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Original Article

MicroRNA-185 overexpression sensitizes breast cancer cells to ionizing radiation: a potential therapeutic role in breast cancer

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Abstract: Solid tumor cells can be resistant or develop resistance to radiation therapy. The aim of this study was to investigate whether microRNA-185 (miR-185) involved in cell radioresistance and could sensitize breast cancer cells resistant to radiotherapy. We analyzed the expression level of miR-185 in radioresistant cell lines (MDA-MB-231RR and SKBR3RR) in comparison with cell lines in normal condition. Furthermore, we also assessed the impact of miR-185 overexpression on the expression levels of AKT1, RAD52, and Bcl-2, and the proteins involved in the mechanism of radioresistance. The result showed that miR-185 was downregulated in radioresistant breast cancer cells and contrary to AKT1 and RAD52 expression. Furthermore, overexpression of miR-185 reduced the expression of AKT1, RAD52, and Bcl-2. Interestingly, miR-185 overexpression suppressed cell proliferation and increased radiosensitivity in radioresistant cells. Silencing the expression of Bcl-2 using Bcl-2 siRNA in combination with miR-185 inhibitor also suppressed cell proliferation and sensitizes breast cancer cells to radiotherapy. In conclusion, our results demonstrated that decreased expression of miR-185 conferred radioresistance and restoration of miR-185 baseline expression sensitizes breast cancer cells to radiotherapy. These data suggested that miR-185 might be a potential sensitizer to radiotherapy.

Keywords: microRNA-185, radioresistance, breast cancer, AKT, RAD52, Bcl-2

Introduction

Breast cancer is one of the most common cancer and the most common cause of cancer-related deaths of females worldwide [1]. Surgical resection has always been the major therapeutic option for the primary tumor [2, 3]. The addition of adjuvant radiotherapy further improves the prognosis of disease [4]. Currently, adjuvant radiotherapy after mastectomy is the gold standard of care for patients with solid tumors [5]. About half of cancer patients had received radiation therapy during the treatment [6]. However, tumor hypoxic microenvironment might lead to the development of radioresistance that tumor cells could resistance to radiotherapy [7]. This radioresistance impeded the radiotherapy to kill tumor cells during tumor treatment [8]. The molecular mechanism of radioresistance is not clearly known owing to the involvement of many genes and complex cellular genetic response to radiation. It suggests that modulators that could regulate multiple target genes simultaneously were involved in radioresistance [8].

MicroRNAs (miRNAs) are small, short, non-coding RNAs, which regulate expression of protein-coding genes at post-transcriptional level [9, 10]. miRNAs are involved in various cellular functions such as development, differentiation, proliferation, and apoptosis [10-12]. It has been showed that the upregulation and downregulation of miRNAs might play the critical role in progression of cancer [13-16]. A single miRNA can simultaneously target multiple genes and regulate various signal pathways. Thus, miRNAs might be modulators of radioresistance response of cancer cells and have potency to be used for sensitize radioresistant breast cancer cells to radiotherapy [8].

There were many studies examined the possible roles of miRNAs in radioresistance in various cancer cells. It was found that miRNA let-7 overexpression made cancer cells sensitive to radiotherapy [17]. It was also been found that miR-521 participated in radiation modulation [18]. Furthermore, miR-101 targeted DNA-PKcs and ATM to sensitize tumors to radiotherapy [19]. The involvement of miRNAs in radioresistant breast cancer had also been investigated and indicated that miR-302 overexpression made breast cancer cells sensitive to radiotherapy through reducing the expression of AKT1 and RAD52 [8]. The miR-185 was demonstrated to suppress tumor growth and progression by targeting the Six1 oncogene in human cancers, including breast cancer, ovarian cancer, hepatocellular carcinoma, and pediatric malignancies such as rhabdomyosarcoma and Wilms' tumor [20]. The role of miR-185 in radioresistance of renal cell carcinoma has also been investigated and indicated that miRNAs could be used both as direct cancer therapeutic agents and tools to sensitize tumor cells to radiotherapy [21].

In the present study, we investigated the role of miR-185 in breast cancer, the effect of miR-185 expression on radioresistance and the relationship of miR-185 expression and susceptibility of breast cancer cells to radiotherapy.

Materials and methods

Cell culture

The human breast cancer cell lines MDA-MB-231 and SKBR3 (Invitrogen, USA), were respectively cultured in Roswell Park Memorial Institute (RPMI) 1640 medium and Dulbecco's Modified Eagle Medium (DMEM) medium, supplemented with 10% fetal bovine serum, 100 units/mL penicillin sodium, and 100 μg/mL streptomycin sulfate (Invitrogen) at 37°C in 5% CO_2.

Induction of radioresistant breast cancer cells

MDA-MB-231 and SKBR3 cells were irradiated at the dose of 2 Gy/day for 20 days. Surviving cells were selected and cultured for the next experiments. Radioresistant MDA-MB-231 and SKBR3 cells were designated as MDA-MB-231RR and SKBR3RR, respectively.

Cell transfection

The radioresistant breast cancer cells (MDA-MB231RR and SKBR3RR) were transfected with miR-185 mimic, miR-185 inhibitor, or scramble control using Lipofectamine RNAiMAX (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions.

Cell viability assay

The cell viability was determined using 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-Htetrazolium bromide (MTT) assay. Cells were seeded at 5×10^4 cells per well in 12-well plates. At 48 h after miR-transfection, the cells were collected and re-seeded in 96-well plates at 2×10^3 cells per well and cultured for 1-5 days. For every time point, cells were selected and added 20 μl/well MTT (Sigma, St. Louis, MO, USA) and incubated for another 4 h at 37°C. Then, cells were added 150 μl dimethyl sulfoxide (DMSO, Sigma, USA) for every well and the plates were shaken for 10 min. After removing the medium, the absorbance was measured at 570 nm (OD_{570}). Each experiment was repeated three times.

Western blot analysis

Cells after been treated were washed with phosphate buffer saline (PBS) and lysed with the solution containing Tris (20 mM, pH 7.4), EDTA (0.1 mM), NaCl (150 mM), 1% NP-40, NaF (10 mM), Na_3VO_4 (1 mM) (Sigma, St. Louis, MO, USA), and protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland). Protein samples were boiled for 5 min in sodium dodecyl sulfate (SDS) sample buffer. Then samples were separated on a SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to nitrocellulose membranes (Whatman, Dassel, Germany). Blots were blocked for 45 min in 5% nonfat dry milk in 1 × TBS-T, incubated with the appropriate primary antibodies overnight, and then incubated for 1 h with horseradish peroxidase (HRP)-conjugated secondary antibodies. Protein bands were imaged using the WEST-ZOL-plus Western Blot Detection System (iNtRON Biotechnology, Seoul, Korea).

Cell clonogenic survival assays

After 48 h of miR-transfection, MDA-MB-231RR and SKBR3RR cells were seeded in 6-well

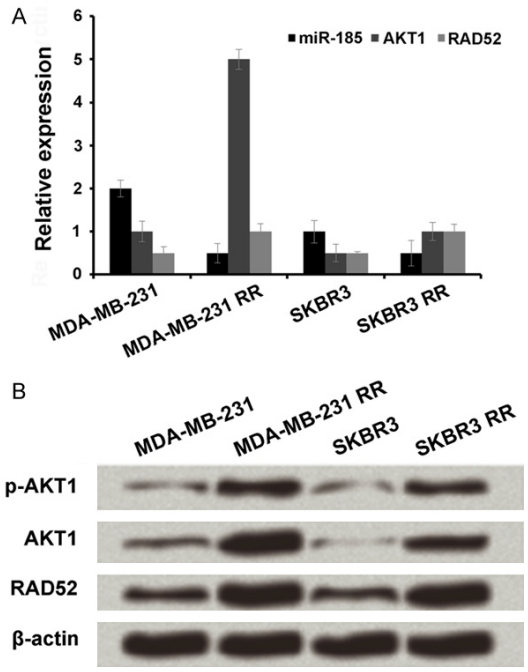

Figure 1. miR-185 expression was downregulated in radioresistant breast cancer cells. A. Expression of miR-185, total AKT1, and RAD52 in normal breast cancer cells (MDA-MB-231 and SKBR3) and radioresistant breast cancer cells (MDA-MB-231RR and SKBR3RR). qRT-PCR was used to determine the expression of miR-185, which was normalized to U6 expression to obtain relative expression, and mRNA expressions of AKT1 and RAD52 were normalized to β-actin expression to obtain relative expression. B. Western blot analysis results for the expressions of phosphorylated AKT1 (p-AKT1), total AKT1 and RAD52 in MDA-MB-231, SKBR3, MDA-MB-231RR, and SKBR3RR cells. β-actin acted as an internal control. qRT-PCR: quantitative reverse transcription polymerase chain reaction.

plates at specific cell densities in triplicate, followed by exposing to radiation at indicated doses (1-6 Gy) using 6 MV X-rays generated by linear accelerators (Varian 2300EX; Varian, Palo Alto, CA). The treated cells were incubated for 10 to 16 days at 37°C, then cells were fixed with 100% methanol and stained with 1% crystal violet (Sigma Aldrich Co., St. Louis, MO, USA). The colonies containing more than 50 normal-looking cells were inspected using a microscope (Olympus IX71; Olympus, Tokyo, Japan). The surviving fraction was calculated as follows: number of colonies/number of plated cells. The sensitization enhancement ratio with a survival fraction of 10% (SER10) was also calculated. Each experiment was performed at least three times.

RNA extraction and quantitative real-time PCR

Total RNA was extracted from cultured MDA-MB-231, SKBR3, MDA-MB-231RR, and SKBR-3RR cells by using Trizol reagent, following the manufacturer's instructions (Invitrogen). Reverse transcription was performed using the All-in-One miRNAs reverse transcription kit (Genecopoeia, MD, USA). cDNAs were subjected to quantitative real-time polymerase chain reaction (qRT-PCR) using SYBR Premix Ex Taq (Takara, Tokyo, Japan) on Stepone plus (Invitrogen). RNU6B (U6) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as controls for normalization of expressions of miR-185 and protein kinase C epsilon type (PKCe), respectively. Relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method. All experiments were performed in triplicate. Primers for miR-185 and U6 were purchased from GeneCopoeia (CA, USA).

Statistical analysis

All experiments were repeated three times. All data are expressed as mean ± standard deviation (SD). All statistical analysis were performed using the SPSS 19.0 software. The significance of differences in mean values within and between multiple groups was evaluated using an ANOVA followed by a Duncan's multiple range test. Student's t-test was used to evaluate statistical significance of differences between two groups. The $P<0.05$ was considered as statistically significant.

Results

miR-185 was downregulated in radioresistant breast cancer cells and inversely correlated with AKT1 and RAD52

We analyzed the expression levels of miR-185, AKT1, and RAD52 in two types of breast cancer cell lines (triple-negative MDA-MB-231 and Her2/neu positive SKBR3), and also in their radioresistant sublines (MDA-MB231RR and SKBR3RR) using qRT-PCR. Our data showed that miR-185 mRNA expression levels were downregulated in radioresistant breast cancer cells while mRNA expression levels of AKT1 and RAD52 were upregulated compared to their expression levels in normal condition (**Figure 1A**). Furthermore, protein expression levels of phosphorylated AKT1 (p-AKT1), total AKT1, and

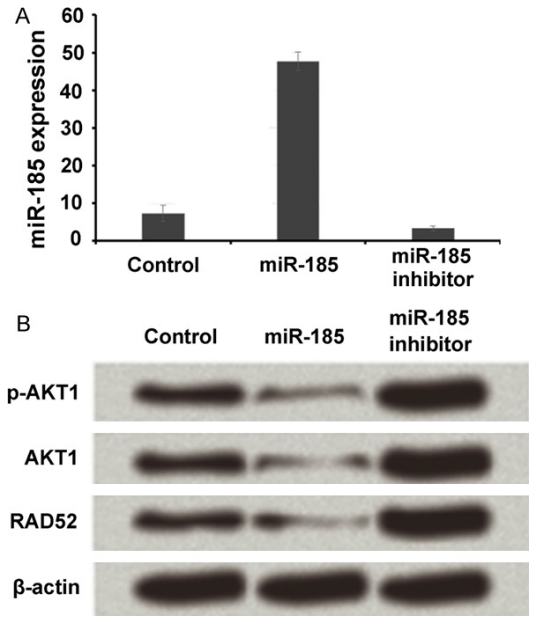

Figure 2. Overexpression of miR-185 reduced the expression of AKT1 and RAD52. A. mRNA expression of miR-185 mimics, inhibitors, and control in MDA-MB-231RR cells. The transfection efficiency of miR-185 mimics, inhibitors, and control were evaluated by RT-PCR. B. Expression of p-AKT1, total AKT1 and RAD52 in MDA-MB-231RR cells transfected with miR-185 mimics, inhibitors, or control. β-actin acted as an internal control. qRT-PCR was used to determine the expression levels. qRT-PCR: quantitative reverse transcription polymerase chain reaction.

RAD52 were also upregulated in radioresistant breast cancer cells (**Figure 1B**). These results demonstrated that expression level of miR-185 was downregulated in radioresistant cells and were inversely correlated with the expression levels of AKT1 and RAD52.

miR-185 reduced the expression of AKT1 and RAD52 in radioresistant cells

MDA-MB231RR cells were transfected with miR-185 mimic, miR-185 inhibitor, or scramble control. As shown in **Figure 2A**, miR-185 expression levels were upregulated in miR-185 mimic transfected cells. Conversely, miR-185 expression was suppressed in miR-185 inhibitor transfected cells. In **Figure 2B**, miR-185 overexpression decreased the expression levels of AKT1 and RAD52 in MDA-MB-231RR cells. Results also showed that p-AKT1 expression was downregulated in MDA-MB-231RR cells with miR-185 mimic transfection. In contrast, all these results were opposite in miR-185 inhibitor transfection. It suggested that restored expression of miR-185 affected AKT1 activity and the expression of RAD52.

miR-185 overexpression sensitized radioresistant cells to ionizing radiation

To investigate whether overexpression of miR-185 could make the radioresistant cells sensitive to ionizing radiation, The MDA-MB-231RR and SKBR3RR cells were transfected with miR-185 mimic. The results of surviving rate showed that significantly lower survival fractions were observed in the miR-185-transfected cells compared to control (**Figure 3A** and **3B**). It suggested that the miR-185 transfected radioresistant cells were significantly sensitive to radiation therapy compared to control. MTT assay was performed to exclude effect of miR-185 overexpression on cell viability. Compared with the control, the cell viability of radioresistant cells was reduced by miR-185 mimic transfection (**Figure 3C** and **3D**), while showed no significant effect on growth of radioresistant cells.

miR-185 regulated Bcl-2 expression in radioresistant cells

We also analyzed the impact of miR-185 overexpression on Bcl-2 expression in radioresistant cells. We transfected MDA-MB-231RR and SKBR3RR cells with miR-185 mimic, miR-185 inhibitor, or scramble control. As shown in **Figure 4A**, **4B**, miR-185 overexpression reduced the expression of Bcl-2, and conversely miR-185 suppression increased the expression of Bcl-2 compared to the control. These results suggested that miR-185 also regulated the anti-apoptotic protein Bcl-2.

Silencing the expression of Bcl-2 sensitized radioresistant cells to ionizing radiation

As miR-185 regulated the expression of Bcl-2, we analyzed the impact of Bcl-2 silencing on radioresistant cell viability and their sensitivity to ionizing radiation. MDA-MB-231RR and SKBRRR cells were transfected with miR-185 inhibitor, miR-185 inhibitor + Bcl-2 siRNA, or scramble control. As shown in **Figure 5**, treated with miR-185 inhibitor + Bcl-2 siRNA remarkably decreased the survival rate of radioresistant cells as compared with miR-185 inhibitor alone or control (**Figure 5A** and **5B**). Additionally, silencing the expression of Bcl-2 also suppressed the growth of radioresistant

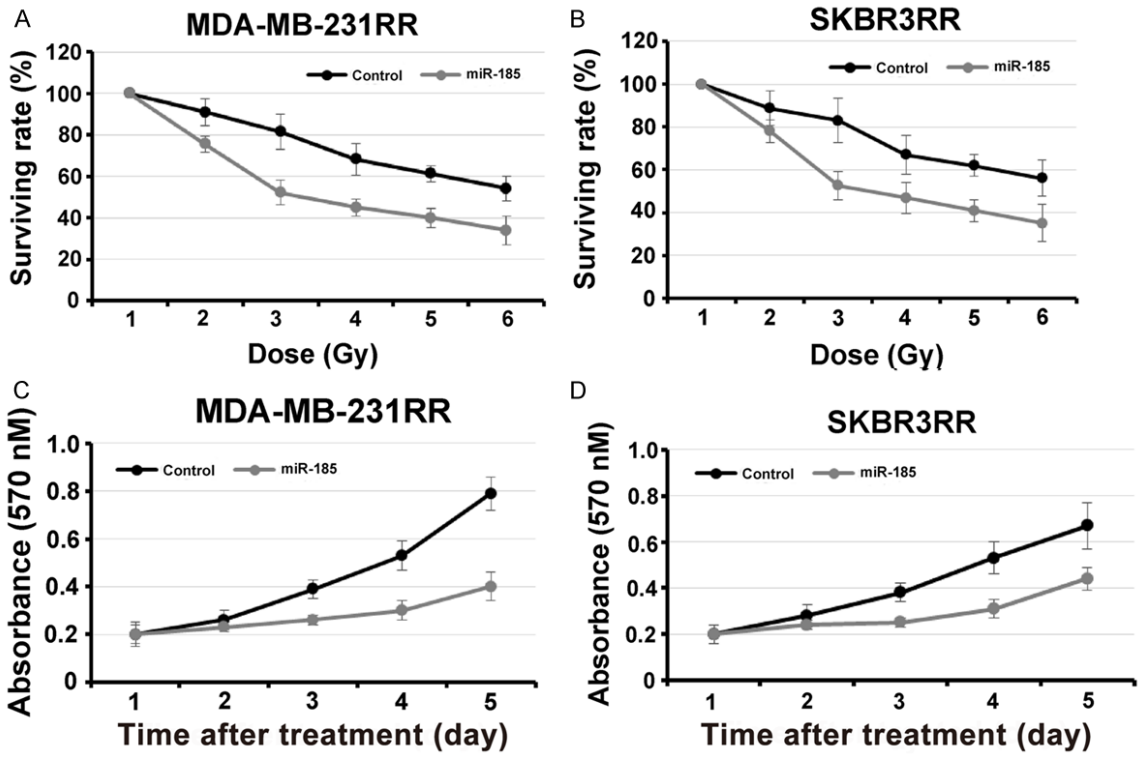

Figure 3. miR-185 suppressed radioresistant cells viability and sensitized cells to ionizing radiation. A and B. Clonogenic survival assays of MDA-MB-231RR and SKBR3RR cells treated with miR-185 mimic (miR-185) or scramble control (Control) with the increasing doses of radiation. Surviving fractions were calculated as number of colonies/number of plated cells. SER10, sensitizer enhancement ratio at 10% survival. C and D. Cell viability was determined after MDA-MB-231RR and SKBR3RR cells were transfected with miR-185 mimic (miR-185) or scramble control (Control) for 1-5 days. Values are presented as the mean ± standard deviation.

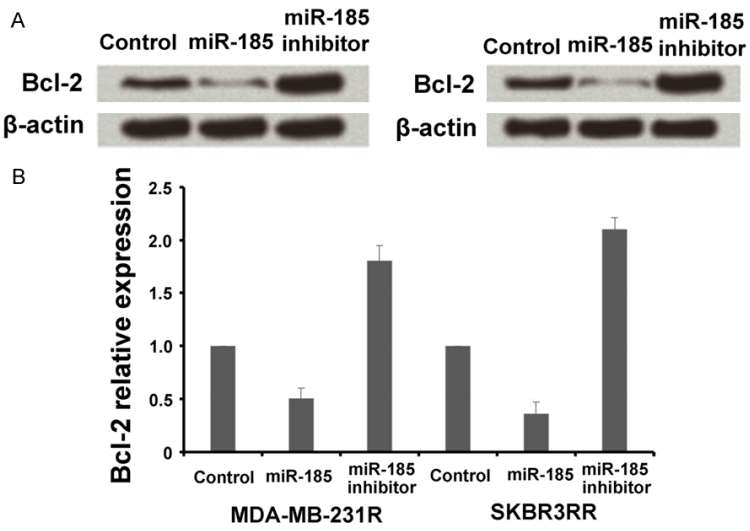

Figure 4. miR-185 regulated Bcl-2 expression in radioresistant cells. A. Western blot analysis results for Bcl-2 expression in radioresistant cells. B. Relative expression of Bcl-2 in MDA-MB-231RR and SKBR3RR cells transfected with miR-185 mimic, miRNA-185 inhibitor, or scramble control. β-actin was acted as an internal control.

cells in 1-5 days (**Figure 5C** and **5D**). These results showed that Bcl-2 played an important role in radioresistance rate of cancer.

Discussion

The involvement of miRNA in cancer resistance to radiotherapy has not been well explored, though there were many evidences indicated the critical role of miRNAs in cancer [22]. In the present study, we demonstrated that miR-185 was downregulated in radioresistant breast cancer cells. In addition, overexpression of miR-185 sensitized radioresistant breast cancer cells to ionizing radiation by

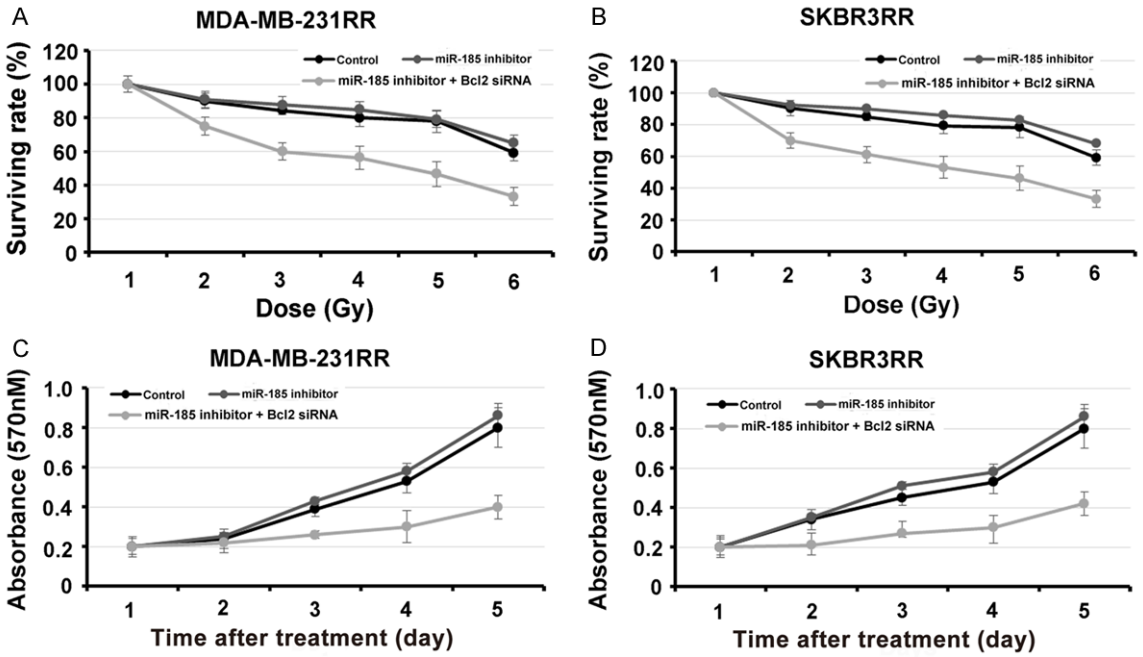

Figure 5. Silencing the expression of Bcl-2 suppressed radioresistant cell viability and sensitized cells to ionizing radiation. A and B. Clonogenic survival assays of MDA-MB-231RR and SKBR3RR cells treated with miR-185 inhibitor, miR-185 inhibitor + Bcl-2 siRNA or negative control with the increasing doses of radiation. Surviving fractions were calculated as number of colonies/number of plated cells. C and D. Cell viability was determined after transfection of miR-185 inhibitor, miR-185 inhibitor + Bcl-2 siRNA or negative control for 1-5 days in MDA-MB-231RR and SKBR3RR cells. Values are presented as the mean ± standard deviation.

regulating the expression of AKT1, RAD52, and Bcl-2.

We found that miR-185 expression was inversely correlated with the expression of AKT1 and RAD52, that overexpression of miR-185 downregulated the expression of AKT1 and RAD52. It has been showed that the PI3K/AKT pathway play critical role in the mechanisms of radioresistance [23-25]. RAD52 was involved in DNA double-strand break repair and homologous recombination [26, 27]. In addition, RAD52 also interacted with DNA recombination protein RAD51 [28, 29], which indicated that RAD52 was involved in RAD51-related DNA recombination and repair. Thus, downregulation of miR-185 might lead to development of radioresistance in breast cancer cells, mainly due to the expression of AKT1 and RAD52.

More than half of miRNA genes are frequently located at fragile sites and cancer-associated genomic regions that further strengthens the role of miRNAs in cancer [30, 31]. Despite the challenges, miRNAs are attractive candidates as sensitizers for radioresistance compared to other types of nucleic acids or proteins. Except few specific miRNAs, most miRNAs are downregulated in tumors [16, 32]. Suppression of miRNA increases cellular transformation and tumorigenesis [33]. In recent studies, viral let-7 miRNAs inhibited tumor growth in a mouse model of lung adenocarcinoma [34, 35]. These results suggested that re-expression of even a single miRNA in cancer cells could provide significant therapeutic benefits.

We also analyzed the impact of miR-185 overexpression on Bcl-2. Bcl-2 was chosen due to its possible role in resistance. It has been showed that dysregulation of Bcl-2 promoted innate or acquired treatment resistance in breast cancer [36]. We found that miR-185 regulated Bcl-2 expression by decreasing its expression in the radioresistant breast cancer cells. In addition, we also found that the expression silence of Bcl-2 sensitized the radioresistant breast cancer cells to ionizing radiation. These results proved that Bcl-2 was also involved in radioresistance of breast cancer cells. As miR-185 regulated Bcl-2, the role of miR-185 in the radioresistance of breast cells was further strengthened by these results.

In conclusion, this study revealed that overexpression of miR-185 efficiently sensitized radioresistant breast cancer cells to ionizing radiation by directly downregulating expression of AKT1, RAD52, and Bcl-2. Our results might provide a new efficient radiotherapy strategy in breast cancer treatment.

Disclosure of conflict of interest

None.

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