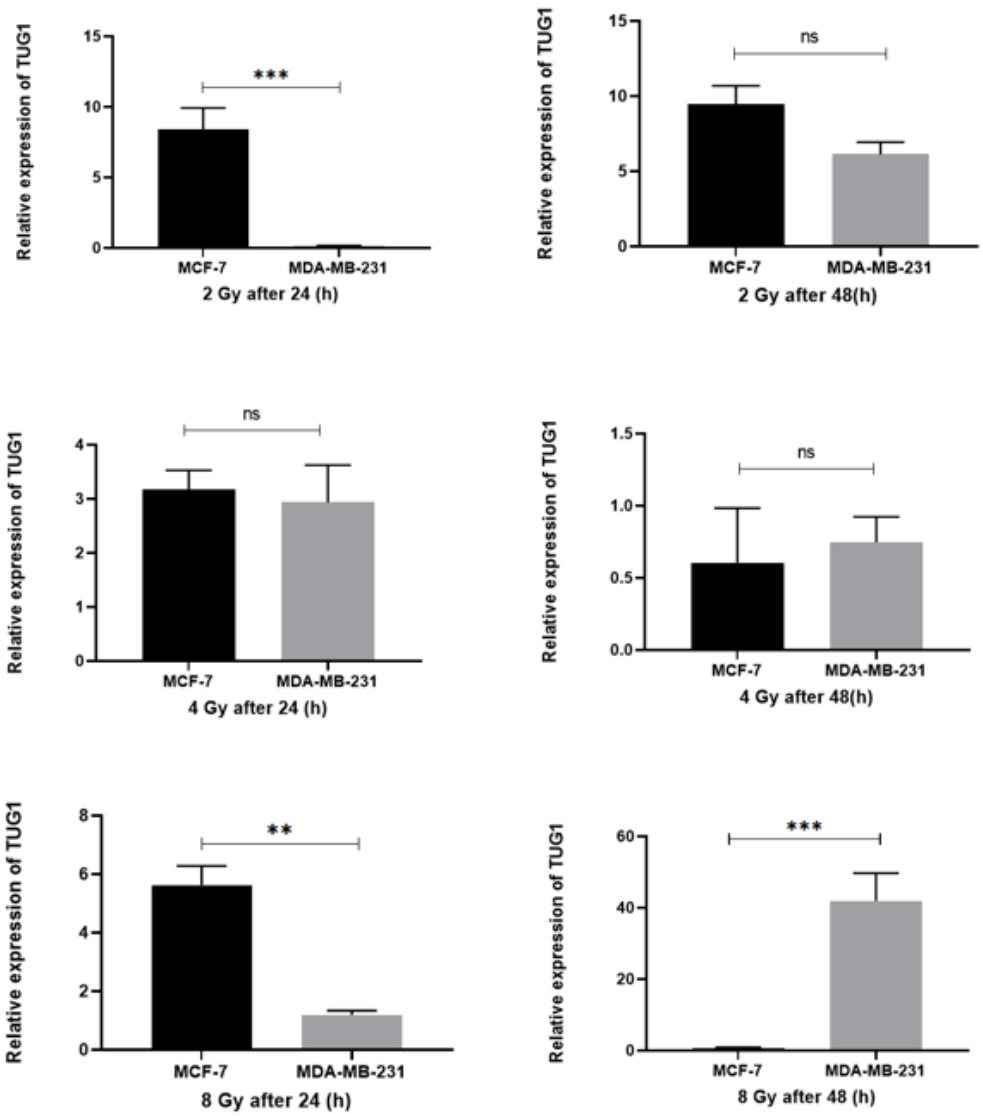

Figure 4. A Comparison between the Relative Expressions of *TUG1* LncRNA between the MCF-7 and the MDA-MB-231 Cell Lines in each Dose of Radiation after 24 h and 48 h. *p<0.05; **p<0.01, ***p<0.001.

study with melanoma, it was shown that *DANCR* could suppress miR-335, which plays a role in radiosensitivity of melanoma.[55]

Regarding expression changes of *DANCR* under the effect of radiation, one study showed its role in proliferation and radiation resistance of nasopharyngeal carcinoma by increased expression level.[52]

TUG1 is a lncRNA with oncogenic or tumor suppressor function in many cancers.[26, 27] Recent studies have demonstrated that upregulation of *TUG1* is presumably a predictor of survival in human cancer and is closely associated with increased tumor size, advanced pathological stage, and distant metastasis.[56,57] It is shown that this lncRNA is activated upon DNA damage in a *p53* dependent pathway and is regulated by *p53*[58] or itself targets p53 via miRNAs.[59] Since the tumor suppressor gene *p53* regulates cellular processes after IR, its function in cancer cells closely relates to radiotherapeutic efficacy.[60,61] As a target gene of *p53*, *TUG1*, by promoting cell cycle arrest and apoptosis by regulating some miRNAs, including miR-221, increases cell radiosensitivity.[61] *TUG1* has a suppressor function on miR-221, this miRNA plays role in promotion of DNA repair pathway and cause radioresistance.[62,63] Also, it was shown that *TUG1* could activate *HMGB1*,[64] that plays role in DNA repair pathway[65] cause radiosensitivity and is a radiosensitizer in breast cancer.[66]

In prostate cancer it was shown that *TUG1* represses *SMCA1*, a gene involved in radioresistance.[67, 68]

Moreover, it was shown that similar to *DANCR*, *TUG1* could regulate *EZH2*,[69] a key regulator of DNA damage response and also *p53*.

It was shown that irradiation could increase its expression level of *TUG1* in MCF-7 and MDA-MB-231 cell lines After 24 and 48 h after irradiation. Doses of 2 and 8 Gy were the most effective dose causing upregulation in both cell lines. Moreover, for it was shown that the expression level of *TUG1*

after irradiation of both cell lines was more significant at 24 h sampling time compared to 48 h sampling time.

To date, there is limited or no report regarding expression level of *TUG1* in response to radiation in breast cancer, but a study done on breast cancer tissues showed that expression level of *TUG1* was significantly down-regulated compared to corresponding normal tissues.[70] However, one study in relation to the role of lncRNA *TUG1* on radiosensitivity of prostate cancer showed that its expression was remarkably increased after irradiation.[68] Also, the qRT-PCR analysis revealed the upregulation of *TUG1* in response to radiation in bladder cancer cell lines in a time- and dose-dependent manners.[64] Another study done by Wang et al., showed a significant up-regulation of *TUG1* in radiosensitive ESCC tissues and cell lines compared to the resistant ones.[71]

In a study by Lucian Beer et al., showed a significant upregulation of *TUG1* after irradiation of peripheral blood mononuclear cells of healthy individuals. Also, this study revealed a strong time dependent regulation of a variety of lncRNAs, including *TUG1*.[60]

Conclusion

In conclusion, the present study showed that irradiation causes a significant increase in *TUG1* and *DANCR* lncRNAs expression in MCF-7 and MDA-MB-231 cell lines in a dose and time dependent manner. Also, it is demonstrated that *DANCR* and *TUG1* expression significantly differ between these two breast cancer cell lines. These results in addition to the recent evidences about the role of *DANCR* and *TUG1* in DNA repair pathways are indicative of response to radiation in breast cancer cells. Therefore, there might be a possibility that *DANCR* and *TUG1* could be regarded a biomarker for response to radiation.

Authors' Contributions

MN performed experiments, samples collection, data curation, data analysis, and wrote the manuscript draft. HM designed and supervised the research plan prepared, data analysis, edited and approved the final manuscript.

Funding

This research has been supported by Tarbiat Modares University Tehran, Iran by grant number IG-39711 provided to Prof. H. Mozdarani.

Conflict of Interest Disclosures

The authors declare that they have no conflicts of interest.

Acknowledgment

This study was supported by the Research Department of the Tarbiat Modares University (grant number: IG-39711), Tehran, Iran. The authors also thank Mr. Hassan Nosrati for irradiation of samples.

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