

Analysis of microRNAs expression changes in human breast cancer cell lines following exposure to ionizing radiation

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ABSTRACT

► Short Report

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Revised: May 2016

Accepted: June 2017

Int. J. Radiat. Res., July 2018;
16(3): 383-388

DOI: 10.18869/acadpub.ijrr.16.3.383

Background: The expression alterations of specific miRNAs were analysed in response to gamma-irradiation in two breast cancer cell lines MCF-7 and MDA-MB-231 with low and high BCSCs content to design probable strategies for sensitizing breast cancer stem-like cells MDA-MB-231 to radiation. **Materials and Methods:** The expression levels of hsa-miR-34a, hsa-let-7i and hsa-miR-21 were assessed by QRT-PCR in MCF-7 and MDA-MB-231 breast cancer cells at three time points after gamma irradiation using three different doses. **Results:** The results showed that the over-expression of hsa-miR-34a in MCF-7 cells was much more significant than MDA-MB-231 cells. Furthermore, there was a considerable over-expression of hsa-miR-21 in MDA-MB-231 cells following exposure to ionizing radiation (IR). The hsa-let-7i expression changes were different, depending on radiation dose, time post irradiation and cell type. **Conclusion:** According to the results, we may be able to sensitize stem-like MDA-MB-231 breast cancer cells to radiation by increasing miR-34a and decreasing miR-21 expression levels simultaneously.

Keywords: miR-34a; miR-21; let-7i; ionizing radiation; breast cancer.

INTRODUCTION

The most frequent carcinoma and second reason of cancer-related mortality in women, is breast cancer ⁽¹⁾. Today, the main clinical obstacle to the successful therapy of breast cancer is treatment resistance such as radio-resistance, which results in metastasis and relapse. Growing evidence show that one of the most important reasons behind this is the presence of a rare population of stem-like cells called breast cancer stem cells (BCSCs). Therefore finding a way to reduce BCSCs radio-resistance results in better clinical outcomes for patients with breast cancer ⁽²⁾.

MicroRNAs (miRNAs) constitute a group of evolutionarily conserved endogenous, single stranded and non-coding RNA molecules consisting of approximately 22 nucleotides in length. MicroRNAs have critical roles as

post-transcriptional regulators for gene expression in the cell cycle, DNA damage response, and apoptosis pathway. It should be noted that the mRNA degradation or translational inhibition functions of these molecules are exerted through their sequence-specific binding to the 3' un-translated regions of target mRNAs ⁽³⁾. Therefore, specific miRNAs are often up- or down-regulated in response to irradiation; hence more information about miRNAs regulation and function will be critical to develop radiation therapy ⁽⁴⁾.

MicroRNA 21 (miR-21) regulates some tumour suppressor genes, such as phosphatase and tensin homolog (PTEN), programmed cell death 4 (PDCD4), tropomyosin 1 (TPM1) and cell division cycle 25A (Cdc25A) ^(5,6). So it could be involved in the cellular response to IR and promote radio-resistance of breast cancer cells by inducing G2/M cell cycle arrest ⁽⁷⁾. In contrast,

miR-34a is a tumour suppressor, which targets several genes involved in DNA damage response (DDR) such as *Bcl-2*, *Notch1*, high mobility group A2 (*HMGA2*), *Cyclin D1*, *Cyclin E2*, cell division cycle 2 (*CDK1*), cyclin dependent kinase 4 and 6 (*CDK4* and *CDK6*), *MET* and sirtuin 1 (*SIRT1*)⁽⁸⁾ and may be used as a marker and therapeutic target for cell response to radiation⁽⁹⁾. Also, *miR-34a/Notch1* is involved in regulating breast cancer stem cell⁽¹⁰⁾. *Let-7* is an important miRNA family consisting of 12 members that are frequently down-regulated in a number of human cancers⁽¹¹⁾. They target *RAS*, *CDK6*, *Cyclin D2*, and *Cdc25A* genes which their over-expression lead to a pro-survival phenotype in response to IR⁽¹²⁾. *Let-7* effect on several breast stem cell-like properties by inhibiting more than one target⁽¹³⁾.

We hoped that the study of miRNA expression changes in response to IR in breast cancer stem-like cells could be a starting point to a better radiotherapy in breast cancer disease. Thus, we decided to focus on three miRNAs, *hsa-miR-21*, *hsa-miR-34a*, and *hsa-let-7i* since they are associated with the modulation of cell damage response pathways^(7,9,12). Furthermore, we analysed these miRNAs expression changes after gamma-irradiation in the MDA-MB-231 cells with the highest CD44⁺/CD24^{-/low} stem-like population, at approximately 98%⁽¹⁴⁾ and MCF-7 cells which harbour just 3.5% of CD44⁺/CD24^{-/low} phenotype⁽¹⁵⁾. The aim of this study was to evaluate the role of miRNAs, related to cancer stem cells in treatment by radiation. The specific miRNAs (*miR-21*, *miR-34a*, *Let-7i*) expression were investigated in response to gamma radiation in MCF7 (with low BCSCs content) and MDA-MB1-231 (with 80% BCSCs). According to the results obtained from miRNAs expression, we designed probable strategies for sensitizing MDA-MB-231 cells to radiation.

MATERIALS AND METHODS

Cell culture

The human breast adenocarcinoma cell lines MCF-7 (IBRC C10082, MCF-7 type) and MDA-MB

-231 from National Center of Genetic and Biological Reservoirs (NCGBR, Iran) and Pasteur Institute of Iran (IPI) were maintained in DMEM/F12 and RPMI 1640 medium respectively, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. All cells were incubated in a humidified atmosphere of 5% CO₂ at 37°C. The culture medium, penicillin-streptomycin solution and FBS were purchased from Gibco™ Life Technologies (Carlsbad, CA, USA), all other supplements were from Sigma Aldrich (St Louis, MO, USA).

Ionizing radiation treatment

Adjusted number of 3×10⁵ monolayer cells in the logarithmic growth phase was irradiated at a dose rate of 0.89 Gy/min with gamma-rays from a Co⁶⁰ source (Theratron 780C, Theratronix, Canada) available at Cancer Institute of Iran. The cells received a total dose of 2; 4 or 8 Gy^(9,16) for the definite time at room temperature and corresponding controls were sham irradiated. The exposed and sham irradiated cells were subsequently incubated in humidified atmosphere at 37°C under 5% of CO₂ and harvested after indicated time points (4, 8 and 24 hours after irradiation) to extract RNAs. The experiment was repeated for each dose in duplicate.

Quantitative Real-time PCR amplification

After irradiation, total RNAs including miRNAs total were isolated from all samples using miRCURY™ RNA Isolation Kit (Exiqon A/S, Vedbaek, Denmark) according to the manufacturer's instructions. The cDNAs were made from the isolated RNAs using the miRCURY™ LNA Universal cDNA synthesis kit II (Exiqon A/S, Vedbaek, Denmark) according to the manufacturer's protocol.

For the detection of specific miRNAs expression, the ExiLent SYBR® Green master mix kit (Exiqon A/S, Vedbaek, Denmark) was applied and the QRT-PCR reactions were prepared as below-mentioned protocol. 4 µl diluted RT products (80x) were used as the templates in a 10 µl reaction containing 1 µl

primer sets and 5 µl PCR Master Mix. All primer sets specific for mature *hsa-miR-34a*, *hsa-let-7i*, *hsa-miR-21* (table 1) and U6 snRNA (internal control) were purchased from Exiqon Company (Vedbaek, Denmark). The expression of mature *hsa-miR-21*, *hsa-miR-34a* and *hsa-let7i* were measured by a Rotor-Gene 3000TM Real-Time PCR Detection System (Corbett, Sydney, Australia). The reaction conditions were as following: denaturing at 95°C for 10 min and 40 cycles of denaturing at 95°C for 10 sec and annealing at 60°C for 60 sec followed by a melting curve analysis. Endogenous control U6 snRNA was used for normalization. The results were presented as fold change of each miRNA in each specific treated cell relative to

corresponding un-treated cells. The experiments were repeated twice, and all reactions were run in duplicate.

Data and statistical analysis

Data analysis was performed using the software supplied with the real-time PCR instrument to obtain raw threshold cycle (Ct) values. Melting curves were determined for each primer set following all RT-PCR runs using the Rotor-Gene 3000 software. Ct values for each microRNA were normalized to the respective Ct values for the reference gene U6 snRNA. Data were obtained as fold change in each miRNA gene expression using the $2^{-\Delta\Delta C_t}$ method (17) and were expressed as log2 values.

Table 1. MicroRNAs for QRT-PCR analysis in radiated cells.

miRNA	miR Base accession	sequence
<i>hsa-miR-34a-5p</i>	MIMAT0000255	UGGCAGUGUCUUAGCUGGUUGU
<i>hsa-miR-21-5p</i>	MIMAT0000076	UAGCUUAUCAGACUGAUGUUGA
<i>hsa-let-7i-5p</i>	MIMAT0000415	UGAGGUAGUAGUUUGUGCUGUU

RESULTS

MDA-MB-231 and MCF-7 cells growing in steady state were treated with 2, 4 and 8 Gy doses of gamma rays and investigated at 4, 8, and 24 hours post irradiation for the specific microRNAs (*miR-34a*, *miR-21*, and *Let-7i*) expression changes using Real-Time PCR.

The miRNAs expression after gamma irradiation in MCF-7 cells

The expression of *miR-21* was induced in the MCF-7 cell line only 4 h after radiation, with a decline in expression at later time points in all doses. So, the *miR-21* expression was not considerably changed at different irradiation doses during the experiment times compared to *miR-34a* and *let-7i*, in this cell line. Accordingly, the results illustrated that *miR-34a* was the most up-regulated miRNA post irradiation in MCF-7 cells. Also, the expression of *miR-34a* increased to the maximum level at 24 h after radiation in all three doses. In fact, excluding 2Gy, 8h treatment there was a significant, progressive,

permanent increase of *miR-34a* expression, in a dose- and time dependent fashion in the cells. Consequently, we observed considerable changes in expression of *let-7i* miRNA, at higher doses of 2 Gy, which peak at 8 h time point in 4 and 8 Gy transiently, with a decline in its expression at later time point (figure 1).

The miRNAs expression after gamma-irradiation in MDA-MB-231 cells

The results showed a similar expression pattern for *miR-34a* and *miR-21* at 2 and 4 Gy in MDA-MB-231 breast cancer cell line. Accordingly, these two miRNAs expression induced at 4 and 8 hours post irradiation but decreased at 24 hour. In 8 Gy, the expression of all three miRNAs especially *miR-21* were strongly induced at 4 h post-irradiation. In addition, *Let-7i* microRNA expression was different depending on dose and time post irradiation (figure 2). Interestingly, the *miR-21* increase was more significant in MDA-MB-231 cells compared to MCF-7 cells.

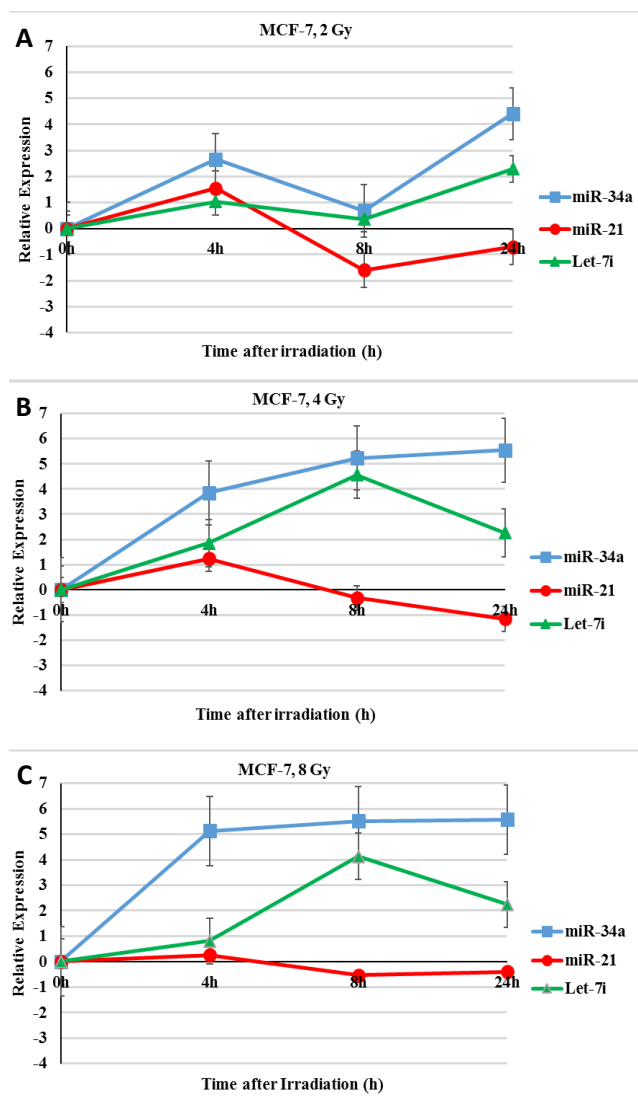


Figure 1. Relative expression of miR-34a, miR-21 and let-7i microRNAs in MCF-7 cells after exposure to different doses of γ -rays compared to the un-irradiated sham corresponding control. The miRNAs expression was assessed at 0, 4, 8, and 24 h post irradiation. The data is expressed as Log2 values. A. 2 Gy dose, B. 4 Gy dose, C. 8 Gy dose. (■) miR-34a, (●) miR-21, (▲) let-7i.

DISCUSSION

Today, the main reason of tumour treatment resistance is the breast cancer initiating cells (BCICs). It has been reported that 98 and 3.5 percentage of stem-like cells existed in MDA-MB-231⁽¹⁴⁾ and MCF-7⁽¹⁵⁾ cell lines, respectively. Since three specific miRNAs, *miR-21*, *miR-34a* and *let-7i* are involved in DNA repair pathways,

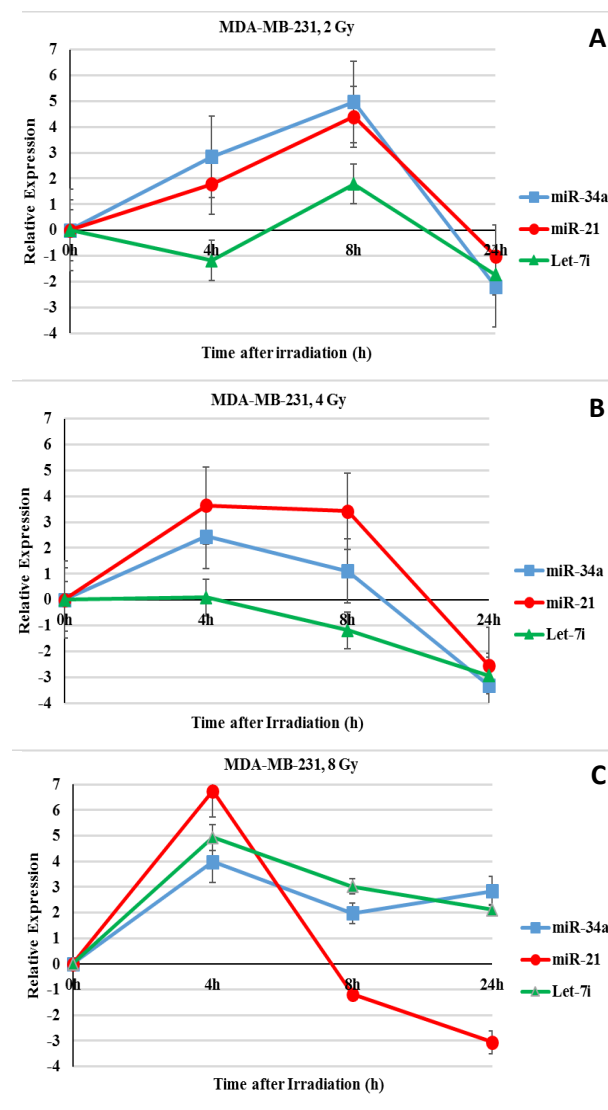


Figure 2. Relative expression of miR-34a, miR-21 and let-7i microRNAs in MDA-MB-231 cells after exposure to different doses of γ -rays compared to the un-irradiated sham corresponding control. The miRNAs expression was assessed at 0, 4, 8, and 24 h post irradiation. The data is expressed as Log2 values. A. 2 Gy dose, B. 4 Gy dose, C. 8 Gy dose. (■) miR-34a, (●) miR-21, (▲) let-7i.

apoptosis and cell cycle arrest^(7,9,12), in this study, we analysed expression changes of them after gamma radiation in MDA-MB-231 and MCF-7 (IBRC C10082, MCF-7 type) cells, as breast cancer stem cells (BCSCs) and non-BCSC models, respectively.

It has been reported that IR-Induced *miR-21* facilitates the repression of *PTEN* as its target, and the activation of PI3K/AKT to enhance the

DDR and cell survival ^(6, 7). In the study conducted by Wang and colleagues found that decreased expression of *Cdc25A* by *miR-21* led to cell cycle inhibiting to repair radiation-related cell damage ⁽¹⁸⁾. Therefore, *miR-21* can act as a radio-resistant miRNA when transiently over-expressed after radiation treatment ⁽⁶⁾. Also, we detected *miR-21* over-expression in both MCF-7 and MDA-MB-231 cells post irradiation. Interestingly, up-regulation of *miR-21* was more significant in irradiated MDA-MB-231 than MCF-7, which was only observed in primary hours.

Liu et al. (2011) reported that *miR-34a* could be induced in several mice organs by radiation. They also showed that induction of *miR-34a* by radiation was in a *p53* dependent manner, followed by down-regulation of anti-apoptotic molecule *Bcl-2* that leads to apoptosis, cell-cycle arrest or senescence. On the other hands, the transcription factor encoded by the *p53* tumour suppressor gene is post-transcriptionally activated by DNA damaging agents/radiation ⁽⁹⁾. Accordingly, we found that *miR-34a* expression level was up-regulated by gamma irradiation in breast cancer cells (MCF-7 and MDA-MB-231). Interestingly, the over-expression of *miR-34a* was much more significant in MCF-7 than MDA-MB-231, since MCF-7 cells harbour a wild type functional *p53* gene.

Chaudhry (2009) found that the expression levels of several members of the *let-7* family miRNA were increased after ionizing radiation treatment in Jurkat cells but were decreased in TK6 cells ⁽¹⁹⁾. Taylor (2014) presented that elevated expression of *let-7* in human malignancies elicits down-regulation of *Ras* family members, *HMGA2*, and *c-Myc*, thereby suppressing the development, progression, and therapeutic resistance of human tumours ⁽²⁰⁾. Also, we observed *let-7i* microRNA expression changes following exposure to gamma irradiation were different as up- or down regulation depending on dose, time post irradiation and cell type, similar to other studies that reported up- or down-regulation of *let-7* family miRNAs expression in different gamma irradiation exposed cells ⁽¹⁹⁾.

In conclusion, with regards to our findings,

we may be able sensitize somewhat stem-like MDA-MB-231 breast cancer cells to radiation by increasing *miR-34a* and decreasing *miR-21* expression levels simultaneously.

ACKNOWLEDGEMENTS

This study was funded by Tarbiat Modares University grant. Also, the authors wish to thank Department of Radiation Oncology of Tehran University of Medical sciences for supporting this research.

Conflicts of interest: Declared none.

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