Original Article
MiR-122-3p sensitizes breast cancer cells to ionizing radiation via controlling of cell apoptosis, migration and invasion

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Abstract: Nowadays, radio-resistance remains a grim problem occurs in patients with breast cancer. This study was aimed to explore the role of microRNA-122-3p (miR-122-3p) on breast cancer cells radio-sensitivity. Human breast cancer cells line MDA-MB-231 was used and transfected with miR-122-3p mimic, inhibitor or control. Transfected cells were exposed to various doses of irradiations, and then the surviving rate, apoptosis, cells migration and invasion were detected by clonogenic survival assay, flow cytometry, and transwell assay respectively. Furthermore, Western blot analysis was used to detect the expression changes of phosphatase and tensin homolog/phosphatidylinositol-3-kinase/serine/threonine kinases (PTEN/PI3K/AKT) pathway and epithelial-mesenchymal transition (EMT) related proteins in transfected cells after irradiation. The surviving rate of MDA-MB-231 cells was significantly decreased by miR-122-3p overexpression \((P < 0.05 \text{ or } P < 0.01)\). Apoptosis was induced by miR-122-3p overexpression \((P < 0.001)\) while cells migration and invasion were inhibited by miR-122-3p overexpression \((P < 0.001)\). Besides, miR-122-3p overexpression significantly down-regulated the expressions of p-AKT, AKT, Vimentin and Snail 1 \((P < 0.01 \text{ or } P < 0.001)\), whereas significantly up-regulated the levels of PTEN and E-Cadherin \((P < 0.01)\). MiR-122-3p suppression showed the inversed impacts on the surviving rate, apoptosis, cells migration and invasion, as well as these proteins expressions. MiR-122-3p enhanced the radio-sensitivity of breast cancer cells via controlling of cell apoptosis, migration and invasion. PTEN/PI3K/AKT pathway and EMT related proteins were implicated in this enhancement impacts.

Keywords: MicroRNA-122-3p, breast cancer, radio-sensitivity, apoptosis, migration, invasion

Introduction
Breast cancer is the most common cancer and it is the leading cause of cancer death in females worldwide [1]. To date, the exact cause of breast cancer is unclear, while it is believed genetics and environmental factors contribute to the carcinogenesis and progression of breast cancer [2, 3]. For the treatment, multimodal therapy often includes surgery, radiotherapy, chemotherapy, hormone therapy and biologic therapy [4]. Radiotherapy is always given after surgery to destroy microscopic tumor cells that may have escaped surgery, and this therapeutic strategy can greatly reduce the risk of recurrence [5, 6]. However, radio-resistance remains a serious obstacle to kill tumor cells successfully [7]. Thus, finding effective radio-sensitizers will be helpful for us to improve the treatment, as well as improve the outcomes of patients with breast cancer.

MicroRNAs (miRNAs) are a large family of short non-coding RNAs consisting with about 22 nucleotides [8]. MiRNAs post-transcriptionally modulate gene expression through negative regulation of the stability or translational efficiency of their target mRNAs [9, 10]. An increasing number of studies have demonstrated that miRNAs play pivotal roles in the tumorigenesis and development of breast cancer [11, 12]. MiR-206 has been found inhibit the stemness and metastasis of breast cancer via targeting megakaryoblastic leukemia (translocation) 1 (MKL1)/interleukin-11 (IL-11) pathway [13]. MiR-644a has been reported as an inhibitor of both cell survival and epithelial-mesenchymal transition (EMT) whereby acting as pleiotropic therapy-sensitizer in breast cancer [14].

MiR-122-3p is a family member of miRNAs locates at chromosome 18q21.31. Down-regulation of miR-122-3p has been found in multiple
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Figure 1. MiR-122-3p sensitized MDA-MB-231 cells to ionizing radiation. MDA-MB-231 cells were transfected with miR-122-3p mimic, inhibitor or control, and exposed to various doses of irradiations. Afterward, surviving rate was measured by clonogenic survival assay. MiR-122-3p, microRNA-122-3p; *, P < 0.05; **, P < 0.01.

diseases, including hepatocellular carcinoma, idiopathic asthenospermia, rheumatoid arthritis and hepatitis C [15-18]. However, to our knowledge, none literature has reported the role of miR-122-3p in the radio-resistance of breast cancer cells. In the present study, we aimed to examine the role of miR-122-3p in breast cancer. Human breast cancer cells line MDA-MB-231 was used and transfected with miR-122-3p mimic, inhibitor or control. Transfected cells were exposed to various doses of ionizing radiations, and then the surviving rate, apoptosis, migration and invasion were determined respectively, aiming to explore the functional impacts of miR-122-3p on the radiosensitivity of breast cancer cells. In addition, the expressions of phosphatase and tensin homolog/phosphatidylinositol-3-kinaseserine/threonine kinases (PTEN/PI3K/AKT) pathway and EMT related proteins in cells were detected to reveal the possible underlying mechanism of miR-122-3p in breast cancer cells.

Materials and methods

Cell culture and transfection

Human breast cancer cells line MDA-MB-231 was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA), 100 U/mL penicillin, and 100 U/mL streptomycin (Invitrogen, Carlsbad, CA, USA) [19]. Cells were incubated in a humidified 5% CO2 incubator at 37°C.

Cells were seeded into 6-well culture dishes at a density of 2 x 105 cells/well and cultured for 24 h at 37°C. The cells were then transfected with miR-122-3p mimic, inhibitor or control (RiboBio, Guangzhou, China) by using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the manufacturer’s instructions [20]. After 48 h of transfection, cells were collected for the forthcoming analyses.

Clonogenic survival assay

After transfection, cells were seeded into 6-well plants at a density of 1 x 105 cells/well and followed by exposure to the indicated doses of radiations (2, 4, 6, 8 or 10 Gy), by using 6 MV X-rays generated by linear accelerators (Varian 2300EX; Varian, Palo Alto, CA) at a dose rate of 3 Gy/min [21]. After 14 days of incubation at 37°C, colonies were fixed with ice-cold ethanol/aceton (1:2) for 10 min, and then stained with 1% crystal violet (Sigma-Aldrich, St. Louis, MO, USA) for 5 min. Microscopic inspection (Olympus IX71; Olympus, Tokyo, Japan) was used for the colonies observation. Colonies containing ≥ 50 normal-looking cells were counted and clonogenic survival was calculated as previous described [22].

Apoptosis assay

Apoptotic cells were quantified by using an Annexin V-FITC/PI Kit (4 A Biotech Co. Ltd., Beijing, China) according to the manufacturer’s protocol. Transfected cells were exposed to 10 Gy X-rays for 12 h at 37°C, and then cells were collected and re-suspended in 200 μL binding buffer containing 10 μL Annexin V-FITC and 5 μL PI. After incubate in the dark at room temperature for 20 min, the samples were analyzed on the flow cytometry (FACS Calibur, Becton Dickson, San Jose, CA, USA) [23]. The number of intact cells, apoptotic cells and necrotic cells were discriminated by counting the cells directly.

Migration and invasion assays

Cell migration and invasion were measured by transwell assay. Briefly, transfected cells were harvested and seeded into the upper chamber of transwell inserts (8-μm pore size and 24-well insert; BD Biosciences). DMEM media containing 10% FBS was added to the lower
chamber as the attractant. For cell invasion assay, each upper chamber was coated with Matrigel (Becton-Dickinson Labware, Bedford, MA, USA) before cells were seeded. Cells were treated with continuous 10 Gy X-rays for 12 h at 37°C. Afterward, cells on the upper surface of the filter were removed by cotton swabs. Then the migrated and invaded cells in the lower surface were permeabilized with 100% methanol for 15 min and stained with Giemsa (Leagene, Beijing, China) for 30 min [24]. Microscopic inspection (Olympus IX71; Olympus, Tokyo, Japan) was used for viewing and photographing stained cells. Cell numbers were counted from ten different fields.

**Western blot**

Transfected cells were exposed to 10 Gy dose of radiation for 12 h at 37°C. Cellular protein was extracted by lysis buffer (Beyotime, Shanghai, China) and proteins in each sample were quantified using BCA Protein Assay Kit (Beyotime), according to the manufacturer’s instructions. Equal amounts of protein were loaded in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred into polyvinylidene fluoride (PVDF) membranes. The membranes were blocked within 5% nonfat dry milk for 1 h at room temperature. Then membranes were incuba-
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Ted overnight in primary antibodies for E-cadherin (sc-8426), Vimentin (sc-373717), Snail 1 (sc-393172), p-AKT (sc-514032), AKT (sc-5298), PTEN (sc-7974) or Actin (sc-8432) (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C. The secondary antibody horseradish peroxidase (HRP) conjugate (sc-516087; Santa Cruz Biotechnology) was used to incubate membranes for 1 h at room temperature. Blots were visualized by ECL Plus Western Blotting Substrate (Thermo Scientific).

Statistical analysis

All data were expressed as mean ± standard deviations (SD) from three independent experiments. Statistical analysis was performed using Student t tests analysis of variance in GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA, USA). A value of $P < 0.05$ was considered as statistical difference.

Results

MiR-122-3p sensitized MDA-MB-231 cells to ionizing radiation

To evaluate the role of miR-122-3p in the radiosensitivity of breast cancer cells, MDA-MB-231 cells were transfected with miR-122-3p mimic, inhibitor or control, and then surviving rate was calculated. As results showed in Figure 1, overexpression of miR-122-3p significantly decreased the surviving rate when cells were exposed to 4-10 Gy doses of radiations ($P < 0.05$ or $P < 0.01$). However, miR-122-3p suppression displayed the inverse results, that it significantly increased the surviving rate at the same radiation doses ($P < 0.05$). These data evidenced that miR-122-3p could sensitize breast cancer cells to ionizing radiation.

MiR-122-3p induced apoptosis while suppressed migration and invasion in MDA-MB-231 cells after irradiation

In order to investigate the detailed function of miR-122-3p on breast cancer cells, MDA-MB-231 cells were first transfected with miR-122-3p mimic, inhibitor or control and then exposed to 10 Gy dose of irradiation. Cells apoptosis, migration and invasion were determined respectively. As results showed in Figure 2A and 2B, miR-122-3p overexpression significantly increased the apoptotic cells rate after irradiation ($P < 0.001$), while miR-122-3p suppression displayed the inverse effect ($P < 0.05$). Besides, transwell assay showed that (Figure 3A and 3B), miR-122-3p overexpression significantly inhibited cells migration and invasion after irradiation ($P < 0.001$). Unsurprisingly, miR-122-3p suppression significantly enhanced cells migration and invasion ($P < 0.05$ or $P < 0.001$). Thus, we inferred that miR-122-3p sensitized breast cancer cells to ioniz-

Figure 3. MiR-122-3p suppressed migration and invasion in MDA-MB-231 cells after irradiation. After MDA-MB-231 cells were transfected with miR-122-3p mimic, inhibitor or control, and exposed to 10 Gy of irradiation, cell migration and invasion were detected by transwell assay. MiR-122-3p, microRNA-122-3p; *, $P < 0.05$; ***, $P < 0.001$. 


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MiR-122-3p sensitized MDA-MB-231 cells to ionizing radiation via regulating PTEN/PI3K/AKT pathway and EMT related proteins

To explore the possible molecular mechanisms of miR-122-3p on breast cancer cells, miR-transfected MDA-MB-231 cells were exposed to 10 Gy dose of irradiation, and then the expression levels of PTEN/PI3K/AKT pathway and EMT related proteins in cells were detected. As results showed in Figure 4A and 4B, miR-122-3p overexpression significantly down-regulated the levels of p-AKT (P < 0.001) and AKT (P < 0.01), whereas up-regulated the level of PTEN (P < 0.01). However, miR-122-3p suppression showed the contrary regulatory impacts (P < 0.01). Figure 5A and 5B showed that, up-regulation of E-Cadherin (P < 0.01) and down-regulation of Vimentin (P < 0.001) and Snail 1 (P <
0.01) were found in miR-122-3p overexpressed cells. As expected, miR-122-3p suppression regulated these proteins expression in the reversed direction ($P < 0.05, P < 0.01$ or $P < 0.001$). These data evidenced that miR-122-3p sensitized breast cancer cells to ionizing radiation might be via regulating these protein expressions.

**Discussion**

Radiation is a part of therapy for breast cancer and is often provided following surgery. Nevertheless, radio-resistance remains a grim problem occurs in patients with breast cancer, and the underlying mechanism of radio-resistant remains unclear. In this study, we found that after ionizing radiation, miR-122-3p overexpression significantly decreased the surviving rate of MDA-MB-231 cells. In addition, miR-122-3p overexpression induced apoptosis, and suppressed migration and invasion after cells were exposed to irradiation. Further, down-regulation of p-AKT, AKT, Vimentin and Snail 1, as well as up-regulation of PTEN and E-Cadherin was found in miR-122-3p overexpressed cells.

The role of several miRNAs in the radio-resistance of breast cancer has been elucidated. Anastasov et al. reported that miR-21 expression in breast cancer cells contributed to radiation resistance by compromising cell cycle progression [25]. Sun et al. demonstrated that miR-200c enhanced radio-sensitivity in breast cancer cells by targeting Ubiquilin 1 (UBQLN1) [26]. Here, our results showed that overexpression of miR-122-3p significantly suppressed the surviving rate, after breast cancer cells were exposed to irradiation. These data provided the first evidence that miR-122-3p functioned as a radio-sensitizer in breast cancer.

Cancer cells tend to be resistant to radiotherapy partly due to defects in apoptosis [27]. Moreover, studies have showed that altered miRNA expression may play an important role in the radiotherapy of cancer cells by impairing cellular responses that affect apoptosis [28]. Gwak et al. found that silencing of miR-21 conferred radio-sensitivity in malignant glioma cells through the regulation of apoptosis [29]. Studies in vivo and in vitro have revealed that overexpression of miR-34a could significantly enhance the radio-sensitivity by inducing cell apoptosis [30]. Similar with these previous studies, our study displayed that miR-122-3p overexpression increased the apoptotic cells rate after irradiation, suggesting that miR-122-3p enhanced radio-sensitivity of breast cancer cells via controlling of apoptosis. PTEN is widely known as both tumor suppressor gene and apoptosis regulator that is implicated in breast cancer [31]. Besides, negative regulation of PI3K/AKT has been identified as a crucial downstream signaling pathway of PTEN [32]. Previous studies have revealed that deregulation of PTEN/PI3K/AKT pathway was associated with the trastuzumab resistance of breast cancer [33] via blocking apoptosis [34]. However, in this study, miR-122-3p overexpression notably up-regulated PTEN while down-regulated p-AKT and AKT after irradiation. Thus, we hypothesized that PTEN/PI3K/AKT pathway might be involved in the effects of miR-122-3p on irradiation induced apoptosis in breast cancer cells.

Metastasis is one of the most devastating hallmarks of breast cancer that is related alterations in cell adhesion and migration [35]. In addition, radiation has been shown to promote the migration and invasion of different types of tumor cells, including breast cancer cells [36, 37]. Currently, multiple investigations have evidenced that miRNAs constitute a regulatory event in cell migration and invasion [38]. However, little was known about the role of miRNAs in breast cancer cells migration and invasion after irradiation. In the present study, we found that miR-122-3p overexpression remarkably suppressed MDA-MB-231 cells migration and invasion, indicating that miR-122-3p enhanced the radio-sensitivity of breast cancer cells via weakening cells migratory and invasive abilities. EMT has been found to be implicated in the invasive fronts of breast cancer [39]. To probe the underlying mechanism of miR-122-3p in breast cancer cells migration and invasion after irradiation, the expression levels of EMT-related factors were measured. The negative correlation marker of metastasis, E-Cadherin, was up-regulated in the miR-122-3p overexpressed cells. However, the positive correlation marker of metastasis (Vimentin), and the repressor of EMT-activating transcription (Snail 1) were down-regulated by miR-122-3p overexpression. Therefore, we speculated that miR-122-3p inhibited breast cancer cells migration and invasion through regulating EMT related proteins.
In conclusion, we demonstrated that miR-122-3p sensitized breast cancer cells to ionizing radiation via controlling cell apoptosis, migration and invasion. PTEN/PI3K/AKT pathway and ETM-related proteins were implicated in the effects of miR-122-3p on breast cancer cells radio-sensitivity. However, further studies still needed to investigate whether the impacts of miR-122-3p on MDA-MB-231 cells can be reproduced in other types of breast cancer cells.

Disclosure of conflict of interest

None.

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