Therapeutic Potential of miR-29a in Breast Cancer Patients with type 2 Diabetes and its Regulatory Mechanism

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Abstract

MicroRNAs (miRNAs) acting as tumour suppressors or oncogenes (oncomiRs), are a promising new focus of targeted therapeutics for cancer. Diabetes preexisted in approximately 16% of breast cancer patients. This study mainly aims to investigate the therapeutic potential of miR-29a in breast cancer patients with type 2 diabetes (BDM) and its regulatory mechanism. Our results indicated that miR-29a is upregulated in BDM patients, which was correlated with worse prognosis. In human breast cancer cells, miR-29a knockdown reduced cell proliferation, cells migration and invasion in BDM conditions (25 mmol/L glucose + 25 nmol/L insulin). In xenograft mouse model for BDM, miR-29a knockdown suppresses MDA-MB-231 cells tumourigenesis and metastasis. Finally, we demonstrated that miR-29a promoted cell growth and metastasis of BDM via targeting of SIRT1. Collectively, our findings indicated that anti-miR-29a inhibited cell proliferation and invasion in BDM by targeted SIRT1, anti-miR-29a may represent a novel therapeutic approach for the management of BDM patients.

1. Introduction

Both breast cancer and diabetes are common chronic diseases among women, which are the global health issues of great concern [1]. Abundant epidemiological evidence shows that diabetes, especially type 2 diabetes, have been intricately related with breast cancer. Compared with breast cancer of non-diabetic patients, the mortality rate of breast cancer patients with diabetes is significantly higher (RR = 1.53; 95% CI: 1.23 to 1.90) [2]. Previously study has showed that preexisting diabetes is independently correlated with worse overall survival (OS) and disease-free survival (DFS) in female breast cancer patients [3]. Type 2 diabetes can increase the synthesis of sex hormones, high insulin and IGF can reduce sex hormone binding globulin of hepatic synthesis, and IGF-I can be activated and androgen synthesis increase. *Novosyadlyy R*, et al, used MKR mice of type 2 diabetes to verify that type 2 diabetes promotes breast development and breast cancer through hyperinsulinemia [4]. Sirtuin 1 (SIRT1) is a kind of NAD⁺ dependent class III histone deacetylation (HDAC) enzyme, which is involved in energy metabolism and insulin resistance. *Balestrieri*, et al, revealed the decrease of SIRT1 in diabetes patients [5]. SIRT1 deficiency is proved to promote or suppress tumors in breast cancer according to different BC molecular subtypes [6]. Accordingly, SIRT1 may be a critical determinant in the development and metastasis of diabetic breast cancer.

Numbers studies have proved that miRNA dysregulation is causal in variety of cancers, which play a tumour suppressors or oncogenes (oncomiRs) and the miRNAs (antimiRs) of miRNA mimics and molecules targeted have shown promise in preclinical development [7]. Some miRNA targeted therapies are under way in clinical trials, such as the mimics of tumor suppressor miRNA miR-34 has reached phase I clinical trials for the treatment of cancer, and anti-miRs against miR-122 has reached phase II clinical trials for the treatment of hepatitis [8]. MiR-29a, as a dominant member in the miR-29 family, is up-regulated in the serum of type 2 diabetic patients and 3T3-L1 adipocytes cultured with high levels of glucose and insulin [9]. MiR-29a were confirmed to regulate the EMT and metastasis of breast cancer cells [10]. In our previous study have supported that the breast cancer cells can accelerated proliferation

and metastasis via adjust miR-29a expression involved in the insulin signaling pathway[11]. Furthermore, Recent studies have revealed miR-29a targeted to the 3'-untranslated region (UTR) of SIRT1 mRNA [12, 13]. To our knowledge, there is no research to illuminate the regulatory relationship of miR-29a-3p/SIRT1 in human diabetic breast cancer. In this study, we aim to explore the potential and regulatory mechanisms of miR-29a as a biological target for the treatment of diabetic breast cancer.

2. Material And Methods

2.1. Clinical tissue samples

37 cases of BDM patients at the Third Hospital of Nanchang City (Nanchang, China) were enrolled in the study. The control group included 50 age-matched breast cancer patients without type 2 diabetes mellitus (NBDM) at the same period, based on the oncologist confirmation and negative familial history of diabetes. All patients with primary breast cancer who underwent surgical resection from December 2012 to October 2016. The fresh tumor tissue specimens were collected and frozen in liquid nitrogen immediately and stored at -80°C until needed. All the patients were female, and the postoperative histologically diagnosed with invasive breast cancer. After surgery, all enrolled patients were followed up every 3 months and the survival time was recorded. The cut-off for the follow-up period was December 31, 2019.

The baseline tumor characteristics are listed and compared between BDM group and NBDM group (Supplementary data, Table S1). This study was conducted in accordance with the Helsinki Declaration and was approved by the Ethics Review Committee of Third hospital of Nanchang. Written informed consent was obtained from all breast cancer patients in our study.

2.2. Cell culture

The MDA-MB-231 breast cancer-derived cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and maintained in Dulbecco's Modified Eagle's medium (DMEM, Gibco BRL, Grand Island, NY, USA) containing normal glucose (5.6 mM/L), supplemented with 10% fetal bovine serum (FBS; Gibco), 100 U/ml penicillin and 100 mg/ml streptomycin (Invitrogen, USA) at 37°C in a humidified incubator under 5% CO₂-95% air.

2.3. Plasmids, lentivirus production and transduction

miRZip-29a, an shRNA-miR-29a structure cloned in a SBI's pGreenPur™ lentivector, was purchased from System Biosciences (Cat No, MZIP29a-PA-1; Palo Alto, CA), lentivirus used to stably overexpress miR-29a in breast cancer cells were purchased from Genechem Company (Shanghai, China), and the Scr control plasmid was a generous gift from Professor F-C Zhang at Ruijin Hospital of Shanghai Jiaotong University.

MiRZip-29a plasmid or Scr control plasmid was transfected into 293T cells by Lipofectin Reagent (Invitrogen) according to the manufacturer's protocol. Pseudoviral particles were applied on MDA-MB-

231 cells. 24 hours later, Virus-infected cells were subjected to drug selection (1 µg/ml puromycin) for 7 days to achieve stable cell subclones. They were labeled as blank group, anti-miR29a and vector group. SIRT1 plasmid (5'-CCGGATTGAAGAATGTTGG-3', 5'-ATCTGCTCCTTTGCCACTC T-3') cloned into pcDNA3.1 vector to increase expression of SIRT1 in MDA-MB-231 cells (named as pc-SIRT1 and pcDNA3.1). After drug-selection, cells of normal conditions (5.6 mM/L glucose + 5 nM/L insulin) or BDM (25 mM/L glucose + 25 nM/L insulin) conditions were cultured in vitro in different experiments.

2.4. TaqMan miRNA analysis

The RNA extracted from the tissues and cells using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's protocol. MiRNAs were detected by real-time reverse transcriptase polymerase chain reaction (qRT-PCR). The cDNAs were produced by reverse transcription kits (Takara, Dalian, China) and miRNA specific bulg-loop™ miRNA RT primers (Ribobio Co, Guangzhou, China) (Supplementary data, Table S2). Real-time PCR was performed on a StepOnePlus Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) using an SYBR Green I Real-Time PCR kit (Takara, Dalian, China). Using the comparative delta CT ($2^{-\Delta\Delta Ct}$) method to estimate the relative expression of miR-29a-3p. U6 small nuclear 2 (RNU6) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as endogenous control in this study.

2.5. Western blot

Proteins collected from the relevant cells are electrophoretic on a polyacrylamide gel and transferred to a PVDF membrane. Then, 5% fat free dry milk was used to seal the membrane in TBS-T for 1 hour. The primary antibodies were applied to the cell membrane at 4°C overnight and then washed off with TBS-T. The membrane was then cleaned with TBS-Tween and incubated with a secondary antibody at 1:10,000. The Clarity Western ECL substrate (Bio-Rad, Hercules, CA, USA) was used for protein detection. The relevant data were analyzed using GraphPad Prism 5 software (GraphPad software, San Diego, CA, USA). Repeat Western blots at least three times.

2.6. Immunohistochemistry

For immunohistochemistry, paraffin sections were treated with primary antibody against SIRT1 (1:50 dilution, DF6033, Affinity Biosciences) at 4°C overnight. After incubating with secondary antibody and DAB substrate for visualization, the sections were subjected to fluorescence microscopy (CKX53, Olympus) for imaging.

2.7. Luciferase reporter assay

The wild-type 3'UTR of SIRT1 mRNA and the mutant type 3'UTR of SIRT1 mRNA (UGGUGCU to ACCACGA) were amplified and inserted into pmiRGLO vectors (Promega Corporation, Madison, WI, USA). The HEK-293T cells were co-transfected with the reporter plasmids and miR-29a-3p mimics or miR-NC using Lipofectamine 3000 (Invitrogen). After cells transfection at 48 h, the cells were collected and Renilla luciferase activity was detected by using the Dual Luciferase Reporter Assay System (Promega Corporation, Madison, WI, USA).

2.8. CCK-8 assays

The cells of different groups were seeded in 96-well plates and cultured for 12, 24, 48 or 72 h. Cell proliferation was detected with Cell Counting Kit-8 (CCK-8) Kit (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). reference the manufacturer' instructions. The absorbance at 450 nm was examined using a microplate reader (Bio-Rad, Berkeley, CA, USA). Triplicate independent experiments were performed.

2.9. Flow cytometry analysis

The cells were cultured in the DMEM medium with insulin (25 nmol/L) plus high glucose (25 mmol/L) for 48 h, then the cells were harvested and washed twice with PBS and fixed overnight at -20 °C with 70% ethanol. After washed with PBS, the cells were stained with 5 ul of propidium iodide solution (10 ug/ml) and 100 ul of RNase (100 ug/ml) in PBS and incubated for 30 min at room temperature. The analysis was performed using FACS Calibur (Becton Dickinson, Franklin Lakes, NJ) and CellQuest Pro software (Becton Dickenson).

2.10. Wound healing assay

In order to determine MDA-MB-231 cells migration, the different groups of those cells were seeded onto 6-well plates, and cultured in BDM condition per well overnight. Wounds were made by gently scraping with a disposable pipette tip, and those MDA-MB-231 cells were photographed using a phase contrast microscope (Olympus, Japan) at 0 and 48 hours. Distance migration was measured by calculating the area of the wound using Image J software and represented in the form of bar graph. All experiments in this study were repeated independently three times with similar results.

2.11. Invasion assay

Cell migration assays were performed in 24-well transwell chambers (8.0 µm; Corning Incorporated, Corning, NY, USA) to detect the migration and invasion of transfected cells. Every 200 µl of cell suspensions were pre-coated with 50 µl of 1.0 mg/ml Matrigel (Millipore, Billerica, MA, USA), which obtain from serum-free media with the density of 2×10^4 cells/ml and seeded into the upper chamber of transwells. After, the chemo-attractant (600 µl of medium containing 10% serum) was added to the lower chamber with 24 hours of incubation. The cells on the upper side of the transwell were removed with a cotton swab which were fixed with 100% methanol (30 min), than staining using Crystal Violet Staining Solution (Beyotime Institute of Biotechnology)

2.12. Xenograft mouse model for breast cancer with type 2 Diabetes Mellitus

All female BALB/c mouse with 4-week-old which were purchased from the Animal Research Center of Shanghai Jiaotong University. The mouse were fed ad libitum a high-fat diet (HFD) containing 60 kcal % fat for 10 weeks and acclimated to it for at least 7 days before the experiment. After 2 weeks of HFD treatment, the mouse was intraperitoneally injected for 5 consecutive day with small dose of 40 mg/kg streptozotocin (STZ) (Sigma-Aldrich, Beijing, China) dissolved in 100 μ l of 0.05 M citrate buffer (pH 4.5). The STZ solution is prepared a few minutes before use. 3 days after STZ administration, the random blood glucose of mice was detected. The model of T2DM mice was established successfully if the blood glucose was more than 16.7 mmol/l for two days.

MDA-MB-231 cells with Scr control plasmid or MiRZip-29a plasmid were washed with DPBS and resuspended in Matrigel (10 mg/mL). All mouse received s.c. injections at the fourth mammary fat pad with 0.1 mL of cell suspension (5×10^5 cells/mL). The mouse were sacrificed 8 weeks after inoculation. As the Tumor volumes formula: $\text{volume} = 0.5LS^2$, which were assessed by measuring the longest (L) and shortest (S) diameter of the tumor under a microscope. The number of tumor nodules in lung was also counted after 8 weeks. Each tissue was excised and embedded in paraffin for histopathological examination. The schematic diagram of animal model construction process was shown in Fig. 1. This animal study was approved by Ethics Committee for animal experimental research of Third hospital of Nanchang.

2.13. Statistical analyses

T test, mann-whitney U test, bidirectional analysis of variance and correlation analysis were used to compare the differences between variables in this study. The Kaplan–Meier analysis was performed to compare Disease-specific Survival (DSS) and Recurrence-free Survival (RFS) between the groups. $P < 0.05$ was considered statistically significant. All analyses were performed using SPSS. The mean $\pm$ SD is displayed in the figures.

3. Results

3.1. *MiR-29a-3p is upregulated in BDM and correlated with poor prognosis*

The expression of mi-29a in BC cell lines and tumor tissues were measured by qRT-PCR. As shown in Fig. 2A, the expression of miR-29a-3p was increased in BDM tissues than NBDM tissues. The expression of miR-29a in BC cell lines cultured in BDM conditions was also higher than that cultured in normal conditions (Fig. 2B).

The expression of miR-29a was correlated with the clinicopathological characteristics of BDM. As shown in Table 1, the high level of miR-29a expression was significantly correlated with proliferation index (ki-67 $\geq 30\%$), distant metastasis and lymph node metastasis. The Kaplan-Meier analysis suggested that BDM

with high miR-29a expression had significantly worse RFS and DSS than those with low miR-29a-3p expression (Fig. 2C, 2D).

Table 1
Association between miR-29a-3p and clinicopathological features of breast cancer patients with type 2 diabetes mellitus

Variable	Case,n	miR-29a-3p expression, n		P Value
		Low (n = 19)	High (n = 18)	
menopausal status				
Pre-menopausal	18	11	7	0.248
Post menopausal	19	8	11	
Histologic type				
Special type	6	4	2	0.357*
Non special type	31	15	16	
Axillary lymph node status				
Metastasis	14	4	10	0.031
No	23	15	8	
Tumor size				
≥ 2CM	23	10	13	0.219
< 2CM	14	9	5	
ER				
positive	25	11	14	0.197
negative	12	8	4	
PR				
positive	21	10	11	0.603
negative	16	9	7	
HER-2				
negative	27	12	15	0.312*
positive	10	7	3	
Ki67				
≥ 30%	20	6	14	0.005
< 30%	17	13	4	
distant metastasis				

Variable	Case,n	miR-29a-3p expression, n		P Value
		Low (n = 19)	High (n = 18)	
Yes	12	3	9	0.026
NO	25	16	9	

3.2. Anti-miR-29a inhibited cell proliferation in BDM condition

In order to research the effect of miR-29a knockdown on the proliferation of breast cancer cells in BDM condition, we constructed an anti-sense miR-29a vector (“shRNA-miR-29a”, named as “anti-miR-29a”). We established a stable MDA-MB-231 cells constitutively-expressing “anti-miR-29a” by utilizing this vector. A qRT-PCR assay showed that miR-29a levels were significantly descended in the stable cells (Fig. 3A). The CCK8 assay showed cell proliferation abilities of anti-miR-29a group were sharply decreased compared with control vector group or blank control group (Fig. 3B). Flow cytometric analysis indicated that knockdown of miR-29a in MDA-MB-231 cells could significantly down-regulate the G1 phase ratios and increase G2 phase ratios (Fig. 3C,3D). These data suggested that miR-29a knockdown of breast cancer cells in BDM condition could inhibit cells growth and proliferation.

3.3. Anti-miR-29a reduced cells migration and invasion in BDM condition

To investigate the role of miR-29a knockdown on migration and invasive potential of MDA-MB-231 cells, we established wound healing assay and matrigel invasion assay, respectively. The results of a wound healing assay have showed that MDA-MB-231 cells of anti-miR-29a group displayed a lower migration ability (4.67 ± 0.33) compared with that in control vector group (10.17 ± 0.17) or blank control group (10.83 ± 0.17) (Fig. 4A,4B). As the Transwell invasion assays showed that the invasion cell numbers of MDA-MB-231 cells in anti-miR-29a group was 4.67 ± 0.88 , less than 47.67 ± 5.44 in control vector group and 50.33 ± 2.91 in blank control group (Fig. 4C,4D). These data suggested that miR-29a knockdown of breast cancer cells in BDM condition reduced cells invasion and migration.

3.4. Anti-miR-29a suppress breast cancer tumorigenesis and metastasis in T2DM mice

The diabetic mice were induced by STZ. Subsequently, MDA-MB-231 cells with anti-miR-29a and control vector-transfected cells were implanted into the fourth mammary fat pads of these diabetic mouse (n = 6). These mouse were followed for 8 weeks post transplantation, and the tumor volume and tumor weight were calculated. Interestingly, the group of anti-miR-29a had an obviously reduced tumor growth compared with control vector group (Fig. 5A,5B,5C). Metastatic activity is detected by examining organs at necropsy. Histological analysis of lungs isolated at necropsy suggested only one visible lung metastatic nodules

were observed in the anti-miR-29a group, whereas visible lung metastatic nodules were observed in all of the control vector group mice, and 83.3% of them were multiple metastases (5/6) (Fig. 5D,5F). That is to say, BDM animals with miR-29a knockdown had less metastasis in the lung compared with control vector-transfected cells. Together, these results indicated that miR-29a knockdown suppresses MDA-MB-231 cells tumorigenesis and metastasis in BDM animals.

3.5. SIRT1 is a direct target of miR-29a-3p in BDM

Based on the analysis with the miRNA online database including TargetScan Human7.2 and miRBase, we found SIRT1 to be a predicted target of miR-29a-3p (Fig. 6A). To test the direct interaction between miR-29a-3p and the 3' UTR of SIRT1, we performed dual-luciferase reporter assay using HEK-293T cells. Not unexpectedly, a significant reduction in luciferase activity was observed in the miR-29a-overexpressing cells with WT reporter, while no difference was noted with the MUT reporter (Fig. 6B).

To illuminate the significance of SIRT1 expression in BDM conditions, western blot method was carried out in BC cells and immunohistochemistry (IHC) was performed in tumor tissues. Interestingly, SIRT1 protein expression in MDA-MB-231 cells decreased by 33% after 72 hours of BDM culture compared with that in normal conditions (Fig. 6C). The IHC results showed that the positive rate of SIRT1 in BDM tissues was significantly lower than that in NDBM tissues ($P < 0.05$, Fig. 6D).

To investigate the effects of miR-29a on SIRT1 expression in MDA-MB-231 cells cultured in BDM conditions, we performed the rescued experiments that miR-29a-overexpressing MDA-MB-231 cells were transfected with pcDNA3.1-SIRT1 plasmid. The results showed that the protein expressions of SIRT1 were sharply downregulated in the miR-29a-overexpressing group compared with the control vector group, while the protein of SIRT1 was remarkably restored after transfection with pcDNA3.1-SIRT1 plasmid (Fig. 6E). Furthermore, the proliferation, migration and invasion ability of MDA-MB-231 cells were obviously increased in the miR-29a + SIRT1 group compared with the miR-29a group (Fig. 6F,6G,6H). After that, we also found the positive expressions of SIRT1 in BDM tissues were associated with miR-29a levels (Fig. 6I). Taken these data together, we demonstrated that SIRT1 might be a direct target gene of miR-29a, miR-29a mediated SIRT1 in human diabetic breast cancer.

4. Discussion

Women with type 2 diabetes has been identified as an independent risk factor for breast cancer (BC) [14]. *Wei, et al* have found that the MCF-7 cells under high glucose and high insulin conditions were promoted the proliferation and invasion through activating the Ras/Raf/ERK pathway and upregulating IRS1 [15]. Recent studies revealed breast cancer patients with diabetes mellitus (BDM) have a greater risk of death and tend to present at later stages and receive altered treatment regimens [16, 17]. In addition, compared with their nondiabetic counterparts, BDM patients had a 51% shorter OS time and 28% shorter DFS [3]. Therefore, it is an important clinical problem to find a more reasonable scheme and accurate target to treat BDM patients. High level of MiR-29a expression was found in serum with type 2 diabetic patients and 3T3-L1 adipocytes cultured with high glucose and high insulin [9]. MiR-29a is up-regulated and

tristetraprolin is down-regulated in metastatic breast cancer patients, including invasive ductal carcinomas tissues and invasive cell lines [10, 18]. MiR-29a play a tumor activator induces breast cancer cell proliferation and EMT via targeting TET1[19]. MiR-29a also promotes epithelial mesenchymal transformation, migration, and invasion of breast cancer cells by down-regulating histone H4K20 trimethylation [20]. Our previous study supported miR-29a was over-expressed in MCF-7 and T47D cells cultured in high insulin medium, and promoted cells growth and invasion involved in the insulin signaling pathway [11]. This study showed miR-29a is upregulated in tumor tissues of BDM patients and BC cell lines cultured in BDM conditions. Kaplan-Meier analysis also confirmed miR-29a was associated with poor prognosis with BDM patients. In a word, our results and previously studies in favor of the concept that mir-29a promotes breast cancer growth and progression under BDM conditions, and also a promising target for the treatment of BDM patients.

Sirtuin-1 (SIRT1) is often described as “regulator of regulators” and reported to play an important role in glucose metabolism, DNA repair, genome maintenance and cancer development[5, 6]. SIRT1 is the selective modulators in breast carcinogenesis, which may act as a tumor-suppressor or promotor [6]. Our data indicated SIRT1 was downregulated in breast cancer with diabetes mellitus. According to the analysis of biological information and the literature reports, it is believed that SIRT1 is the target of miR-29a [12, 13]. It is up-regulated in MDA-MB-231 cells and promotes tumor invasion [21]. we demonstrated that miR-29a promoted cells proliferation and invasion in BDM by targeted SIRT1. This may be a novel regulatory mechanism of breast cancer with diabetes mellitus.

As mentioned above, miR-29a plays the role of oncogene in breast cancer with diabetes mellitus. Therefore, knockdown of miR-29a have a promising therapeutic strategy for this sub-type of breast cancer. Several evidences have suggested miR-29a knockdown promote apoptosis of MCF-7 cells cultured in normal RPMI-1640 medium [22] or in Dulbecco's modified Eagle's medium [23], which indicated that miR-29a may be play a promising strategy for interferes with the tumor cell apoptosis. Consistent with the present results, miR-29a knockdown inhibits breast cancer cells growth and proliferation, reduced cells invasion and migration in BDM condition.

According to the methods reported in the previous literature [24, 25], female mice were induced type 2 diabetes and then the MDA-MB-231 anti-miR-29a cells or MDA-MB-231 control vector cells were injected in the mammary fat pad of these diabetic mice. As expected, anti-miR-29a-3p inhibited tumor growth, total tumor volume and weight of these BDM xenograft. Interestingly, this treatment also reduced visible lung metastatic nodules of BDM xenograft. Many nano-liposomes encapsulating the RNAi strategy have entered clinical trials in many cancers [26], showing that targeting miR-29a with anti-miRNAs as a novel therapy for the treatment of breast cancer with type 2 diabetes is worth further exploration.

5. Conclusion

Taken together, high level of miR-29a expression in breast cancer patients with diabetes mellitus and associated with poor prognosis. MiR-29a promoted cells proliferation and invasion in BDM by targeted

SIRT1. Anti-miR-29a inhibits breast cancer cells growth and proliferation, reduced cells invasion and migration in BDM condition, also reduce tumor growth of these BDM xenograft. Therefore, targeting miR-29a with anti-miRNAs as a novel therapy for the treatment of breast cancer with type 2 diabetes is worth further exploration.

Abbreviations

BDM Breast cancer with type 2 diabetes mellitus

NBDM Breast cancer without type 2 diabetes mellitus

BC Breast cancer

T2DM type 2 diabetes mellitus

RFS Recurrence-free Survival

DSS Disease-specific Survival

OS Overall Survival

DFS Disease Free Survival

DMEM Dulbecco's modified Eagle's medium

STZ streptozotocin

HFD high-fat diet

EMT Epithelial-Mesenchymal Transition

Declarations

Ethics approval

The institutional ethics committee of Third hospital of Nanchang ,Nanchang, China approved the study.

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding authors on reasonable request.

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Authors' contributions

Z.H.L And K.D.H contributed to conception and design of the study. *L.L.M, L.C, Y.Y.H, W.J.L and C.L* performed the experiments and analyzed the data. Z.H.L drafted the manuscript. X.Q.Y and K.D.H provided the useful suggestions and help in editing the manuscript. All the authors read, and approved the submitted version.

Conflicts of Interest

There is no conflict of interest.

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Figures

Figure 1

The schematic diagram of animal model construction process. (A) Fed ad libitum a high-fat diet (HFD) containing 60 kcal% fat. (B) The model of T2DM mice was established intraperitoneally injected streptozotocin (STZ). (C) MDA-MB-231 cells with MiRZip-29a plasmid or Scr control plasmid were implanted into the mammary fat pads, Xenograft mouse model for breast cancer with T2DM was established Successfully.

Figure 2

MiR-29a is upregulated in BDM and correlated with poor prognosis. (A) Relative expression of miR-29a in BDM tissues compared with in NBDM tissues ($P < 0.001$). (B) Relative expression of miR-29a in BC cell lines cultured in BDM conditions compared with that cultured in normal conditions. (C) Kaplan-Meier analysis of recurrence-free survival in BDM patients with high and low miR-29a expression. (D) Kaplan-Meier analysis of disease-specific survival in BDM patients with high and low miR-29a expression.

* $P < 0.001$.

Figure 3

Anti-miR-29a inhibits cell proliferation in BDM condition. (A) miR-29a downregulation as a consequence of miRZip enrichment was assessed by qRT-PCR. (B) CCK8 assay was used to detect the ability of cell proliferation in anti-miR-29a group and empty vector group or blank control group. (C-D) cell cycle was estimated by flow cytometry assay in anti-miR-29a group and empty vector group or blank control group. ** $P < 0.01$ versus empty vector group or blank control group.

Figure 4

Anti-miR-29a inhibit cells migration and invasion in BDM condition. (A-B) Migration ability was measured by wound healing assay and relative wound recovery was presented as % recovery of the wound distance at 48 hours relative to 0 h (magnification, $\times 100$). (C-D) Cellular invasion ability was assessed using transwell invasion assays (magnification, $\times 400$). ** $P < 0.01$.

Figure 5

Anti-miR-29a suppress breast cancer tumourigenesis and metastasis in T2DM mice. (A) Xenograft tumors were obtained from MDA-MB-231 cells with anti-miR-29a and control vector-transfected cells in situ. (B-C) Tumor weight and volume were observed and recorded in the groups indicated above. (D) Histological analysis of lungs isolated at necropsy. (E) The percentage of mice with visible lung metastatic nodules was determined. (F) The average number of visible lung metastatic nodules / each mice was determined. ** $P < 0.01$.

Figure 6

MiR-29a promoted cells proliferation and invasion in BDM by targeted SIRT1. (A) Bioinformatics analyses indicate that SIRT1 is a target gene of miR-29a-3p. (B) Luciferase reporter assays verified the direct interaction of miR-29a-3p and the 3'UTR of SIRT1. (C) The protein levels of SIRT1 in MDA-MB-231 cells were compared in BDM condition and normal condition. (D) The expression of SIRT1 in BDM tissues and NBDM tissues were detected by IHC. (E) The protein expression of SIRT1 in miR-29a-overexpressing MDA-

MB-231 cells were examined by Western blotting ,and then detected in these cells transfected with pcDNA3.1-SIRT1 or blank pcDNA3.1 vector. (F-H) CCK8 assay, wound healing assay and transwell invasion assays used to measure the ability of cell proliferation in miR-29a-overexpressing +SIRT1 group and with the miR-29a-overexpressing group.(I) The relationship between miR-29a expression levels and the expression of SIRT1 in BDM tissues.The bars represent the mean $\pm$ SD of three independent experiments. *P < 0.05.