MiR-27a Modulates Radiosensitivity of Triple-Negative Breast Cancer (TNBC) Cells by Targeting CDC27

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Background: MiR-27a is significantly overexpressed in triple-negative breast cancer (TNBC). However, the exact biological function of MiR-27a in TNBC is not fully understood. In this study, we verified miR-27a expression in TNBC cells and explored how its overexpression modulates radiosensitivity of the cells.

Material/Method: qRT-PCR analysis was performed to study miR-27a expression in TNBC lines MDA-MB-435 and MDA-MB-231 and in normal human breast epithelial cell line MCF10A. Dual luciferase assay was performed to verify a putative downstream target of miR-27a, CDC27. CCK-8 assay was used to assess the influence of miR-27a-CDC27 axis on cell proliferation under irradiation (IR) treatment.

Results: We confirmed significantly higher miR-27a expression in 2 TNBC cell lines – MDA-MB-435 and MDA-MB-231 – than in human breast epithelial cell line MCF10A. miR-27a could modulate proliferation and radiosensitivity of TNBC cells. CDC-27 is a direct target of miR-27a and its downregulation conferred increased radioresistance of the cells.

Conclusions: The miR-27a-CDC27 axis might play an important role in modulating response to radiotherapy in TNBC cells. Testing miR-27a expression might be a useful way to identify a subgroup of patients who will benefit from an IR-based therapeutic approach.

MeSH Keywords: Apc3 Subunit, Anaphase-Promoting Complex-Cyclosome • MicroRNAs • Radiotherapy, Adjuvant • Triple Negative Breast Neoplasms

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Background

Triple-negative breast cancer (TNBC), which is defined as the absence of estrogen receptor, progesterone receptor, and HER-2 expression [1], is difficult to treat since most chemotherapies target just 1 of the 3 receptors. Therapies combined with adjuvant radiotherapy help to improve survival of TNBC patients. However, this strategy is challenged due to the emergence of radioresistance of cancer cells [2]. Therefore, understanding the underlying mechanism of radioresistance of TNBC is quite beneficial in developing more effective therapeutic strategy.

miRNAs are a group of small, conservative, non-coding RNAs degrading or repressing the translation of target mRNAs by directly binding to the 3’-UTR [3]. Altered miRNA expression was observed in TNBC and some of the miRNAs play important roles in modulating proliferation, migration, or even radiosensitivity of the cancer cells [4,5]. For example, overexpression of miR-155 in TNBC cancers reduces the levels of RAD51, a central protein in homologous recombination modulating cellular response to irradiation (IR) [2]. MiR-31 can directly target protein kinase C Epsilon and thus enhance apoptosis of TNBC cells under IR treatment [6]. MiR-27a is significantly overexpressed in TNBC [7]. In fact, miR-27a has long been recognized as an oncomiR in breast cancer. Its overexpression is associated with endothelial differentiation of breast cancer stem like cells [8]. It can also regulate specificity protein transcription factors and the G2-M checkpoint in TNBC cell line MDA-MB-231 [9], thereby affecting cell proliferation and apoptosis. Since IR induced apoptosis is highly related to cell cycle arrest, miR-27a is highly possible involved in regulation of radiosensitivity of TNBC cells. CDC27 is a core component of anaphase-promoting complex (APC) and is involved in regulation of mitotic checkpoint to ensure chromosomal integrity [10]. Several study observed that APC or CDC27 is downregulated in breast cancer [11–13]. However, its upstream regulation in breast cancer is still not clear.

In this study, we verified miR-27a expression in TNBC cells and explored how its overexpression modulates radiosensitivity of the cancer cells.

Material and Methods

Cell culture

Triple negative breast cancer cell line MDA-MB-435 and MDA-MB-231, normal human breast epithelial cell line MCF10A and HEK293T cells were obtained from ATCC. The two breast cancer cells were cultured in RPMI-1640 medium and HEK293T cells were cultured in Dulbecco's modified Eagle's (DEME) medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin. MCF10A cells were grown in Ham’s F12: DMEM (50:50) medium supplemented with 2 mM L-glutamine, 20 ng/ml epidermal growth factor (EGF) (Sigma), 0.1 mg/ml cholera toxin (CT) (Sigma), 10 mg/ml insulin (Sigma), 500 ng/ml hydrocortisone (Sigma) and 5% horse serum (Atlanta Biologicals). All cells were cultured in an incubator with humidified atmosphere and 5% CO₂ at 37°C.

Reagents and cell transfection

MiR-27a mimics, anti-miR-27a (antagomiR-27a), CDC27 siRNA and the negative controls were purchased from RiboBio (China). To overexpress or knockdown or miR-27a, cells were transfected with 75 nM miR-27a mimics or 200nM antagomiR-27a respectively by using lipofectamine 2000 (Invitrogen). To knockdown the expression of CDC27, cells were transfected with 50nM CDC27 siRNA using Oligofectamine (Invitrogen). CDC27 lentiviral expression vectors (without 3’-UTR region) were purchased from GENECHEM (China). To generate adequate lentiviral particles for infection, the lentiviral vectors were transfected to HEK293T cells in combination with the packaging mix. At 48 h after infection, the culture supernatant was collected and the viral titer was determined. To overexpress CDC27, the cells were treated with viral supernatants with the presence of 8 μg/ml Polybrene (Sigma-Aldrich).

Ionizing radiation (IR) (γ-ray)

At 24 h before irradiation treatment, MDA-MB-435 and MDA-MB-231 cells were plated in 25 cm² polystyrene flasks to form a subconfluent monolayer. Then the cells were irradiated by using a Gamma Cell 40 Exactor (Nordion International) at a dose rate of 2.4 Gy/min.

Quantitative RT-PCR

Total RNAs from MDA-MB-435 and MDA-MB-231 cell samples were extracted with TRizol reagent (Invitrogen). The absorbance of the RNA samples was determined with a UV-visible spectrophotometer (NanoDrop Technologies) and the samples with A260/A280>2 were used for following studies. Taqman miRNA Assays was used to measure miR-27a expression. RNU6B was used as an internal control. To quantify CDC27 mRNA expression analysis, first strand cDNA was synthesized by using RevertAid first strand cDNA synthesis kit (Fermentas) according to the manufacturer’s instructions. Then qRT-PCR analysis was performed by using Power SYBR Green PCR Master Mix and CDC27 specific primers: (F) 5’- AGAAGTTATGTTGTGGCCTTGG-3’ and (R) 5’-AGGTACAACAGCAGCATGGTTC-3’. All qRT-PCRs were performed with ABI Prism 7300 sequence detection system (Applied Biosystems Inc.).
Western blot analysis of CDC27 protein expression

MDA-MB-435 and MDA-MB-231 cells were lysed with lysis buffer (Beyotime, China). The extract samples were separated by 10% SDS-PAGE gel and then transferred to NC membrane for a conventional Western blotting analysis. The membranes were probed with primary antibodies to CDC27 (1:3000, ab72214, Abcam) and GAPDH (1:2000, ab125247, Abcam) and then incubated with corresponding HRP-conjugated secondary antibody (1:10000, anti-rabbit IgG (HRP) ab191866, Abcam; 1:10000, anti-mouse IgG (HRP), ab6728, Abcam). The signals were visualized by using ECL Western Blotting Substrate (Thermo Scientific Pierce) and the signal intensity was quantified by using Image-J software.

MTT assay of cell proliferation

Cells after different treatments were plated at 5×10^3 cells/well in 96-well plates. Cells were cultured for 48 h and then cell viability was measured by Cell Counting Kit-8 (CCK-8) (Dojindo) according to manufacturer’s instruction. Each test was performed with 3 repeats.

Flow cytometry analysis of apoptotic cells

At 48 h after treatments, cells were plated in 6-well plates at 4×10^5 cells/well. The proportion of apoptotic cells was determined by using Fluorescein Active Caspase 3 Staining Kit (88-7004, eBioscience) with a flow cytometry (FACScalibur, BD Biosciences). The results were analyzed by using ModFit (BD Biosciences).

Dual luciferase analysis

The binding site between miR-27a and CDC27 was predicted by using TargetScan 6.2. With the predication, DNA oligonucleotides with wide-type and mutant sequence of CDC27 3’-UTR were designed and chemically synthesized: WT: forward, 5’-aaacTTACCTGTATTTGTTAGTCAAGCTGTGAAAATAAGGTGGATt-3’; reverse, 5’-ctagaATCCACCTTATTTTCACAGCTTGACTAACAAATACAGGTAgttt-3’; MUT: forward, 5’-aaacTTACCTGTATTTGTTAGTCAAGGACACTTAATAAGGTGGATt-3’; reverse, 5’-ctagaATCCACCTTATTTTCACAGCTTGACTAACAAATACAGGTAgttt-3’. These oligonucleotides were inserted into the downstream of the firefly luciferase gene in pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega) between PmeI and XbaI sites to construct dual luciferase reporters, named as pmirGLO-CDC27-WT and pmirGLO-CDC27-MUT respectively. HEK-293T and MDA-MB-435 cells were co-transfected with 150ng reporter plasmids and 50 nM miR-27a mimics. Both firefly and Renilla luciferase activities were measured 24 h after transfection using the Dual-Luciferase Reporter Assay System (Promega) and the GloMax™ 20/20 Luminometer (Promega).

Statistically analysis

Quantitative variables with normal distribution were reported as median ±SD. Between group comparison was performed using unpaired t-test. p < 0.05 was considered as statistically significant. *** **** and ***** donates significance at 0.05, 0.01 and 0.001 level, respectively. All statistical analyses were performed using SPSS for Windows 17.0 (SPSS, Inc).

Results

MiR-27a is significantly increased while CDC27 expression is significantly decreased in TNBC cells

To assess the role of miR-27a in TNBC, TNBC cell line MDA-MB-435 and MDA-MB-231 and normal human breast epithelial
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Figure 2. MiR-27a is involved in radiosensitivity of TNBC cells. (A–D) qRT-PCR analysis of miR-27a expression in MDA-MB-435 (A, C) and MDA-MB-231 (B, D) cells after transfection of 200 nM antagomiR-27a (A, B) or 75 nM miR-27a mimics (C, D). (D–G) CCK-8 assay of cell proliferation of MDA-MB-435 (D, F) and MDA-MB-231 (E, G) cells transfected with 200 nM antagomiR-27a with/without treatment of IR (8 Gy) (E, F) or transfected with 75 nM miR-27a mimics with/without treatment of IR (8 Gy) (G). (I, J) MDA-MB-435 cells with miR-27a knockdown or overexpression were stained with caspase-3 staining kit 24 h after IR (8 Gy) treatment. Then, flow cytometry analysis was performed to measure the proportion of cells with active caspase-3 staining. Representative flow cytometry images of the cells with active caspase-3 (I). Quantification of apoptotic cells with active caspase-3 signal in Figure 2I (J). Data are shown as mean ± S.D. by 3 independent experiments. * P<0.05, ** P<0.01, *** P<0.001.

MiR-27a is involved in radiosensitivity of TNBC cells

To further explore the biological function of miR-27a, MDA-MB-435 and MDA-MB-231 cells were transfected for miR-27a knockdown (Figure 2A, 2B) and overexpression (Figure 2C, 2D), respectively. In both of the cell lines, miR-27a knockdown significantly associated with TNBC cells, while its expression is negatively correlated with CDC27 expression.
significantly inhibited cell proliferation and also enhanced IR-induced proliferation inhibition (Figure 2E, 2F). In contrast, miR-27a overexpression significantly promoted cell proliferation and also alleviated IR-induced proliferation inhibition (Figure 2G, 2H). To further verify the role of miR-27a in radiosensitivity, we assessed how its overexpression and knockdown affect IR induced apoptosis in MDA-MB-435 cells. miR-27a overexpression significantly reduced the proportion of active caspase 3-positive cells, while its knockdown enhanced the apoptosis (Figure 2I, 2J). These results suggest that miR-27a is involved in the regulation of radiosensitivity of TNBC cells.

MiR-27a directly targets CDC27 mRNA and regulates its expression in TNBC cells

Although the regulative role of miR-27a in TNBC cells has been verified, its downstream targets are still not clear. Through searching and comparison in online bioinformatics databases, we found CDC27 has a highly conserved 3’UTR sequence with miR-27a in mammals (Figure 3A). Then, we verified this binding using dual-luciferase assay in both HEK-293T (B) and MDA-MB-435 (C) cells co-transfected with 150 ng reporter plasmids and 50 nM miR-27a mimics. Both firefly and Renilla luciferase activities were measured 24 h after transfection and the firefly luciferase activity was normalized to the Renilla luciferase activity. (D, E) Western blot analysis of CDC27 protein expression in MDA-MB-435 transfected with miR-27a mimics or siCDC-27 (D) or transfected with antagoniR-27a or infected with CDC-27 lentiviral particles (E). Data are shown as mean ±S.D. by 3 independent experiments. * P<0.05, ** P<0.01, *** P<0.001.

MiR-27a modulates radiosensitivity of TNBC cells through targeting CDC27

Because the regulative role of miR-27a on CDC27 was verified in TNBC cells, we further studied whether this axis is involved in regulation of radiosensitivity. Transfection of CDC27 in both MDA-MB-435 and MDA-MB-231 cells could inhibit cell proliferation. In addition, CDC27 overexpression also enhanced IR-induced inhibition of cell growth (Figure 4A, 4B). At the same time, knockdown of CDC27 in these 2 cell lines
conferred increased cell growth, with or without IR treatment (Figure 4C, 4D). Transfection of CDC27 could significantly abrogate miR-27a-induced growth promotion (Figure 4E, 4F). However, knockdown of CDC27 could rescue cell growth inhibition induced by miR-27a knockdown (Figure 4G, 4H). These results suggest that miR-27a can modulate radiosensitivity of TNBC cells through targeting CDC27.

**Discussion**

MiR-27a is one of the miRNAs significantly upregulated in TNBC patients [7]; its high expression is associated with poor overall patient survival [14]. Its oncogenic mechanisms have been widely reported in breast cancer. miR-27a can directly target FOXO1 and thus regulate transformation or maintenance of an oncogenic state in breast cancer cells [15]. It can also regulate specificity protein transcription factors and the G2-M checkpoint in TNBC cells MDA-MB-231 [9], thereby affecting cell proliferation and apoptosis. However, since a miRNA usually has multiple targets and the targets may vary in different pathological process, how miR-27a affects TNBC needs to be further studied. In the current study, we confirmed significantly higher miR-27a expression in 2 TNBC cell lines – MDA-MB-435 and MDA-MB-231 – than in normal human breast epithelial cell line MCF10A. In addition, we also observed that miR-27a could modulate proliferation and radiosensitivity of TNBC cells. However, the exact mechanism is not clear.

CDC27 is a core component of the anaphase-promoting complex (APC) and is involved in regulation of mitotic checkpoints to ensure chromosomal integrity [10]. APC plays a critical role in promoting metaphase-anaphase transition through ubiquitizing and degrading securin. It also promotes the M-to-G1 transition by ubiquitinating cyclin-B and accelerating its degradation [16]. Therefore, as a core component of APC, dysregulation of CDC27 may significantly affect the function of the polymeric protein complex. Due to its importance in mitotic checkpoints or spindle assembly checkpoint (SAC) control, this molecule is considered as a target of some anticancer drug [17]. It has been reported that APC or CDC27 is downregulated in breast cancer [11]. However, how it is dysregulated and the exact biophysical function of this protein in breast cancer is not fully understood.

In this study, we verified a highly conserved putative binding site between miR-27a and CDC27 and demonstrated that miR-27a can directly regulate CDC27 expression in TNBC cancer cells. There is emerging evidence showing that TNBC cells are characterized by altered distribution of cell cycles, checkpoints of chromosomal integrity, and control of DNA damage repair [18,19]. A recent study reported that lower expression
of protein predicted shorter survival time for breast cancer patients [11]. Therefore, it might act as a tumor suppressor in breast cancer. Due to the important role of CDC27/APC in the mitotic regulatory pathway, it might also be involved in regulation of IR-induced cell death. A previous study reported that lower expression CDC27 was associated with poorer response to radiotherapy in squamous cell cervix carcinoma [20]. In the current study, we explored the role of CDC27 in regulation of radiosensitivity of TNBC cells and found its downregulation conferred increased radioresistance of the cells. Therefore, the miR-27a-CDC27 axis might play an important role in modulating response to radiotherapy in TNBC cells. In fact, radiosensitivity might be regulated by a complex network. For example, overexpression of miR-155 in human breast cancer cells can reduce RAD51 expression, a central protein in homologous recombination, thereby affecting the cellular response to IR [2]. MiR-31 can also enhance apoptosis of TNBC cells under IR treatment [6]. More studies are required to explore the underlying mechanism of radioresistance in TNBC.

Conclusions

The MiR-27a-CDC27 axis might play an important role in modulating response to radiotherapy in TNBC cells. Testing miR-27a expression might be a useful practice to identify subgroup of patients who will benefit from an IR-based therapeutic approach.

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