Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org

Original Research

Construction of SIRT1 gene shRNA lentivirus vector and its effect on the proliferation of breast cancer cells

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Received December 10, 2019; **Accepted** May 6, 2020; **Published** June 5, 2020

Doi: http://dx.doi.org/10.14715/cmb/2020.66.3.33

Abstract: Current experiment aimed to investigate the construction of the SIRT1 gene shRNA lentivirus vector and its effect on proliferation of breast cancer cells. Altogether 80 cases of breast cancer tissues and 80 cases of normal adjacent tissues were collected. qPCR was used for detecting SIRT1 expression. Western blot was used to detect the expression of EMT marker protein. The effect of lentivirus infected sh-SIRT1 on the cell biological function of SK-BR-3 and MDA-MB-231 cells was detected. MTT assay was used to detect cell activity, Transwell cell was used to detect cell invasion and migration, and cell apoptosis detected by flow cytometry. Compared with normal tissues adjacent to cancer, the expression of SIRT1 in cancer tissues increased significantly. Compared with human breast epithelial cells (MCF 10A), SIRT1 expression in breast cancer cells (MDA-MB-231, SK-BR-3) increased significantly. The above results showed that SIRT1 was significant greatly expressed in breast cancer. Compared with the sh-Control group, the cell activity, invasion and migration of the sh-SIRT1 group were enhanced, while cell apoptosis was weakened. In the sh-SIRT1 group infected by lentivirus, cell activity, cell invasion and migration decreased, while cell apoptosis increased. Compared with sh-Control, the expression of α-catenin, PTEN and E-cadherin in the sh-SIRT1 group in SK-BR-3 and MDA-MB-231 cells was down-regulated, while the expression of N- cadherin, β-catenin and Vimentin was up-regulated. Compared with sh-Control, the expression of α-catenin, PTEN and E-cadherin in the sh-SIRT1 group infected by lentivirus was up-regulated, while the expression of N- cadherin, β-catenin and Vimentin was down-regulated. To sum up, SIRT1 is highly expressed in breast cancer cells. The proliferation of breast cancer cells was inhibited after lentivirus infection with sh-SIRT1.

Key words: shRNA lentivirus vector; SIRT1; Breast cancer; Proliferation.

Introduction

Breast cancer has been known as the most common female malignant tumor, and even life-threatening when severe, has a major impact on women's physical and mental health (1-3). Based on statistics, the incidence rate of breast cancer in the world is showing an increasing trend (4). Since most surgical patients are already in the advanced stage of breast cancer at the time of treatment, breast cancer is likely to metastasize even if the cancerous tissue is removed (5, 6). In recent years, researchers have paid more and more attention to the role of molecular targeted therapy in breast cancer. Gene target therapy has progressively become a hot spot in breast cancer studies (7, 8).

Silent information regulator 1 (SIRT1), the first member of the Sirtuin family, participates in the occurrence of various diseases such as tumors and affects the development and drug resistance of diseases (9-11). In recent years, researchers have paid attention to the role of SIRT1 in the tumor. Studies on the relationship between SIRT1 and cervical cancer showed that the cell migration ability of cervical cancer cells transfected with siRNA-SIRT1 is inhibited after interfering with the expression of SIRT1 in cervical cancer cells (12). So et al. found that cisplatin combined with SiRNA-SIRT 1 can reduce the expression level of drug-resistant genes in cervical cancer cells, thus enhancing the sensitivity of cervical cancer cells to cisplatin. Therefore, SIRT1 may be involved in the occurrence of drug resistance in cervical cancer (13). Most studies believed that SIRT1 is narrowly related to the happening and development of various tumors, and it can promote the occurrence and development of breast cancer, but its exact role in breast cancer has not been clearly defined (14, 15).

Although many studies have described the molecular mechanism of SIRT1 in several cancers, the construction of the SIRT1 gene shRNA lentiviral vector and its specific effect on breast cancer cell proliferation is still unclear. Therefore, this study will explore the role of SIRT1 in breast cancer by constructing shRNA lentiviral vector of the SIRT1 gene and observing the expression of SIRT1 in breast cancer cells.

Materials and Methods

Sample collection

Cancer tissues and adjacent normal tissues were collected from 80 patients with breast cancer diagnosed in our hospital (16). Inclusion criteria: patients diagnosed

with breast cancer. Exclusion criteria were followed: patients with mentally ill; patients complicated with other tumors; patients with previous treatment history such as surgery, chemotherapy, radiotherapy or antibiotic treatment; patients did not cooperate with the treatment. Tissue samples and sections were placed in -80°C liquid nitrogen for testing. Venous blood samples from breast cancer patients and 80 healthy people were supplied on an empty stomach and placed in EP tubes without anticoagulants. The samples were then centrifuged at room temperature for 15 min at 3×10^3 rpm. After centrifugating, the supernatant was taken and placed in an EP tube without an RNA enzyme to continue centrifugation for 5 min at 1.2×10^4 rpm. The supernatant was obtained after centrifugation and placed in liquid nitrogen at -80°C for later use. The patients were fully informed of the study and the study was approved by the Hospital Ethics Committee.

Cell culture and transfection

Breast cancer cell lines (MDA-MB-231 (BNCC337893), SK-BR-3 (BNCC100524)) and human normal breast cell MCF 10A (BNCC100439) were purchased from Bena Culture Collection, and the cell cultured in were incubated in an animal cell at 37°C with 5%CO_2. The culture medium system of breast cancer cell line was 1640 medium (Hyclone company) +10% fetal bovine serum solution (Gibco company) +1% penicillin/streptomycin solution (100X, Solarbio company), and the culture system of MCF 10A cell line is DMEM medium (Hyclone company) +10% fetal bovine serum solution (Gibco company) +1% penicillin/streptomycin solution (100X, Solarbio company). Subsequent experiments would be carried out after cell culture to 80-90% of the coverage rate. The day before transfection, the medium was replaced with fetal bovine serum-free medium, and the cells with 1×10^5 cells/well were inoculated into 6-well plates during transfection. Cell lines were transfected with Lipofectamine 2000 transfection kit (Invitrogen, USA). The procedures referred to the kit instructions. After transfection for 8 h, the fresh culture medium was replaced at 37°C with 5% CO_2.

Packaging of lentivirus empty vector and determination of virus titer

We looked up the SIRT1 gene sequence (NM_001033578.2) from Gen Bank, designed a specific sh RNA sequence for the human SIRT1 gene. Negative control (NC) was used as a control. The shRNA lentivirus expression vector of the SIRT1 gene was reconstructed by Beijing Berry Genomics and the recombinant clone was screened by colony PCR for alignment identification. The empty vector p Lentilox 3.7 containing green fluorescent protein (GFP) and lentivirus packaging plasmid were co-transfected into MDA-MB-231 and SK-BR-3 cells. After transfection for 1day, the culture medium was changed, and after culture for 2 days, MDA-MB-231 and SK-BR-3 cells infected with lentivirus concentrated solution were collected, and GFP expression was observed under a fluorescence microscope to evaluate virus infection efficiency. SK-BR-3 and MDA-MB-231 cells infected with SIRT1 shRNA lentivirus were subcultured in DMEM medium containing 10% FBS, 100U/ml penicillin and 100U/ml streptomycin by conventional fluid exchange. Cells were transplanted into 6-well plates with 2×10^5 cells per well. On the second day, when the cell growth density reached about 50%, the cells were infected with virus solution of the corresponding titer in cell culture solution (10% FBS (fetal bovine serum), 4ug/ml polyamine).

Quantitative polymerase chain reaction (qPCR)

The total RNA of tissue samples or cells was purified using Trizol. The total RNA of exosomes was purified by Total Exosome RNA & Protein Isolation Kit (Item No. 4478545, Invitrogen). The concentration and purity of total RNA were detected by ultraviolet spectrophotometer at 260-280nm, and OD260/OD280> 1.8 was selected for qPCR detection. Fast King one-step reverses transcription-fluorescence quantitative kit (Beijing Tiangen Company, Catalog No. FP314) and ABI PRISM 7000 (Applied Biosystems, USA) instruments were used for reverse transcription, PCR amplification and fluorescence quantitative on total RNA. SIRT1 and mRNA primers were designed and synthesized by Shanghai Sangon Biotech. The reaction system was carried out in strict accordance with the kit instructions (50 L): 1.25 L of upstream and downstream primers, 1.0 L of the probe, 10pg/g of the RNA template, 5 μL of 50×ROX Reference Dye ROX, and RNase-Free ddH_2O was added to the total reaction system of 50 uL. Reaction process: reverse transcription at 50°C for 30 min with 1 circle; pre-denaturation at 95°C for 3 min with 1 circle; denaturation at 95°C for 15 s, annealing at 60°C for the 30s with 40 cycles. The results were analyzed by the ABI PRISM 7000 instrument. The internal reference gene was GAPDH. The primer sequences are shown in Table 1.

Western blot

A 1 ml of cell protein extract was added to the culture plate of the cultured cell line (cell lysate: protease inhibitors: phosphatase inhibitors = 98:1:1, v/v/v). The solution was blown several times and centrifuged at 1.2×10^4 r/min for 15 min. The supernatant was taken; SDS-PAGE electrophoresis was carried out for protein separation. The protein was transferred to NC membrane and placed at room temperature for 1 h (sealed with 5% skim milk -PBS solution). Caspase 3, α-catenin, PTEN, E-cadherin, N- cadherin, β-catenin, Vimentin and β-actin primary antibodies were then added and placed at 4°C overnight. The NC membrane was washed with PBS solution for three times, then goat anti-rabbit secondary antibody (HRP cross-linked) was added, and the mixture was allowed to place for

Table 1. Primer sequence for amplification of SIRT1 and GAPDH.

	Upstream sequence	Downstream sequence
SIRT1	5′-CCATGGCGCTGAGGTATATT-3′	5′-TCATCCTCCATGGGTTCTTC-3′
GAPDH	5′-AGAAGGCTGGGGCTCATTTG-3′	5′-AGGGGCCATCCACAGTCTTC-3′

1h at room temperature. Finally, the NC membrane was washed with PBS solution and visualized by enhanced chemiluminescence method. The internal reference protein was β-actin, and the relative expression level of the protein to be detected = gray value of the band to be detected/gray value of the β-actin band.

Evaluation of cell migration and invasion by Transwell method

Trypsin enzymolysis was used to hydrolyze cells to prepare cell suspension. Cells were inoculated into the migration upper chamber (containing 200L 10% fetal bovine serum +1% DMEM medium) with 2×10^4 cells/well, and DMEM medium (containing 10% fetal bovine serum with a total volume of 500L) was added into the lower chamber. After 24h of cell culture, the upper chamber fluid was removed and the cells on the chamber wall were wiped off. 4% of polymethanol was used to fix Transwell's opposite cells for 20 min. Crystal violet was used for staining for 15 min, PBS buffer solution for washing the Transwell chamber. Images of cell migration were collected under a 200-fold microscope. The cell number was calculated by randomly selecting 3 fields of view, and the average value was taken as the number of transmembrane cells. The experiment was repeated three times. The invasion was paved with 8% matrix glue on the above steps, and the quantity of cells per well was increased to 5×10^4.

MTT assay was used to detect cell activity

Trypsin enzymolysis was used to hydrolyze the transfected cells. The cells were centrifuged to remove the enzyme solution, added with fresh culture medium, and the solution was blown to prepare cell suspension. Four 96-well plates were taken and cells were inoculated into the well plates according to the specification of 5×10^3 cells /100μL per well, with 3 wells in each group. One well plate was taken out every 24 h, 5mg/ml MTT solution was added 10μL/well, the cells were continued to culture for 1h, then the culture medium was removed, and the OD value was measured at 570 nm with an enzyme reader. The experiment was repeated 3 times to visualize the cell activity-time curve.

Flow cytometer

Trypsin enzymolysis was used to hydrolyze the transfected cells. The cells were centrifuged to remove the enzyme solution, added with fresh culture medium, and the solution was blown to prepare cell suspension. Four 96-well plates were taken and cells were inoculated into the well plates according to the specification of 5×10^3 cells /100μL per well, with 3 wells in each group. One well plate was taken out every 24 h, 5 mg/ml MTT solution was added 10μL/well, the cells were continued to culture for 1 h, then the culture medium was removed, and the OD value was measured at 570 nm with an enzyme reader. The experiment was repeated 3 times to visualize the cell activity-time curve.

Statistical analysis

The above index data were input into SPSS20.0 software package (Asia Analytics Formerly SPSS China), and GraphPad Prism 6.0 was used for statistical analysis. Each experiment was repeated 3 times. Measurement data were expressed by Mean ± SD, and counting data were expressed by n. An independent sample t-test was used for the data comparison between the two groups. One-way ANOVA was used for the comparison among multiple groups, LSD t-test for pairwise comparison afterward. The relationship between SIRT1 and clinical characteristics was analyzed by double-sample Student's t-test. All data were tested with a two-tailed test. The value of 95% was taken as a confidence interval, the difference was statistically significant when $P< 0.05$.

Results

SIRT1 was highly expressed in breast cancer

qPCR was used to detect SIRT1 in tissues and serum. In this study, various cases of breast cancer tissues and normal tissues adjacent to cancer were collected. qPCR was used to quantify SIRT1 expression in tissues and cells. Compared with normal tissues adjacent to cancer, SIRT1 expression in cancer tissues was significantly increased. Compared with human breast epithelial cells (MCF 10A), SIRT1 expression in breast cancer cells (MDA-MB-231, SK-BR-3) increased significantly. The above results indicated that SIRT1 is highly expressed in breast cancer (Figure 1).

SIRT1 was related to breast cancer metastasis.

In this study, the expression of SIRT1 in different TNM stages was compared and the correlation between SIRT1 and clinical features was analyzed. SIRT1 is highly expressed in T3/T4, N1 and M1. The results are shown in Figure 2 and Table 2.

Lentivirus infected sh-SIRT1 could inhibit the proliferation of SK-BR-3 and MDA-MB-231 cells

In this study, SIRT1 of SK-BR-3 and MDA-MB-231

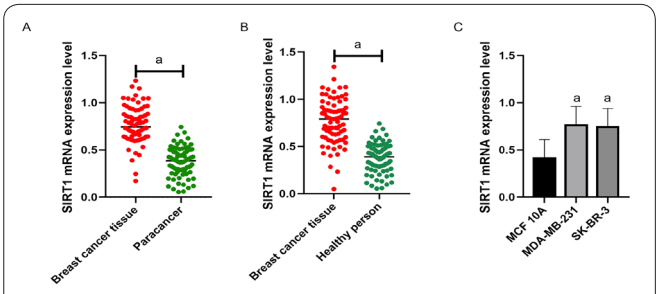

Figure 1. SIRT1 was highly expressed in breast cancer. **A:** SIRT1 was highly expressed in breast cancer tissue, a indicates that $P< 0.001$; **B:** SIRT1 was highly expressed in breast cancer patients, a indicates that $P< 0.001$; C: SIRT1 was highly expressed in breast cancer cells, compared with MCF 10A, a means $P < 0.001$.

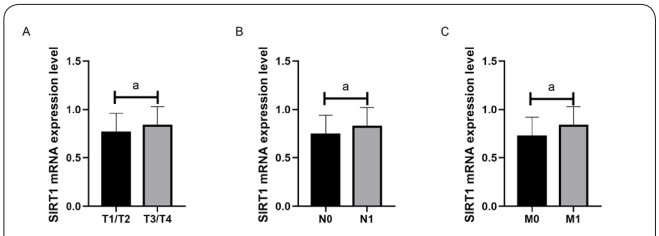

Figure 2. Relationship between SIRT1 and TNM staging. **A:** SIRT1 expression in T1/T2 and T3/T4; **B:** SIRT1 expression in N1 and N0 phases; **C:** SIRT1 expression in M0 and M1 phases.

Table 2. Correlation between SIRT1 and clinical features.

Category	n	SIRT1		χ2	P
		Low	High		
Age				1.270	0.260
≤45	45	25 (62.50)	20 (50.00)		
>45	35	15 (37.50)	20 (50.00)		
Smoking				1.289	0.256
Yes	47	21 (52.50)	26 (65.00)		
No	33	19 (47.50)	14 (35.00)		
Drinking				0.051	0.822
Yes	45	23 (57.50)	22 (55.00)		
No	35	17 (42.50)	18 (45.00)		
T stages				20.830	<0.001
T1/T2	48	34 (85.00)	14 (35.00)		
T3/T4	32	6 (15.00)	26 (65.00)		
N stages				7.231	0.007
N0	45	30 (66.67)	15 (37.50)		
N1	35	15 (33.33)	25 (62.50)		
M stages				21.330	<0.001
M0	50	35 (87.50)	15 (37.50)		
M1	30	5 (12.50)	25 (62.50)		

cells were extracted to observe the effect of SIRT1 on cell biological function. MTT assay was used to detect cell activity, Transwell cell was used to detect cell invasion and migration, and the cell apoptosis was detected by flow cytometry. Compared with a sh-Control group, the cell activity, cell invasion and migration of the sh-SIRT1 group were enhanced, while cell apoptosis was weakened. Cell activity, cell invasion and migration decreased while cell apoptosis increased in the sh-SIRT1 group infected by lentivirus. These results indicated that lentivirus infection with sh-SIRT1 can promote the apoptosis and SIRT1 expression of SK-BR-3 and MDA-MB-231 cells, and it can inhibit cell proliferation (Figures 3 and 4).

Effect of lentivirus infected sh-SIRT1 on EMT of breast cancer cells

Compared with sh-Control, the expression of α-catenin, PTEN and E-cadherin in the sh-SIRT1 group in SK-BR-3 and MDA-MB-231 cells was down-regulated, while the expression of N- cadherin, β-catenin and Vimentin was up-regulated. Compared with sh-Control, the expression of α-catenin, PTEN and E-cadherin in the sh-SIRT1 group infected by lentivirus was up-regulated, while the expression of N- cadherin, β-catenin and Vimentin was down-regulated (Figure 5).

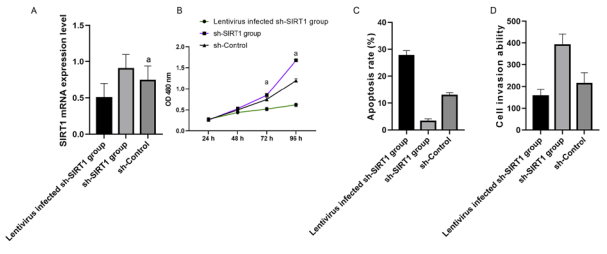

Figure 3. Lentivirus infected sh-SIRT1 could inhibit SK-BR-3 cell proliferation. **A:** Lentivirus infected sh-SIRT1 inhibited SIRT1 expression; **B:** SK-BR-3 cell activity; **C:** apoptosis; **D:** cell migration. Compared with sh-Control, a means P< 0.00.

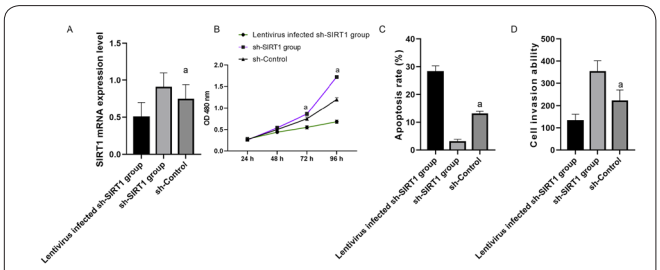

Figure 4. Lentivirus infected sh-SIRT1 could inhibit the proliferation of MDA-MB-231 cells. **A:** Lentivirus infected sh-SIRT1 inhibited SIRT1 expression; **B:** MDA-MB-231 cell activity; **C:** apoptosis; **D:** cell migration. Compared with sh-Control, a means P< 0.001.

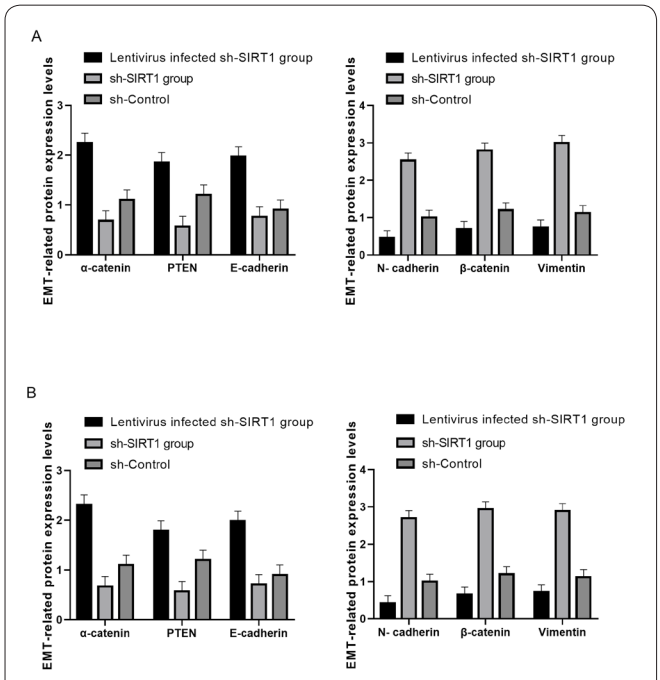

Figure 5. Effect of lentivirus infected sh-SIRT1 on EMT of breast cancer cells. **A:** EMT of SK-BR-3 cells after lentivirus infection. **B:** EMT of MDA-MB-231 cells after lentivirus infection.

Discussion

Lentiviral vectors are currently the most suitable vector tools for in vivo gene transformation and even gene therapy (17, 18). Relevant reports indicated that the lentivirus vector is a gene therapy vector developed based on immunodeficiency virus, and it is the suicide virus (19). Lentiviral vectors can produce high titer viruses expressing shRNA, induce stable functional silencing of gene expression, and become cell gene silencing. Lentiviral vector-mediated RNA interference technology is of great significance to the continuous development of gene therapy (20, 21).

In this study, we first found that SIRT1 is highly expressed in breast cancer, with the highest SIRT1 levels in SK-BR-3 and MDA-MB-231. Therefore, SIRT1 was isolated from SK-BR-3 and MDA-MB-231 cells and cultured with MCF 10A cells. The results showed that over-expression of SIRT1 could promote the proliferation, migration and invasion of SK-BR-3 and MDA-MB-231 cells, and inhibit cell apoptosis. SIRT1, as a histone deacetylase family, participates in acetylation modification of various proteins in various cells (22). Some studies have shown that SIRT1 can inhibit cell growth, proliferation and transformation by regulating glutamine metabolism, thus playing its role in promoting cancer. The analysis showed that SIRT1 is abnormally expressed in various malignant tumors and is closely related to tumor development and cell apoptosis (23). Some studies have shown that SIRT1 changes cell activity and affects its intracellular function by deacetylating the target protein in the nucleus (24), invasion and metastasis of malignant tumors are closely related to the up-regulation of SIRT1 expression, and down-regulation of SIRT1 inhibits the growth of liver cancer and ovarian cancer cells (25). In other studies, the inhibitory effect of shRNA lentivirus vector on CCR3 gene expression could effectively reduce the migration, infiltration and degranulation of mast cells in local tissues, and reduce inflammation in allergic rhinitis mice (26). The results in this study further supplemented the biological function of SIRT1 on breast cancer cells by establishing a shRNA lentivirus vector.

Next, we observed the effect of lentivirus infected sh-SIRT1 on EMT process of breast cancer cells by interfering SIRT1 expression in SK-BR-3 and MDA-MB-231 cells. The results showed that the expression of EMT-related proteins α-catenin, PTEN and E-cadherin in breast cancer cells was down-regulated, while the expression of Bcl-2, N-cadherin and β-catenin was up-regulated. Cancer studies have reported that SIRT1 mRNA and protein levels are reduced, thereby inhibiting EMT and affecting EMT-related molecules, including PTEN and E-cadherin (27, 28). Qi et al. confirmed that SIRT1 is the direct target of miR-448 through double luciferase reporter gene detection. Activation of SIRT1 would reverse the EMT growth inhibition ability of non-small cell lung cancer cells induced by miR-448 mimetics and play a destructive role (29). The above results showed that after lentivirus infected sh-SIRT1 interferes with SIRT1 and down-regulates expression, the expression level of adhesion molecules which aggregate among cells such as α-catenin, PTEN, and E-cadherin was up-regulated, and finally the proliferation and EMT of breast cancer cells were inhibited.

The results of this study showed that lentivirus infected sh-SIRT1 interferes with SIRT1 to promote proliferation, migration, invasion and inhibit apoptosis of breast cancer cells. However, in this study, the relationship between SIRT1 lentivirus vector and breast cancer cell proliferation is only preliminarily studied. In the future experimental design, the signal pathways involved in SIRT1 can be studied to supplement the SIRT1 carcinogenic network. In addition, the clinical value of SIRT1 in breast cancer can also be discussed (30-35).

To sum up, SIRT1 was highly expressed in breast cancer cells by studying the expression mechanism of SIRT1 in breast cancer in this study. The proliferation of breast cancer cells was inhibited after lentivirus infection with sh-SIRT1. Therefore, the SIRT1 gene shRNA lentivirus vector has possible application value in targeted therapy of breast cancer. Finally, cancer is influenced by a number of environmental and genetic factors, and each one needs to be properly researched and studied (36-45).

Acknowledgements
Not applicable.

Funding
Natural Science Foundation of Hubei Province (2019CFC854).

Availability of data and materials
The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions
YY, LH and XL conceived and designed the study, and drafted the manuscript. YY, LH, MT and XL collected, analyzed and interpreted the experimental data. LH and XL revised the manuscript for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate
The study was approved by the Ethics Committee of The First Affiliated Hospital of Baotou Medical College. Signed written informed consent was obtained from the patients and/or guardians.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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