The roles of microRNA-328-3p on proliferation and radiotherapy in breast cancer

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Research Article

Keywords: miR-328-3p, breast cancer, radiotherapy, PTEN

DOI: https://doi.org/10.21203/rs.3.rs-742188/v1

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Abstract

Objectives MicroRNAs regulates varieties of molecular pathways and involve in breast carcinogenesis. Here both breast cancer cell lines and human breast cancer tissues were used to investigate the roles of miR-328-3p in breast cancer. Methods The impact of miR-328-3p on proliferation of MDA-MB-231 and T47D cells was determined by MTT assay. transwell migration and matrigel invasion assays were performed to evaluate effects of miR-328-3p on migration and invasion of breast cancer cells. Caspase 3/7 activities were measured to examine the impact of miR-328-3p on radiotherapy-induced apoptosis in breast cancer cells. The possible binding site of miR-328-3p was verified by dual-luciferase reporter assay. Quantitative real-time polymerase chain reaction was performed to detect miR-328-3p expression level in breast cancer tissues. Western blot and immunohistochemical studies were used to examine protein expression in breast cancer cells and breast cancer tissue, respectively. Results miR-328-3p involved growth, migration and invasion in breast cancer cells and was associated with radiotherapy sensitivity. MiR-328-3p enhanced radiation-induced apoptosis in breast cancer cells by regulating BAX and Bcl-2 expression. Meanwhile, aberrant expression of miR-328-3p was associated with altered expression of PTEN and p-AKT in breast cancer cells. Further study showed miR-328-3p bound to 3’-UTR of PTEN. In addition, breast cancer tissues showed higher level of miR-328-3p than normal breast tissue and higher level of miR-328-3p was seen in lower stage in breast cancer. Conclusions miR-328-3p displayed essential functions in breast carcinogenesis and might be used to predict radiotherapy response and prognosis in breast cancer.

Introduction

In the past several decades, the average survival rate of breast cancer has significantly improved [1]. However, breast cancer remains the most common type of malignancy that affects females under 40 year-old. About 276,480 new breast cancer patients will be diagnosed and 42,170 patients will die from this disease in 2020[2]. Thus, breast cancer is still a big challenge.

Radiotherapy uses high-energy particles or rays to damage cancer cells DNA and eventually kill cancer cells [3]. Radiotherapy is an effective treatment in breast cancer, especially when combined with other treatment such as surgeries and chemotherapy. However, radiation resistance has been seen in breast cancer patients for many years [4]. Varieties of factors contribute radiotherapy resistance in breast cancer. For example, breast cancer stem cells become enriched and resist to radiation [5]. Interestingly, recent data have demonstrated microRNAs play important roles in radiotherapy resistance. Luo et al. reported miR-668 improved radiotherapy resistance in breast cancer cells by regulating NF-κB [6]. Apparently, different miRNAs affect radiotherapy resistance by targeting different molecular pathway. Sun et al found miR-200c promoted radiation sensitivity in breast cancer cells by regulating UBQLN1 [7]. Huang and his colleagues reported miR-620 improved radiotherapy resistance in breast cancer by regulating hydroxyprostaglandin dehydrogenase 15-(nicotinamide adenine dinucleotide)[8]. These findings indicate miRNAs play essential roles in tumor radiation sensitivity by regulating cell cycle key components, DNA damage repair and apoptosis ect.
In this study, we investigated the roles of miR-328-3p in growth, migration, invasion and radiotherapy sensitivity using both breast cancer cells and human breast cancer tissues.

**Materials And Methods**

**Breast cancer cell lines**

MDA-MB-231 and T47D breast cancer cells (ATCC, USA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, USA) supplemented with 10% fetal bovine serum (Gibco, USA) and antibiotics (100 units of penicillin/ml and 100 mg of streptomycin/ml, Invitrogen, USA).

**Transfection**

MDA-MB-231 and T47D cells were transfected with either miR-328-3p mimic or miR-328-3p inhibitor (Invitrogen, USA) using lipofectamine 2000 (Invitrogen, USA). The breast cancer cells were incubated with transfection medium for no more than 6 h, then washed with PBS and grown in complete medium for further analyses. The scramble miRNA was used as negative control (Invitrogen, USA).

**Quantitative real-time polymerase chain reaction (qRT-PCR)**

MiR-328-3p level in breast cancer cells after transfection was examined by qRT-PCR. Briefly, breast cancer cells were incubated with cold TRIzol reagent (Invitrogen, USA) and total RNA was extracted. Reverse transcription was performed using High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific, USA). The RT-PCR were performed as follows: 95°C for 60s, 35 cycles at 94°C for 35 s, 60°C for 30 s, followed by a dissociation stage. The primers were synthesized by Sigma (USA) and the sequences were as follow: miR-328-3p forward: 5'- TTCCTAATGTTAAGT-3' and reverse 5'- TCAGCTGAAGCTGCCGTAAGA -3'. U6 was used as the endogenous control.

**Cell proliferation assay**

The impact of miR-328-3p on MDA-MB-231 and T47D cells growth was determined by MTT assay. Briefly, transfected MDA-MB-231 and T47D cells were cultured in complete culture medium overnight at 37°C in a humidified incubator. Then different doses of radiation (0, 1, 2 and 4 Gy) were used to treat these breast cancer cells. On every the other day, MDA-MB-231 and T47D cells were incubated with MTT solution for 4 hours. The OD value was measured on a microplate reader at 570nm following by adding DMSO.

**Caspase 3/7 activity-based apoptosis assay**

The impact of miR-328-3p on radiation-induced apoptosis was determined by detecting caspase 3/7 activities. Briefly, transfected MDA-MB-231 and T47D cells were treated with different doses of radiation. Then Caspase-Glo reagent (Promega, USA) was added into the 96-well plates and incubated at room temperature for 2 hours. The caspase 3/7 activities were measured by luminometer (ThermoFisher, USA).
**Transwell assay**

Transwell assays either with or without Matrigel were performed to evaluate invasion or migration in MDA-MB-231 and T47D cells. Breast cancer cells (20,000 cells/chamber) in upper chamber were cultured with serum free medium, the lower chambers were added 0.7 ml medium with FBS. The invaded breast cancer cells on the membrane were stained and counted at 5 random fields under light microscope (100 x) after removal of the cells in the upper chambers.

**Western blotting**

Protein expression in transfected breast cancer cells was examined by western blot assay. Briefly, cold RIPA buffer was used to extract total protein from transfected breast cancer cells. Then protein was separated based on molecular weights using sodium dodecyl sulfate polyacrylamide gel electrophoresis. Polyvinylidene difluoride (PVDF) membrane was used to bind these proteins and incubated with primary antibodies (Table 1) for 18 hours. The PVDF membrane was washed with PBS and reacted with secondary antibodies for 1 hour. The protein bands are developed and analyzed.

**Luciferase assay**

The possible binding gene of miR-328-3p was identified with miRDB online tool. 3'-UTR of PTEN was amplified from normal human cells to include the possible miR-328-3p binding site. PmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, USA) was used to detect the luciferase activity. MDA-MB-231 cells were transfected with both miR-328-3p and Luc-PTEN and Dual-Luciferase Reporter Assay system (Promega, USA) was used to detect luciferase activities.

**MiR-328-3p levels on human breast cancer tissue**

Breast cancer tissues from 131 patients from 2011 to 2019 were obtained from Jilin University Hospital. The Jilin University ethical committee approved the study protocol. All patients signed the research consents during surgical procedures. RNA was extracted from formalin-fixed paraffin-embedded breast cancer tissue and qRT-PCR was done to determine miR-328-3p expression levels.

**Immunohistochemical analysis**

PTEN and Ki-67 immunostains were performed on automated stainer (Bond RX, USA) using FFPE breast cancer tissue.

**Statistical analysis**

Statistical difference was analyzed by one-way analysis of variance (ANOVA) coupled with Tukey correction and student's t test. Differences were considered significant at \( P<0.05 \).

**Results**
MiR-328-3p level in transfected breast cancer cells

Overexpression of miR-328-3p was detected in MDA-MB-231 cells (Fig. 1A) and T47D cells (Fig. 1B) after miR-328-3p mimic transfection. Low expression level of miR-328-3p was detected in MDA-MB-231 cells (Fig. 1C) and T47D cells (Fig. 1D) after miR-328-3P inhibitor transfection.

MiR-328-3p inhibited growth of breast cancer cells with or without radiation

Overexpression of miR-328-3p decreased growth of MDA-MB-231 cells (Fig. 2A) and T47D cells (Fig. 2E) without radiation treatment (p<0.05). Overexpression of miR-328-3p inhibited survival rate in MDA-MB-231 cells (Fig. 2B-D) and T47D cells (Fig. 2F-H) with radiation treatment (p<0.05). However, low expression level of miR-328-3p improved growth of MDA-MB-231 cells (Fig. 3A) and T47D cells (Fig. 3E) without radiation (p<0.05). Low level of miR-328-3p enhanced survival rate in MDA-MB-231 cells (Fig. 3B-D) and T47D cells (Fig. 3F-H) with radiation treatment (p<0.05).

MiR-328-3p improved radiation-induced apoptosis in breast cancer cells

High caspase3/7 activities were detected in MDA-MB-231 cells (Fig. 4A) and T47D cells (Fig. 4B) after transfection with miR-328-3p mimic. Furthermore, miR-328-3p decreased Bcl-2 expression and enhanced BAX expression in MDA-MB-231 cells (Fig. 4 C-D) and T47D cells (Fig. 4 E-F).

MiR-328-3p altered invasion and migration in breast cancer cells

Overexpression of miR-328-3p decreased migration and invasion in MDA-MB-231 cells (Fig. 5 A-B) and T47D cells (Fig. 5 E-F). Low level of miR-328-3p improved migration and invasion in MDA-MB-231 cells (Fig. 5 C-D) and T47D cells (Fig. 5 G-H).

MiR-328-3p targeted PTEN in breast cancer cells

High level of miR-328-3p increased PTEN expression, but inhibited p-AKT expression in MDA-MB-231 cells and T47D cells (Fig. 6 A-C). However, low level of miR-328-3p inhibited PTEN expression, but increased p-AKT expression in MDA-MB-231 cells and T47D cells (Fig. 6 D-F).

MiR-328-3p directly bound to PTEN

PTEN was recognized as possible binding site of miR-328-3p (Fig. 7A). No significant changes were seen in luciferase activity in MDA-MB-231 cells transfected with both mu-PTEN-3’-UTR-pGL3 and miR-328-3p (Fig. 7B). On the contrast, luciferase activity was dramatically inhibited in MDA-MB-231 cells transfected with both wt- PTEN-3’-UTR-pGL3 and miR-328-3p(p<0.05) (Fig. 7C).

MiR-328-3p expression on human breast cancer tissues

Compared with normal breast tissue, higher level of miR-328-3p was detected in breast cancer tissue. Meanwhile, miR-328-3p expression was associated with clinical stage of breast cancer (Fig. 8A, Table 2).
Immunostain for Ki-67 showed a higher proliferation index in breast cancer tissue with low expression of miR-328-3p. Interestingly, breast cancer tissue with low level of miR-328-3p showed higher expression of PTEN (Fig. 8B).

**Discussion**

Studies have indicated miRNAs regulate variety of biological activities such as differentiation, apoptosis and proliferation [9]. Some miRNAs act as either oncogenes or tumor suppressors and can be used to predict prognosis[10]. Pan et al. reported that miR-328-3p decreased growth and inhibited metastasis in colorectal cancer cells by down-regulation of PI3K/Akt signaling pathway [11]. Lu et al. found that up-regulation of miR-328-3p inhibited proliferation and invasion of hepatocellular carcinoma by regulating AKT phosphorylation [12]. Yan et al demonstrated that miR-328-3p inactivated PI3K/AKT signaling pathway and inhibited epithelial-mesenchymal transition in urothelial cancer [13]. Recent study showed that miR-328-3p was up-regulated in triple-negative breast cancer [14]. Further study indicated that miR-328 inversely regulated breast cancer resistance protein (BCRP/ABCG2) and changed drug disposition in breast cancer cells [15–16]. In the current study, overexpression of miR-328-3p inhibited proliferation of breast cancer cells, but low level of miR-328-3p improved proliferation of breast cancer cells. Our results and prior studies suggest miR-328-3p can serve as tumor suppressor in breast cancer.

Some studies have demonstrated that miRNAs play important roles in radiotherapy resistant by regulating different signaling pathways such as decreasing apoptosis, enhancement of cell cycle protein expression and improvement of DNA damage repair [17]. Zhang et al reported that miR-29b might improve radiosensitivity by regulating PTEN express and inhibiting DNA damage repair in cervical cancer [18]. Ray and her colleagues found that miR-191 improved radiation resistance by directly targeting Retinoid X receptor alpha in prostate cancer [19]. Our study showed overexpression of miR-328-3p decreased survival of breast cancer cells after radiation. Low level of miR-328-3p improved survival of breast cancer cells after radiation. These findings indicated miR-328-3p involved in radiotherapy resistance in breast cancer cells. Our study also found miR-328-3p levels were closely related with the radiation-induced apoptosis in breast cancer cells. These results indicated miR-328-3p might regulate radiotherapy resistance through apoptosis pathway.

It is very common for breast cancer to spread to other part of the body such as liver, bone, brain and lungs [20]. Patients with metastatic breast cancer have higher stage and often have poor outcome [21]. Epithelial-to-mesenchymal transition (EMT) is a critical biological process of metastasis in cancer cells [22]. Studies have demonstrated miRNAs involve cancer metastasis and regulate a single step or multiple steps of EMT by targeting different signaling pathways. Liang et al. found that EMT could be improved by miR-221/222 in breast cancer cells by regulating Notch 3 [23]. Arora et al. reported that miR-506 inhibited invasion and migration in breast cancer cells. Meanwhile, the expression of mesenchymal proteins such as Vimentin, Snai2 and CD151 were suppressed by miR-506 [24]. Some miRNAs can inhibit metastasis by targeting EMT regulator. Chen et al reported that miR-200c regulated ZEB2 expression and suppressed metastasis in triple negative breast cancer [25]. In our study, aberrant expression of miR-328-
3p impacted migration and invasion in breast cancer cells. These results indicated miR-328-3p played a role in EMT in breast cancer cells. We further examined impact of aberrant expression of miR-328-3p on protein expression in multiple signaling pathways. We found that miR-328-3p bound to 3'UTR of PTEN in breast cancer cells and overexpression of miR-328-3p enhanced PTEN expression in both breast cancer cells and breast cancer tissues. These results suggested PTEN is target gene of miR-328-3p in breast cancer.

In summary, our study demonstrated miR-328-3p mediated proliferation and radiation sensitivity in breast cancer by regulating PTEN. However, breast cancer is a heterogeneous disease and many factors contribute to radiotherapy resistance in breast cancer. Further studies are required to explore how miR-328-3p interacts with other proteins in regulation breast cancer development and progression.

Declarations

Ethics approval and consent to participate: Ethical committee of Jilin University approved this study. The informed consents were signed by all patients. All methods were performed in accordance with the relevant guidelines and regulations by the Ethical committee of Jilin University.

Consent for publication: Not applicable

Availability of data and materials: The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Competing interests: No potential conflicts of interest

Authors' contributions:

YS and PJ: performed experiments, write manuscript
YS, JL and SX: performed experiments, prepare figures and table
PJ, SX and JL: Collected patient tissue, performed experiments
BL: design the project, write manuscript and statistical analysis

All authors reviewed the manuscript

Funding: Not applicable

Acknowledgement: Not applicable

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Tables

Table 1 The antibodies used in western blot

| Antibody | Vendor                           | Dilution |
|----------|----------------------------------|----------|
| BAX      | Cell signaling Technology, USA   | 1:1500   |
| BCL-2    | Santa cruz Biotechnology, USA    | 1:1000   |
| p-AKT    | Cell signaling Technology, USA   | 1:500    |
| PTEN     | Cell signaling Technology, USA   | 1:1000   |
| AKT      | Cell signaling Technology, USA   | 1:1000   |
| GAPDH    | Cell signaling Technology, USA   | 1:2500   |

Table 2: miR-328-3p expression level and Ki-67 index in different stage breast cancer

Figures
| Stage | Number of patients | miR-328-3p levels in normal breast tissue | miR-328-3p levels in invasive cancer tissue | Ki-67 index (per 2 mm²) |
|-------|--------------------|-------------------------------------------|---------------------------------------------|------------------------|
| IA    | 25                 | 112±11                                    | 481±21                                      | 12±1                   |
| IB    | 28                 | 131±13                                    | 419±26                                      | 11±1                   |
| IIA   | 21                 | 101±15                                    | 389±33                                      | 14±3                   |
| IIB   | 18                 | 87±10                                     | 321±27                                      | 23±3                   |
| IIIA  | 14                 | 97±8                                      | 233±30                                      | 29±4                   |
| IIIB  | 12                 | 121±11                                    | 304±27                                      | 37±3                   |
| IICC  | 7                  | 108±10                                    | 215±31                                      | 39±4                   |
| IV    | 6                  | 109±11                                    | 166±16                                      | 45±3                   |
miR-328-3p level in breast cancer cells transfected either with mimic or inhibitor (A) miR-328-3p level after MDA-MB-231 cells were transfected with miR-328-3p mimic; (B) miR-328-3p level after T47D cells were transfected with miR-328-3p mimic; (C) miR-328-3p level after MDA-MB-231 cells were transfected with miR-328-3p inhibitor; (D) miR-328-3p level after T47D cells were transfected with miR-328-3p inhibitor.
Figure 2

miR-328-3p decreased growth of MDA-MB-231 and T47D cells with or without radiation. (A-D) The growth of MDA-MB-231 cells with or without radiation after transfection with miR-328-3p mimic; (E-H) The growth of T47D cells with or without radiation after transfection with miR-328-3p mimic.
miR-328-3p improved growth of MDA-MB-231 and T47D cells with or without radiation. (A-D) The growth of MDA-MB-231 cells with or without radiation after transfection with miR-328-3p inhibitor; (E-H) The growth of T47D cells with or without radiation after transfection with miR-328-3p inhibitor.
Figure 4

miR-328-3p improved radiation-associated apoptosis in breast cancer cells. (A) Caspase 3/7 activity in MDA-MB-231 cell transfected with miR-328-3p mimic; (B) Caspase 3/7 activity in T47D cell transfected with miR-328-3p mimic; (C-F) Bcl-2 and BAX expression in MDA-MB-231 and T47D cells.
Figure 5

miR-328-3p altered invasion and migration of MDA-MB-231 and T47D cells (A-B) overexpression of miR-328-3p inhibited migration and invasion of MDA-MB-231 cells. (C-D) low level of miR-328-3p improved migration and invasion of MDA-MB-231 cells. (E-F) overexpression of miR-328-3p inhibited migration and invasion of T47D cells. (G-H) low level of miR-328-3p improved migration and invasion of T47D cells.
Figure 6

miR-328-3p changed PTEN/AKT protein expression in breast cancer cells. (A-C) Impact of miR-328-3p overexpression on p-AKT, AKT and PTEN in breast cancer cells. (D-F) Impact of low level of miR-328-3p on p-AKT, AKT and PTEN in breast cancer cells.
miR-328-3p targeted PTEN in breast cancer cells. A. The possible binding site of miR-328-3p in 3'-UTR of PTEN; B. Luciferase activity of MDA-MB-231 cells transfected both miR-328-3p mimic and mut-PTEN-3'-UTR-pGL3; C. Luciferase activity of MDA-MB-231 cells transfected with miR-328-3p mimic with wt-PTEN-3'-UTR-pGL3.
miR-328-3p expression in human breast cancer tissues (A) miR-328-3p levels in different stage of breast cancer tissue and normal breast tissue. (B) Immunohistochemical studies for Ki-67 and PTEN on different levels of miR-328-3p in breast tissues.