

SIRT1 suppresses breast cancer growth through downregulation of the Bcl-2 protein

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Abstract. Silent mating-type information regulation 2 homologue 1 (SIRT1), a member of the class III histone deacetylase (HDAC) family, is the mammalian ortholog of yeast Sir2. It has been reported to play a key role in a variety of physiological processes such as genomic stability, metabolism, neurogenesis and cell survival. The deacetylase function of SIRT1 has been suggested to play a role in prolonging the life of mammals. However, the suggested functions of SIRT1 as a potential tumor promoter have been challenged by observations of their respective downregulation and upregulation in various types of cancer. Breast cancer patients were included in the present study between 2007 and 2008. Their tumor tissues and paired normal breast tissues were collected and used for evaluation of the expression levels of SIRT1 and Ki67. The effects of SIRT1 on human breast cancer cell lines were also investigated. Immunohistochemistry showed that there is a high correlation between SIRT1 and Ki67 expression. Following treatment with sirtinol (inhibitor of SIRT1), the expression of the pro-survival protein Bcl-2 was markedly decreased in both MCF-7 and MDA-MB-231 cell lines, particularly in MDA-MB-231. Results of the present study revealed that inhibition of SIRT1 activity may be a promising chemotherapeutic strategy against breast cancer.

Introduction

Silent mating-type information regulation 2 homologue 1 (SIRT1) is a protomember of the sirtuin family (SIRT1-7) that is involved in a variety of physiological processes such as genomic stability, metabolism, neurogenesis and cell survival due to its ability to deacetylate both histone and numerous

non-histone substrates such as gene silencing, metabolism, neuroprotection, and genomic stability (1,2). SIRT1 acts as a deacetylase not only on histone but also by deacetylating cell cycle regulators such as p53 (3), p73 (4) and Rb (5). With regard to the growth of cancer cells, it has been reported that SIRT1 regulates cell proliferation, survival, and death, and plays a pivotal role in tumorigenesis and longevity.

SIRT1 prompts cell proliferation and plays an important role in the drug resistance of tumor cells, especially during chemotherapy. In humans, several types of cancer, including prostate (6,7), breast cancer (8,9), colon (10,11), glioblastoma (12), lymphoma (13) and acute myeloid leukemia (14) have been demonstrated to have a significantly increased expression of SIRT1. Furthermore, a recent study showed that SIRT1 regulates the DNA damage signaling pathway through deacetylation of NBS1 to promote cellular survival (15). It was reported that SIRT1 upregulates the expression of multidrug resistance 1 (MDR1) in cancer, and this may cause cancer resistance to chemotherapy (16). SIRT1-deficient cells exhibited p53 hyperacetylation following DNA damage and increased ionizing radiation-induced thymocyte apoptosis *in vivo* along with downregulation of MDR1 (16-19). This evidence indicates the oncogenic role of SIRT1.

By contrast, several studies have shown the tumor suppressor role of SIRT1. The reduced transformation capability of c-myc can be compromised in the presence of SIRT1 (20). Ectopic induction of SIRT1 in a β -catenin-driven mouse model of colon cancer significantly reduced colon cancer formation, growth, and animal morbidity (21). Moreover, the mouse embryonic fibroblasts (MEFs) derived from SIRT1^{-/-} mice are prone to spontaneous immortalization, suggesting that SIRT1 behaves as a growth-suppressive gene (22). Analysis of SIRT1 mutant mice revealed that SIRT1^{-/-} MEFs displayed chromosome aneuploidy and repair defects, and that SIRT1 played an important role in the maintenance of the heterochromatin structure through deacetylation of histones *in vivo* (23). Collectively, these previous findings indicated that SIRT1 may possess both pro- and anti-proliferative functions, depending on the cell types. In spite of the controversial role of SIRT1 in tumorigenesis (24,25), it is evident that SIRT1 is significantly involved in the process of tumorigenesis.

Since SIRT1 is involved in both the death and survival of cells, we proposed that SIRT1 may be a critical determinant

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of cell fate during tumorigenesis. To test this hypothesis, we investigated the expression of SIRT1 in tumor breast tissues and matched normal breast tissues of Taiwanese women, and used breast cancer cell lines to examine the role of SIRT1 in breast tumorigenesis.

Materials and methods

Patients. A total of 27 breast cancer patients were included in this study between 2007 and 2008. Tumor tissues and the paired normal breast tissues were immediately frozen after collection. The cases were diagnosed as infiltrating ductal carcinoma (IDC; n=27) according to the World Health Organization classification system (26). Clinical data were collected retrospectively. All patients provided informed consent and the study was approved by the Institutional Review Board of Changhua Christian Hospital.

Antibodies and reagents. The antibodies used for western blotting were: anti-SIRT1 (ab53517; Abcam, Cambridge, UK), anti- β -actin (A-5441) and anti-Bcl-2 (B-3170) (both from Sigma-Aldrich Co., MI, USA). The primary antibodies used for immunohistochemical analysis were: anti-SIRT1 (ab7343; Abcam) and anti-Ki67 (RM-9106-S1; Lab Vision, Fremont, CA, USA). Chemicals and reagents were purchased from Sigma-Aldrich Co., unless otherwise indicated.

Immunohistochemical analysis. The 5- μ m sections of formalin-fixed, paraffin-embedded tissues were deparaffinized, rehydrated and the antigen retrieved. The tissue sections were incubated with hydrogen peroxide for 5 min at room temperature to quench the endogenous peroxidase. After blocking in normal goat serum, the tissue sections were incubated for 1 h at room temperature with the primary antibodies anti-SIRT1 (1:100 dilution) and anti-Ki67 (1:50 dilution), respectively. The slides were then washed with 0.5% Triton X-100 in PBS and incubated with HRP-conjugated secondary antibody for 1 h (Vector Laboratories, Burlingame CA, USA). Nuclei were counterstained with hematoxylin. As a negative control, normal goat serum was substituted for the primary antibody. All slides were examined by two independent investigators who were blind to the clinical data. To evaluate the expression of SIRT1 and Ki67 in tumor breast tissue and matched normal breast tissue, the staining intensity was scored from 0 to 3. The ratio of the positively stained area to the total section area was assessed.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from breast tissue using TRIzol Reagent and cDNA was synthesized by MMLV Reverse transcriptase (both from Invitrogen, Carlsbad, CA, USA). The primer sequences used for PCR are shown in Table I. Synthesized cDNA was amplified and the PCR product was then visualized on 1% agarose gel. The intensity of the PCR product was determined by densitometry using the Gel Image Analysis System (Alpha Innotech Co., San Leandro, CA, USA).

Cell culture. The human breast cancer cell lines and MCF-7 and MDA-MB-231 cell lines were grown in Dulbecco's modified Eagle's medium: Nutrient Mixture F-12 (DMEM/F12)

Table I. Oligonucleotide sequences for RT-PCR.

Primer	Primer sequence
<i>SIRT1</i>	F: 5'-CATCTCTCTGTCACAAATTCATAGCC-3' R: 5'-GTAATTTCTGAAAGCTTTACAGGGTT-3'
<i>GAPDH</i>	F: 5'-GAGTCAACGGATTTGGTCGT-3' R: 5'-GGTGCTAAGCAGTTGGTGGT-3'

SIRT1, silent mating-type information regulation 2 homologue 1; F, forward; R, reverse.

supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin (Gibco, Invitrogen Ltd., Carlsbad, CA, USA) in a humidified atmosphere containing 5% CO₂ at 37°C.

Cell proliferation assay. The cytotoxicity effect of sirtinol on MCF-7 and MDA-MB-231 cells was determined by trypan blue dye exclusion and Alamar-blue assays (AbD Serotec, Kidlington, UK). Briefly, the cells were plated at a density of 2x10⁵ cells in 6-well plates with an increasing dose of sirtinol (10-50 μ M in DMSO) or vehicle alone. Following incubation for 24 or 48 h, cells were collected and an aliquot of cell suspension was mixed with an equal volume of trypan blue, and cells were then counted under the microscope. The cell viability assay was performed following the instructions of the Alamar blue assay kit. In brief, cells were plated at a density of 2x10³ cells in 200 μ l of complete medium in a 96-well plate and treated with increasing doses of sirtinol or vehicle alone. Each treatment was performed in triplicate. The cells were incubated for 24 or 48 h at 37°C in 5% CO₂. Five hours before the end of the culture, the Alamar blue reagent (20 μ l in phenol red free RPMI-1640 medium) was added into each well and incubated up to 24 or 48 h. Absorbance of 590 nm wavelength emissions was recorded.

Flow cytometry analysis. Cell cycle analysis was performed with flow cytometry. Cells were grown at a density of 5x10⁵ cells in 6-well culture plates, treated with different concentrations of sirtinol for 24 and 48 h, and maintained at 37°C in a 5% CO₂ incubator. Following treatment, the cells were gently trypsinized, added to the culture medium, and pelleted by centrifugation. The cell pellet was resuspended in 1 ml PBS, washed at 1,500 x g for 30 min at 4°C, and fixed in ethanol (75% v/v). The cells were then washed with PBS and labeled with propidium iodide/RNase A solution. Labeled cells were analyzed by flow cytometry (Epics XL-MCL Flow Cytometry; Beckman Coulter, Inc., Fullerton, CA, USA) and the analyses were performed using WinMDI 2.9 software.

Western blot analysis. The cells were harvested at 24 and 48 h following sirtinol treatment, as described above, and washed with PBS, respectively. The cells were lysed with ice-cold RIPA lysis buffer (1X TBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.004% sodium azide with protease inhibitor) and harvested. The lysate was centrifuged at 14,000 x g for 30 min at 4°C, and the supernatant

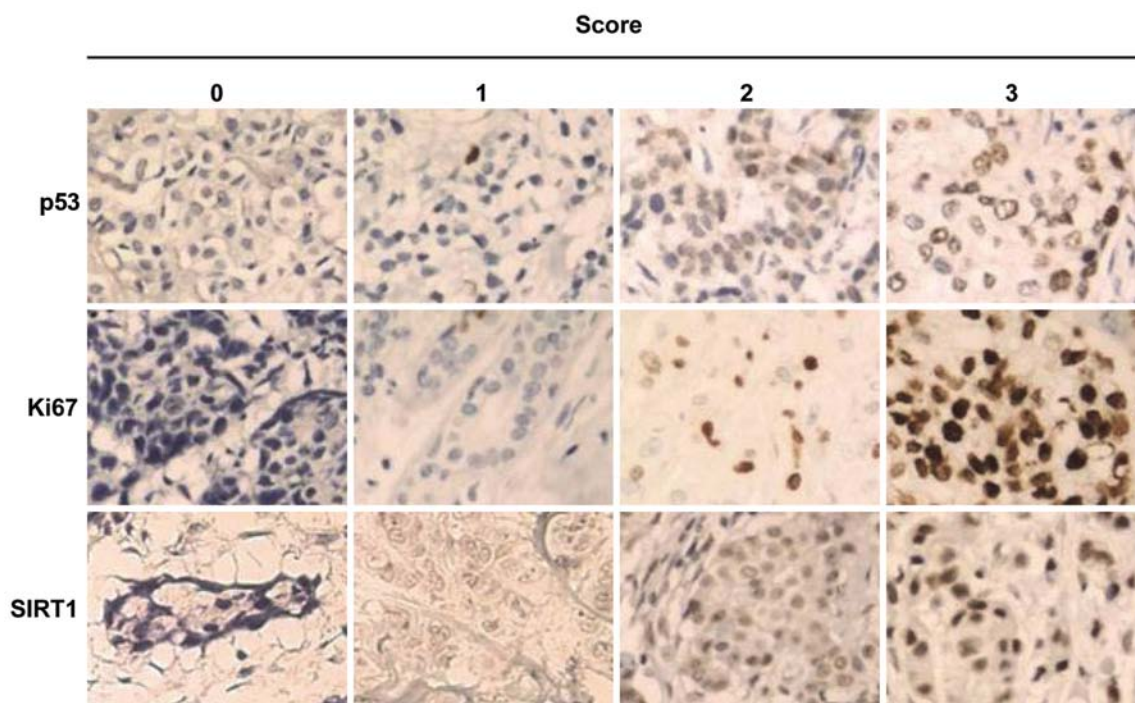


Figure 1. Representative results of immunohistochemical analysis (x200). Top, p53; middle, Ki67; bottom, SIRT1. The staining intensity from negative to high score is 0 to 3 (from left to right panel).

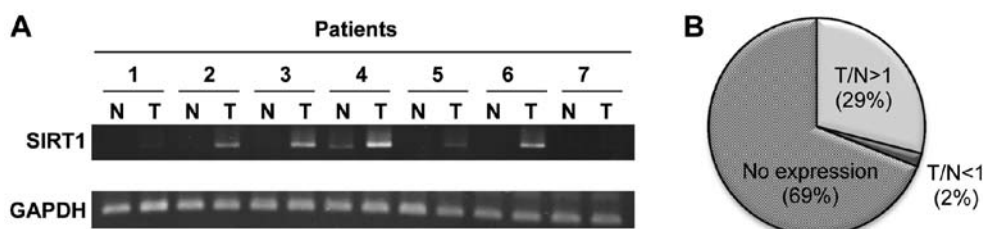


Figure 2. *SIRT1* mRNA expression in breast cancer patients. (A) The *SIRT1* mRNA expression in representative cases. *GAPDH* acted as an internal control. (B) The quantitative results. T, tumor tissue; N, paired normal tissue.

was collected and stored at -20°C until analysis. The protein concentration was determined using the bicinchoninic acid protein assay (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Fifty micrograms of protein were separated by SDS-PAGE and then transferred onto a PVDF membrane ($0.4\ \mu\text{m}$; Millipore, Billerica, MA, USA). Non-specific interactions were blocked by incubating the membrane with 5% non-fat dry milk in 0.5% PBST for 1 h at room temperature. The membrane was then blotted with anti-SIRT1, Bcl-2, or β -actin antibodies at 4°C overnight. HRP-conjugated secondary antibodies were added for 1 h, and the immunoreactive signals were then detected using the SuperSignal West Pico Chemiluminescent Substrate (Pierce). The level of protein expression was analyzed using the Gel Image Analysis System (Alpha Innotech Co.).

Statistical analysis. Statistical analyses were performed using the Student's t-test. A P-value of <0.05 was considered to indicate a statistically significant difference. All data are shown as the means \pm SEM. Statistical calculations were performed using SPSS software (SPSS, Chicago, IL, USA).

Results

Increased gene expression of *SIRT1* in breast cancer. The results of immunohistochemical analysis revealed that SIRT1, Ki67 and p53 were localized in tumor and in normal cells with varying intensities (Fig. 1). The expression of SIRT1 and Ki67 was significantly higher in tumor tissue than in normal tissue ($P<0.001$ and $P<0.005$ for SIRT1 and Ki67, respectively). Twenty-three (82.1%) tumor cases had a higher level of SIRT1 and Ki67 proteins. Furthermore, the RT-PCR results showed that the mRNA level of *SIRT1* was overexpressed in breast tumor tissues (29%), compared to the paired normal tissues (Fig. 2).

Sirtinol induces cell growth inhibition through cell cycle arrest in the G0/G1 phase in breast cancer cells. When cells were treated with sirtinol at higher concentrations ($30\text{--}40\ \mu\text{M}$), cell shrinkage was observed (Fig. 3A). The cell viability was reduced by sirtinol in a time- and dose-dependent manner in both MCF-7 and MDA-MB-23 cells (Fig. 3B). Flow cytometry analysis was used to examine the effects of sirtinol on the cell

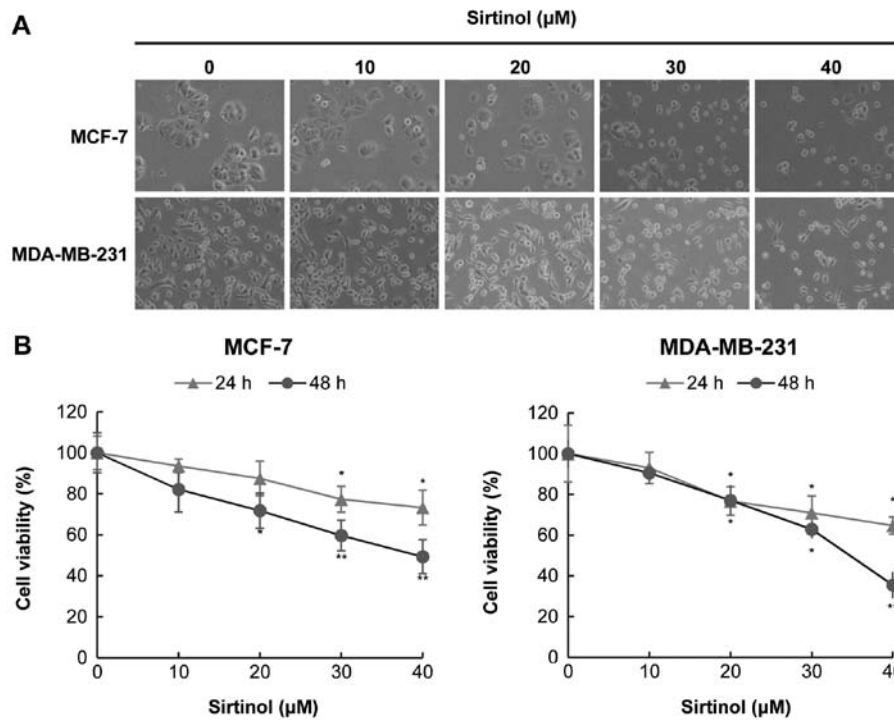


Figure 3. Effects of sirtinol on cell morphology and cellular viability. (A) Cellular morphological changes following sirtinol treatment after 24 h. (B) Cells were treated with the indicated doses of sirtinol for 24 and 48 h, respectively. Prior to each time course, cells were added to a 10% Alamar blue solution in culture medium and incubated for 6 h. Values were obtained from three independent experiments and are expressed as the means \pm SEM. * $P < 0.05$ and ** $P < 0.01$ between control and sirtinol-treated cells.

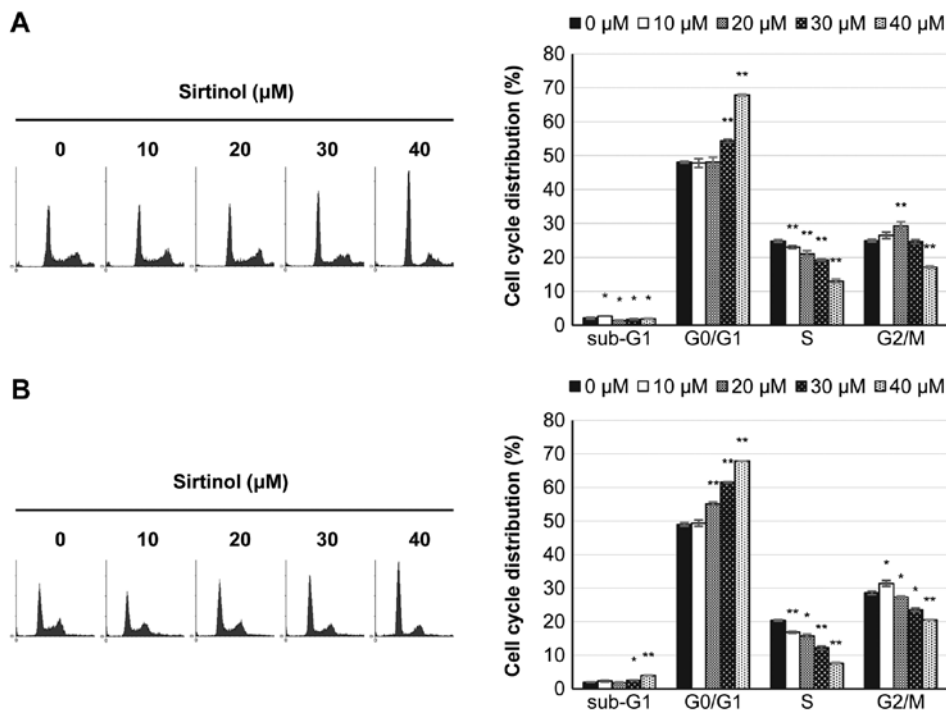


Figure 4. Effect of sirtinol on the cell cycle progression of breast cancer cells. (A) MCF-7 and (B) MDA-MB-231 cells were treated with or without increasing concentrations of sirtinol for 24 h, respectively. Cells were fixed, stained and analyzed for DNA content. Data are presented as the means \pm SD ($n = 3$). * $P < 0.005$ and ** $P < 0.001$ between control and sirtinol-treated cells.

cycle progression of breast cancer cells. As shown in Fig. 4, sirtinol induced a cell cycle arrest in the G0/G1 phase in both cell lines. The proportion of the sub-G1 population also increased slightly in MDA-MB-231 cells, but not in MCF-7 cells.

Sirtinol attenuates the abundance of pro-survival Bcl-2 protein. To characterize the molecular mechanisms underlying these different behaviors, we analyzed the protein expression of Bcl-2 by western blotting. The Bcl-2 protein

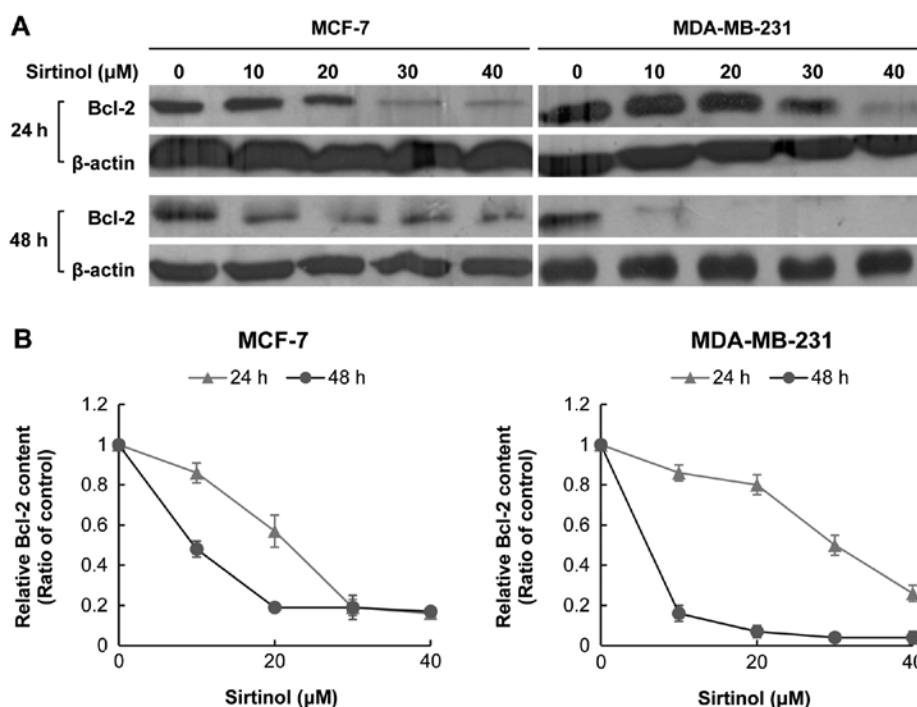


Figure 5. Expression of the Bcl-2 protein in the sirtinol-treated human breast cancer cell lines, (A) MCF-7 and (B) MDA-MB-231. Cells were cultured for the indicated periods with increasing doses of sirtinol (0-40 μM) for 24 and 48 h, respectively. β-actin acted as an internal control.

level in MCF-7 and MDA-MB-231 cells was significantly decreased with sirtinol treatment, particularly with 48-h treatment (Fig. 5).

Discussion

The expression of SIRT1 in breast cancer tissues is controversial. Kuzmichev *et al* (8) and Sung *et al* (27) demonstrated an overexpression of the SIRT1, while Wang *et al* (23) reported a decreased expression of the SIRT1 protein in breast tumor tissues. This study showed that there was an extremely high frequency of overexpressed SIRT1 in breast tumor tissues compared to their paired normal tissues (92.59%, n=27) (Fig. 1). Notably, the immunohistochemistry results indicated that there is a positive and high correlation of Ki67 and SIRT1 expression (Fig. 1A). It was also found that breast cancer tissue with less proliferative potential (Ki67 scores 0 and 1) exerts a lower expression level of SIRT1, suggesting that SIRT1 could be a predictive marker for the prognosis of breast cancer. Therefore, we proposed that an overexpression of SIRT1 may be involved in breast tumorigenesis, particularly in breast tumor promotion.

In general, estrogen receptor (ER)-negative breast cancer is often more aggressive and drug-resistant than ER-positive breast cancer. To examine the roles of SIRT1 in breast cancer cells, two human breast cancer cell lines, MCF-7 (ER-positive) and MDA-MB-231 (ER-negative), were examined. We demonstrated that sirtinol effectively induced proliferation inhibition in both MCF-7 and MDA-MB-231 cells, indicating the promising therapeutic strategy of targeting SIRT1 for breast cancer without having to consider the ER status.

Furthermore, inhibition of SIRT1 causes an accumulation of G0/G1 phase and a decrease in both S and G2/M phases

(Fig. 3), suggesting the role of SIRT1 in cell cycle progression. Furthermore, we explored whether sirtinol inhibits cell proliferation through the apoptosis pathway in MCF-7 and MDA-MB-231 cells. Several studies reported that knockdown of SIRT1 or inhibition of SIRT1 activity, such as sirtinol, splitomycin, and cambinol (28-30), causes an apoptotic death of cancer cells.

Among the proteins involved in the control of the apoptotic pathway, Bcl-2, a pro-survival protein, has been demonstrated to protect cells from apoptosis (31). Bcl-2 inhibits the mitochondrial pathways of apoptosis through local effects at the mitochondrial and endoplasmic reticulum membranes (32). Furthermore, Bcl-2 was reported to be overexpressed in most breast cancer cells (33,34). Therefore, downregulation of Bcl-2 is a potential chemotherapeutic strategy (35). Our data showed that sirtinol promoted apoptosis by decreasing the Bcl-2 expression in both MCF-7 and MDA-MB-231 cells.

The results suggest that inhibition of SIRT1 has therapeutic potential as an antitumor strategy. The present study confirmed the correlation between SIRT1 and the prognosis of breast cancer patients. Markedly, the blockade of SIRT activity causes an anti-breast cancer cell effect through downregulating the Bcl-2 protein. Therefore, the specific targeting of the SIRT1 protein may be a beneficial chemotherapeutic strategy, particularly for ER-negative breast cancer.

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