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Original Article

Significant Impact of let-7d MicroRNA on Breast Cancer Cell Lines Post-Radiation Treatment

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Introduction: Radiotherapy is a common treatment for breast cancer treatment, that induces DNA damage. These DNA damages are addressed through various repair pathways, which regulate the DNA repair systems and confer radio-resistance. Non-coding RNAs are a big proportion of genome transcripts without the potential to encode proteins. Related studies demonstrated that radiation affects the expression of non-coding RNAs. Let-7d, a tumor suppressor in numerous cancers, has some target genes that play a role in the DNA repair system.

Materials and Methods: Human breast cancer cell lines MDA-MB-231 and MCF-7 were cultured in a Dulbecco's Modified Eagle Medium. The exponentially growing cells were treated with some doses of X-rays. After radiation treatment and cell harvesting, RNA was extracted, and cDNA synthesis was done. The let-7d miRNA expression changes were calculated with real-time quantitative reverse transcription polymerase chain reaction.

Results: The results implied that radiation caused increased let-7d expression in breast cancer cell lines after radiation treatment. In addition, the results showed that 24 h after radiation, the expression of let-7d in the radioresistant cell line was higher than the radiosensitive one; 48 h after radiation, the expression of let-7d in the radiosensitive cell line was higher than the other one.

Conclusions: The results demonstrated that radiation treatment increased let-7d miRNA expression in both radiosensitive and radio-resistant breast cancer cell lines. Therefore, let-7d might be involved in the radiosensitivity of breast cancer.

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Introduction

Breast cancer is the second leading cause of cancer deaths and the most common malignancy in women. According to statistics, there is a significant rise in breast cancer incidence, especially in Iran, and the average age of onset in Iranian women is 45 years, while this figure is at least ten years higher in other countries [1, 2]. At present, radiotherapy is the main means for patients with advanced breast cancer [3]. Ionizing radiation has a destroying effect in the cells called Double Strand Breaks (DSBs), so the DNA repair pathways are involved in response to radiotherapy [4]. The most important DNA repair pathways for repairing DNA strand breaks are homologous recombination and non-homologous end joining (NHEJ) [5-7]. Non-coding RNAs constitute a large fraction of genome transcripts without being able to code for proteins. Which can be divided into small (200 nt) and long ncRNAs (lncRNAs) [8, 9]. LncRNAs range from 200 nucleotides to less than 100 kb in length [10, 11]. miRNAs are a class of small non-coding RNA molecules that negatively regulate gene expression at the post-transcriptional level by altering the stability or translational efficiency of its target mRNAs [12, 13]. In the context of the DNA damage response, miRNAs may determine cell fate by dictating the appropriate cellular outcome in response to different levels of DNA damage [14]. In support of this, recent studies have shown expression patterns of miRNAs that are associated with increasing doses of ionizing radiation and ultraviolet [15, 16].

The let-7 family of miRNAs play a vital role in regulating carcinogenesis; one of its members is let-7d, which is located on chromosome 9q22.3, which acts as a tumor suppressor in numerous human cancers [17]. This miRNA has some target genes that play a role in the DNA repair system [18-20]. One study demonstrated that in radioresistant and radiosensitive glioblastoma cell lines, the let-7d expression was higher in radioresistant cell line. Also, they showed that upregulation of let-7d caused radioresistance [21]. In another study, the results suggested the role of let-7d in the radiosensitivity of breast cancer stem cells [22]. Evaluating let-7d expression changes in response to radiation in endothelial cells showed its upregulation after receiving radiation [23]. Also, let-7d plays a role in the sensitivity of hypopharyngeal carcinoma cells to radiation and chemotherapeutic drugs [24]. Also, its shown that let-7d could suppress the Epithelial-mesenchymal transition pathway [25], and its demonstrated that this pathway could cause radioresistance in many cancers [26].

The goal of the present study was to evaluate let-7d miRNA expression changes under the effect of different radiation doses in MCF-7 and MDA-MB- 231 breast cancer cell lines that have somewhat inherent sensitivity and resistance to radiation respectively, to explore let-7d potential as novel markers for irradiation in breast cancer.

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Materials and Methods

Cell Culture

MCF7 and MDA-MB- 231 breast cancer cell lines were obtained from the Pasteur Institute of Iran (Tehran, Iran) and cultured at 37 °C in humidified 5% CO2. All the cell lines were cultured in Dulbecco's Modified Eagle (DMEM Gibco) medium supplemented with 10% fetal bovine serum (FBS) (Bio-Idea, Iran) and 1% penicillin/ streptomycin (PAN-Biotech GmbH).

Irradiation

Cells were irradiated using a 6-MeV linear accelerator (LINAC) (Elekta, Sweden) with 2, 4, and 8 Gy radiation doses, and we had a control flask without receiving any radiation dose. For all the radiation treatments and the control sample, 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. The radiation doses were selected based on related articles. Finally, after trypsinizing, the cells were harvested. We performed the experiments in triplicate.

RNA isolation and quality control

Total RNA was extracted from cells using TRIzol reagent (Geneall, South Korea) according to the manufacturer's instructions. The concentration and quality of the isolated RNA were assessed on a Nanodrop ND-1000 spectrophotometer. All extracted RNAs were stored at -80°C.

Real-time polymerase chain reaction (RT-PCR) and primer designing

For cDNAs synthesis, a reverse transcription kit (Beta Bayern, Germany) was used according to the manufacturer's instructions. Stem-loop primers were used for the reverse transcription of let-7d miRNA.

The RT-PCR was performed using SYBR green with Applied Biosystems instrument in duplicate by adopting the $2^{-\Delta\Delta ct}$ method. The expression levels of the miRNA were normalized to the endogenous control U6. The specific primer sequences are shown in table 1.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 8 software. The data were expressed as means ± SD. The one-way ANOVA test evaluated the difference between groups. For comparing MCF-7 and MDA-MB-231 cell lines, the independent t-test was used for each dose of radiation.

Results

To study the effects of irradiation on let-7d miRNA expression in MCF-7 and MDA-MB-231 cells, we employed Real-time PCR before and after receiving different radiation doses. Finally, the results suggested up-regulation of let-7d miRNA expression after irradiation.

Table 1. The sequence of primers

Gene	Forward primer	Reverse primer
Let-7d	ACGCAGAGGTAGTAGGTTGC	TGCAGGGTCCGAGGTATTCG
U6	CTCGCTTCGGCAGCACATATACT	ACGCTTCACGAATTTGCGTGTC

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Let-7d Expression changes in response to radiation in the MCF-7 cell line

let-7d expression was up-regulated in the MCF-7 cell line at 24 and 48 hours post-irradiation compared to the control sample. Approximately 24 hours after irradiation, the clinically relevant dose of 8 Gy ($p < 0.01$) induced significant changes in let-7d miRNA levels. 48 h after irradiation, there was up-regulation in 2 ($p < 0.001$), 4 ($p < 0.001$) and 8 Gy ($p < 0.0001$) that peaked at 8 Gy (Figure 1 A, B).

Let-7d expression changes in response to radiation in the MDA-MB-231 cell line

As observed in the MCF-7 cell line, let-7d expression was up-regulated in the MDA-MB-231 cell line 24 h and 48h after irradiation in comparison with the control sample. 24 h after irradiation, clinically relevant doses of 2 ($p < 0.001$), 4 ($p < 0.0001$) and 8 Gy ($p < 0.001$) induced significant changes in let-7d miRNA levels. This upregulation peaked at 4 Gy. 48 hours after irradiation, there was up-regulation in 2 ($p < 0.001$) and 4 ($p < 0.05$) Gy that peaked at 2 Gy (Fig.1 C, D).

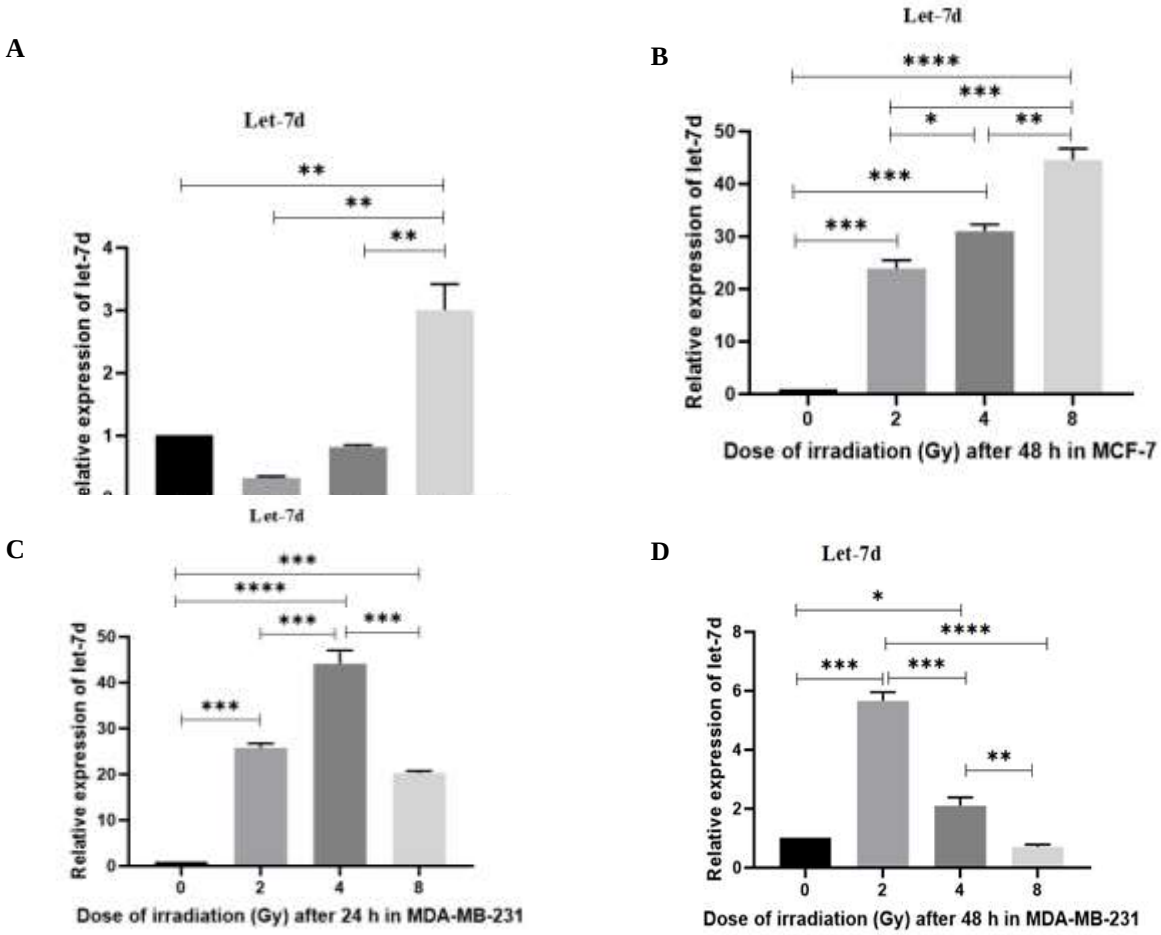

Fig. 1. The let-7d miRNA expression changes after receiving different radiation doses in the MCF-7 cell line after 24 h (A) and 48 h (B). And in the MDA-MB-231 cell line after 24 h (C) and 48 h (D). *p < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

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A comparison between the relative expression of let-7d miRNA in MCF-7 and MDA-MB-231 cell lines

The relative expression of let-7d miRNA 24 h after receiving irradiation in the MDA-MB-231 cell line compared with the MCF-7 cell line was significantly higher for all doses of radiation. This difference was more pronounced at 4 Gy. These findings are demonstrated in Figure 2.

However, 48 h after receiving irradiation the let-7d expression in the MCF-7 cell line was significantly higher than the MDA-MB-231 cell line. At this time, the difference was more pronounced at 8 Gy.

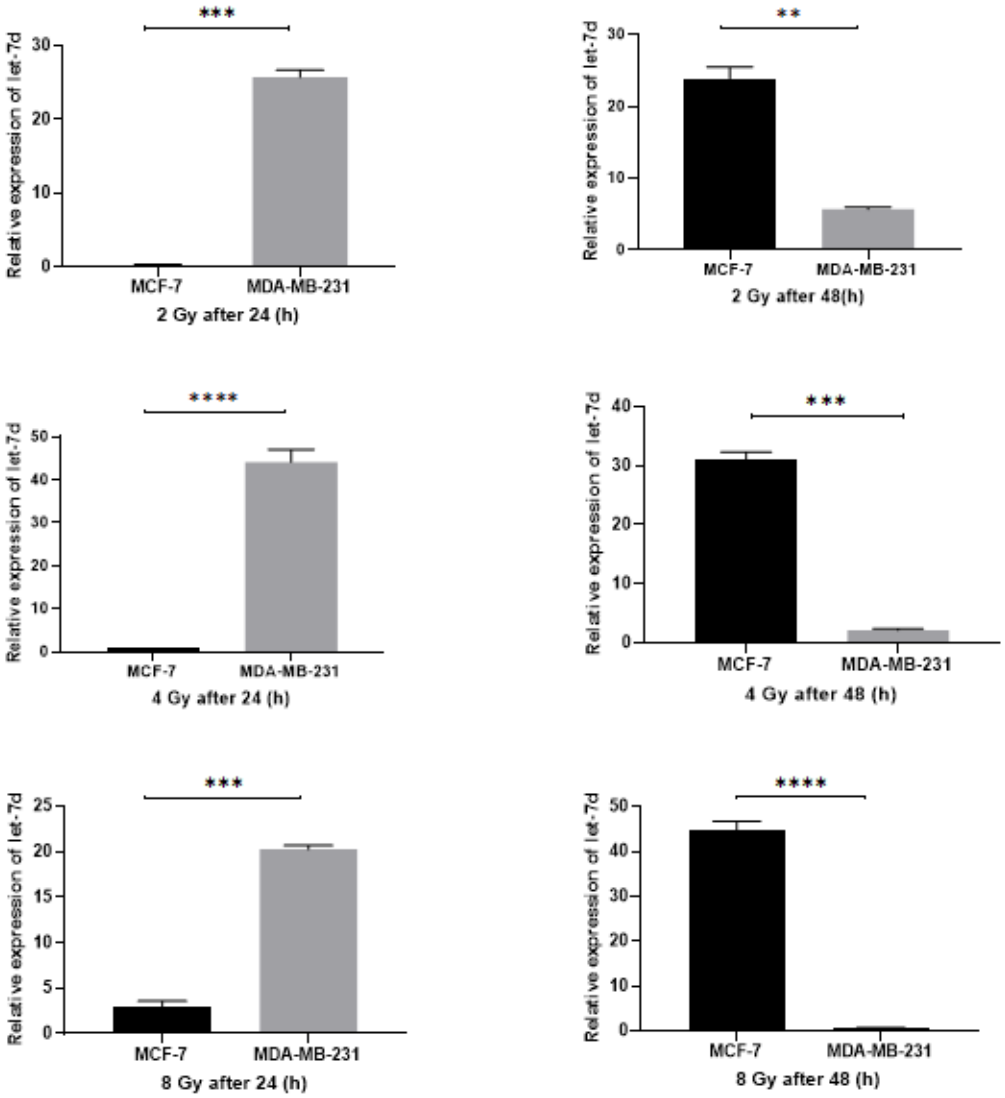

Fig. 2. A Comparison of the relative expressions of let-7d miRNA between MCF-7 and MDA-MB-231 cell lines in each radiation dose 24 h and 48 h after receiving radiation. *p < 0.05, **p < 0.01, ***p < 0.001, ****P < 0.0001.

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Discussion

breast cancer is among the most frequent causes of women's death worldwide. Radiotherapy is one of the mainstream approaches for its treatment. Radiation promotes a series of changes in tumor cells. DNA damage response is one of the main factors which regulate cell survival after radiation [27]. So, insight into the effect of radiation on DNA damage pathways provides an improvement in the prognosis of cancer patients [28].

In recent years, non-coding RNAs, especially miRNAs, as a hot spot, have attracted widespread concern [29]. MicroRNAs are a class of regulatory molecules, and miRNAs have an emerging role in modulating cell physiology and different pathways like DNA repair. It described that miRNAs could be induced by radiation, playing a role as intermediate molecules in the regulation of DNA damage response [30, 31].

Several previous studies have indicated that there is a potential for plasma miRNAs as valuable biomarkers of radiation exposure. A growing body of evidence indicates that let-7d miRNA has expression changes in response to radiation and plays a pivotal role in radio sensitivity or radioresistance of tumor cells.

In order to show let-7d role in the etiology of breast cancer, one study by Wei et al. showed that let-7d was downregulated in breast cancer tissues, and also, they showed that let-7d inhibits growth and metastasis in breast cancer [32].

In the present study, we determined the expression levels of let-7d miRNA in MCF-7 and MDA-MB231 breast cancer cell lines under the effect of radiation and found that let-7d was overexpressed in the irradiated group as compared with controls. Some other studies are showing similar changes in let-7d expression produced on radiated cells. For example, in the study by Lamperska et al., increased levels of this miRNA expression were shown under the effect of radiation in irradiated hypopharynx cancerous cells [24]. In another study, the results suggested an upregulation of let-7d expression after radiation to endothelial cells [23].

To evaluate the relationship between let-7d and radiosensitivity/resistance of breast cancer, a comparison between the expression level between MCF-7 and MDA-MB-231 cell lines was performed. The higher expression level of let-7d in the radioresistant cell line 24 h after receiving radiation and the higher expression level in the radiosensitive cell line 48 h after receiving radiation suggested that besides playing a role in radiosensitivity, let-7d miRNA would contribute to the radioresistance of breast cancer. However, the mechanism of how this miRNA plays these roles is not clear yet. Basic research on its function on radiosensitivity/ resistance of breast cancer is urgently needed. However, probably because of the role of let-7d in both the DNA repair pathway and apoptosis [33], 24 h after receiving radiation, its function in the DNA repair pathway is more activated. Hence, its expression level was higher in radioresistance cell line and 48 h after radiation because of the severity of damage its role in apoptosis is more activated, so the expression level in a radiosensitive cell line is higher than the other one. Different studies are showing the

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role of let-7d in the radiosensitivity/ resistance of various cancers. For example, one study demonstrated that in glioblastoma, its upregulation caused radioresistance [21]. Alternatively, one study suggested the role of let-7d in the radiosensitivity of breast cancer stem cells [22].

Conclusion

This study's results suggested that let-7d miRNA has the potential to be a radiation marker in breast cancer; in addition, maybe it could play a role in the radiosensitivity/resistance of breast cancer patients. However, verifying these findings needs functional studies, in addition to evaluating this miRNA expression in breast cancer patients.

Ethical Considerations

All ethical considerations were followed in compiling this work. This study was supported by the Research Department of Tarbiat Modares University (grant number: IG-39711, ethics code: (IR.MODARES.REC.1399.010), Tehran, Iran.

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Conflict of Interest

The authors declare that there is no conflict of interest regarding this article's publication and financial issues.

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Authors' Contributions

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

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