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Original Research

Knockdown of SIRT1 inhibits proliferation and promotes apoptosis of paclitaxel-resistant human cervical cancer cells

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Abstract: Cervical cancer (CC), a common gynecological cancer, is a primary cause of cancer-related death in women, worldwide. This study investigates the role of Sirtuin 1 (SIRT1) in paclitaxel (PTX)-resistant CC lines. We used qPCR and Western blots to measure SIRT1 mRNA and protein expressions in 10 matched clinical cancer tissues. We compared the expression levels of SIRT1 between sensitive CC cell lines and PTX-resistant cell lines. Subsequently, we used SIRT1 siRNA to knockdown the expression of SIRT1, and then measured cell proliferation, cell apoptosis rate, cell cycle distribution, and expression levels of Bcl-2 and Bax in PTX-sensitive Hela cell line, PTX-resistant Hela and Sila-resistant cell lines. Finally, we detected the location and expression of MRP (multidrug resistance-associated proteins) using immunofluorescence. We found that SIRT1 expression was higher in PTX-sensitive CC tissues than in normal tissues, and significantly higher in PTX-resistant CC tissues than in PTX-sensitive CC tissues. We further demonstrated that knockdown of SIRT1 in PTX-resistant CC cell lines and PTX-sensitive CC cell line inhibited cell proliferation and promoted cell apoptosis. In addition, we observed that blocking SIRT1 expression in PTX-resistant CC cell lines significantly decreased MRP expression. SIRT1 exhibited high expression levels in both PTX-resistant cell lines and patients. Our results suggest that SIRT1 serves as a potential therapeutic target in PTX-resistant CC.

Key words: Cervical cancer; SIRT1; Paclitaxel-resistance; Proliferation; Apoptosis.

Introduction

Cervical cancer (CC), characterized by uncontrolled cell proliferation in the uterine cervix, is a common gynecological cancer prevalent in both developed and developing countries (1, 2). Despite the enormous advances made in diagnostic and therapeutic areas over the past several decades, the incidence of CC worldwide is still increasing yearly. In fact, in 2016, CC accounted for an estimated 12,990 new cases and 4,120 deaths in the United States alone (3). Current treatments include surgery or radiotherapy for patients with early stage CC, and combination therapy with several chemotherapeutic drugs for patients with advanced stage CC (4). Although chemotherapy is a critical therapeutic strategy in various cancers, chemo-resistance is an important factor that influences the therapeutic efficiency of many chemotherapeutic agents (5). Paclitaxel (PTX) is typically used as a first-line chemotherapeutic agent for CC patients, but due to chemo-resistance, its response rates are limited and range between 29 and 63% (6, 7). Therefore, an urgent need exists to explore the underlying molecular mechanisms involved in chemo-resistance of PTX treatments in patients with CC.

Silent mating type information regulation 1 (SIRT1), an important member of the sirtuins family, is a NAD1-dependent class III deacetylase which is involved in numerous biological processes including cell proliferation, apoptosis, and cancer progression (8-10). Although evidence strongly suggests that SIRT1 is closely linked to various cancers, such as prostate cancer, epithelial ovarian cancer, and gastric cancer (11-13), its exact role in chemo-resistance of cancers still remains unclear. Recently, several studies demonstrated that overexpressed SIRT1 contributes to cisplatin resistance in endometrial carcinoma, providing a potential new therapeutic target (14). However, whether SIRT1 is involved in the PTX-resistance of CC remains unknown.

In this study, we first examined the expression patterns of SIRT1 in normal, PTX-sensitive CC, PTX-resistant CC tissues, and in their corresponding cell lines (HeLa, SiHa, HeLa/PTX, and SiHa/PTX). We found that SIRT1 expression was significantly upregulated in PTX-resistant CC tissues and cell lines. Next, we blocked the expression of SIRT1 in PTX-resistant cell lines, and observed that knockdown of SIRT1 inhibited cell proliferation and promoted cell apoptosis. Finally, we measured the expression levels of multidrug resistance-associated protein (MRP) in PTX-resistant cell lines, and found that MRP expression was decreased after knockdown of SIRT1.

Materials and Methods

Patients and tissue samples

Normal, PTX-sensitive, and PTX-resistant human

cervical carcinoma tissues (n = 10) were collected from the First Hospital of Hunan University of Chinese Medicine. Informed consents were obtained from all patients with CC and the study was approved by the Ethics Committee of the First Hospital of Hunan University of Chinese Medicine. All tissue samples were stored at −80°C.

Cell culture and treatment

CC cell lines, HeLa and SiHa, were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). PTX-resistant SiHa/PTX and HeLa/PTX cells were developed from SiHa and HeLa cell lines, respectively, by treating them with different concentrations of PTX in the medium. The concentration of PTX was gradually increased from 0.5 to 20 nM (0.5, 1, 2, 4, 8, 10, 12, 14, 16, 18, 20 nM), until the cells were stably proliferating without any obvious signs of apoptosis. HeLa and SiHa cell lines were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), and 100 U ml^{-1} penicillin, streptomycin in a humidified incubator containing 5% CO_2 at 37°C. PTX-resistant SiHa/PTX and HeLa/PTX cells were cultured in the same medium containing 20 nM PTX.

Immunocytochemistry

SiHa/PTX or HeLa/PTX cells were grown in 6-well plates at 37 °C for 24 h, and then fixed by 4% paraformaldehyde for at least 30 min. After incubating with 10% normal serum to block the nonspecific binding sites , fixed cells were incubated overnight with primary antibodies against MRP (1: 1000, Santa Cruz), followed by incubation with the relevant secondary antibodies (Alexa Fluor 488 goat anti-rabbit IgG, 1: 200, and DAPI 1:10000, Sigma Aldrich, St Louis, MO) at room temperature for 2 h. Cells were washed three times with PBS and images were captured by a Leica NTS confocal laser scanning microscope.

Reverse transcription and real-time Polymerase Chain Reaction (PCR)

Total RNA was prepared from normal, PTX-sensitive CC, PTX-resistant CC tissues, and the corresponding cell lines, using TRIzol reagent (Invitrogen, CA, USA), according to the manufacturer's protocol. Five μg of total RNA was used to reverse transcribe cDNA via RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Waltham, USA). The primers of SIRT1 and GAPDH (internal control) were designed and purchased from Sangon, China. The primers of GAPDH are: forward 5′- CCT CGT CTC ATA GAC AAG ATG GT -3′ and reverse 5′-GGG TAG AGT CAT ACT GGA ACA TG-3′; and the primers of SIRT1 are: forward 5′-GAG TGG CAA AGG AGC AGA-3′ and reverse 5′-TCT GGC ATG TCC CAC TAT C-3′. The mRNA expression levels of SIRT1 were normalized to GAPDH.

Western Blotting

Total protein from tissues and cells were extracted by a lysis buffer (0.1% SDS, 1% Triton X-100, 1mM MgCl2, 10mM Tris-HCl, pH 7.4), and their concentration was measured by the BCA Protein Assay (Pierce, Rockford, IL, USA). About 40 μg of total protein was separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels (SDS-PAGE) after denaturation at high temperature conditions. After separation by SDS-PAGE, proteins were transferred onto nitrocellulose (Millipore, Billerica, MA, USA), and then blocked with 5% low-fat milk powder in Tris-buffer for 2 h at room temperature. The membranes were then incubated with primary antibodies against SIRT1 (1: 1000, Santa Cruz Biotechnology, Inc., CA, USA), Bcl-2 (1: 500, Santa Cruz), Bax (1: 200, Santa Cruz) or GAPDH (1: 500, Santa Cruz). After three washes in Tris-buffered saline (TBS), the relevant horseradish peroxidase-conjugated secondary antibodies were applied to the membranes for 2 h at room temperature, and signals were visualized by enhanced chemiluminescent reagents.

Cell cycle analysis

The percentage of treated HeLa/PTX or SiHa/PTX cells in G1, S and G2 phase were determined by DNA flow cytometry, according to the instructions of the manufacturer. Briefly, cells were seeded into 24-well plates at a density of 1×10^5 cells/well and transfected with SIRT1 siRNA or negative control (NC) siRNA. Then, treated cells were collected and fixed in 80% ethanol, and stored at -20 °C overnight. After three washes with phosphate-buffered saline (PBS), cells were stained with 500 μl PI/RNase staining buffer (BD bioscience, USA) at room temperature for around 30 min and protected from light. The cell cycle distribution was analyzed using FACS-Calibur flow cytometer (Becton Dickinson, USA) and ModFit LT 3.2 Software (Verity Software house, USA).

Cell proliferation analysis

Cell proliferation of treated HeLa/PTX and SiHa/PTX cells was determined by a cell count kit (CCK-8), according to the manufacturer's protocol. Briefly, HeLa/PTX or SiHa/PTX cells transfected with either SIRT1 siRNA or NC siRNA were seeded in 6-well plates at a concentration of 2×10^5 cells/well, and cultured for 24 h. Then, CCK-8 reagents (10 μl/well) were added at 0, 24, 48, or 72 h, and incubated for at least 3 h at 37 °C. Optical density (OD) was detected at 450 nm via a microplate reader (WellscanMK3, Thermo/Labsystems, Finland).

siRNA and transfection

SIRT1 siRNA and negative control (NC) siRNA were designed and obtained from Dharmacon (Lafayette, CO). HeLa/PTX or SiHa/PTX cells (2×10^5) were seeded into 6-well plates containing 2 mL DMEM medium and cultured at 37 °C for 24 h. Then, 20 μL of 30 μmol/L SIRT1 siRNA or siRNA NC were transfected into either HeLa/PTX or SiHa/PTX cells with GeneSilencer siRNA Tranfection Reagent kit, according to the manufacturer's instructions (Gene Therapy Systems, San Diego, CA).

Statistical Analysis

All data is expressed as the mean ±SD, and statistically significant differences among the different groups were analyzed via one-way analysis of variance (ANO-

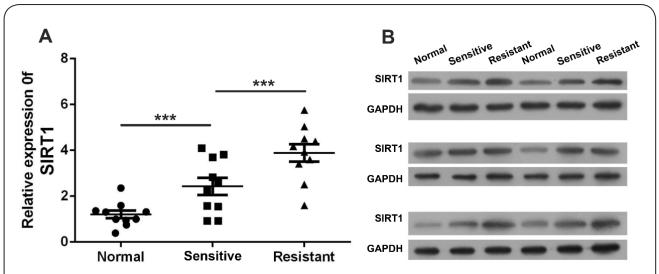

Figure 1. The expression levels of SIRT1 in normal, PTX-sensitive CC, and PTX-resistant CC tissues. (A) SIRT1 expression was detected by qRT-PCR in three groups: normal, PTX-sensitive CC, and PTX-resistant CC tissues (n = 10). (B) Two samples from each group were used to further confirm the expression of SIRT1 by Western blot assay.

VA), and P value <0.05 was considered statistically significant (SPSS 21.0 for Windows).

Results

SIRT1 expression is significantly upregulated in PTX-resistant CC tissues compared to normal tissues or PTX-sensitive CC tissues

To explore the involvement of SIRT1 in the progression and development of PTX-resistant CC, we first measured the expression levels of SIRT1 in three groups (normal, PTX-sensitive CC, and PTX-resistant CC tissues). Results from qRT-PCR showed that SIRT1 expression increased in PTX-sensitive CC tissues compared to the corresponding normal tissues. SIRT1 expression was also significantly upregulated in PTX-resistant CC tissues compared to PTX-sensitive CC tissues (Figure 1A). Western blots further confirmed these results (Figure 1B). These data indicate that SIRT1 is involved in the progression of PTX-resistant CC.

SIRT1 expression is significantly higher in PTX-resistant CC cell lines than PTX-sensitive CC cell lines

To investigate the function of SIRT1 in PTX-resistant CC, we established two PTX-resistant CC cell lines (SiHa/PTX and HeLa/PTX cell lines) by treating CC cell lines (SiHa and HeLa) with daily increasing concentrations of PTX in the cell culture medium. We detected SIRT1 expression in the two PTX-resistant CC cell lines using qRT-PCR and Western blot. We found that SIRT1 expression was significantly upregulated in the SiHa/PTX cells compared to the SiHa cells, and was also significantly higher in HeLa/PTX cells compared to the HeLa cells (Figure 2A and 2B).

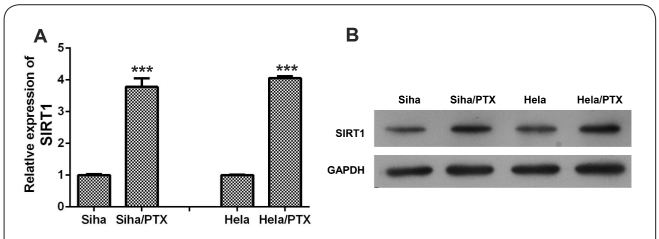

Figure 2. The relative SIRT1 expression in CC cell lines and the corresponding PTX-resistant CC cell lines. The relative expressions of SIRT1 in CC cell lines (SiHa e and HeLa), and PTX-resistant cell lines (SiHa/PTX and HeLa/PTX) were measured by qRT-PCR (A) and Western blot (B).

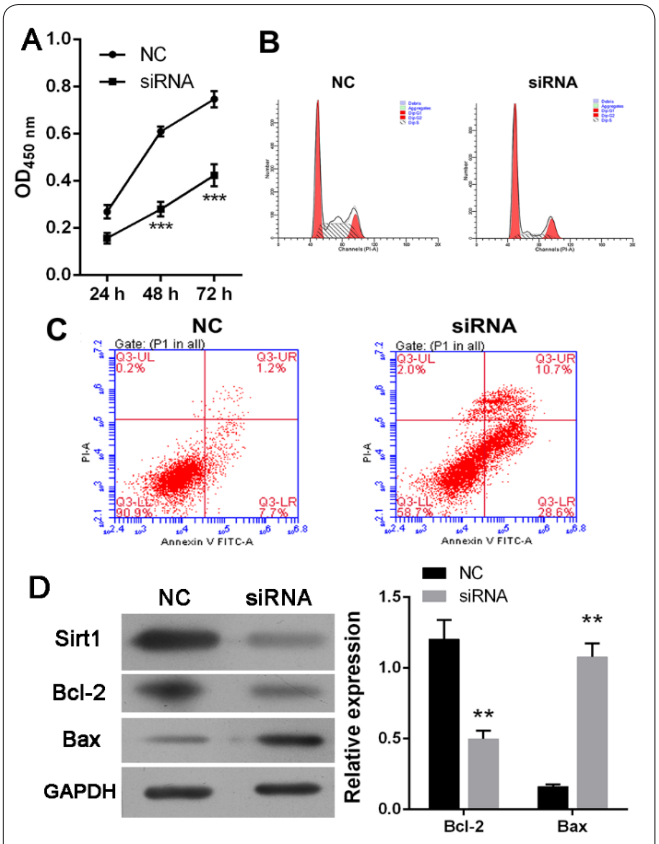

Figure 3. Effects of SIRT1 knockdown on cell apoptosis and the expression profiles of apoptosis-related proteins in PTX-sensitive Hela cell lines. (A) Cell proliferation was monitored with the CCK-8 assay at different time points (24 h, 48 h, and 72 h) in HeLa cells transfected with either NC or siRNA. (B and C) flow cytometry analysis was performed to examine the apoptotic ratio and cell cycle of HeLa after transfection with either SIRT1 siRNA or NC. (D) Apoptosis-related proteins (Bcl-2 and Bax) were measured by Western blot and qRT-PCR in HeLa cells transfected with either SIRT1 siRNA or NC.

Knockdown of SIRT1 inhibited the cell proliferation, and promoted cell cycle arrest and apoptosis of PTX-sensitive CC cells

To further explore the roles of SIRT1 in PTX-sensitive CC, PTX-sensitive CC cells (Hela) were transfected with negative control (NC) and SIRT1 siRNAs (siRNA), respectively. CCK-8 and flow cytometry were performed to detect the cell proliferation, cell cycle and apoptosis abilities. The results indicated that silence of SIRT1 inhibited the Hela cell proliferation ability (Figure 3A); silence of SIRT1 promoted Hela cell cycle arrest (Figure 3B); silence of SIRT1 accelerated Hela cell apoptosis (Figure 3C). and then, expression of SIRT1, Bax, and Bcl-2 protein expression were investigated using Western blot (Figure 3D).

Knockdown of SIRT1 inhibits cell proliferation of PTX-resistant CC cell lines

To further determine the exact role of SIRT1 in PTX-resistant CC, we blocked SIRT1 expression in HeLa/PTX cells and SiHa/PTX cells by transfecting them with SIRT1 siRNA. Western blot analysis and qRT-PCR assay were used to verify the SIRT1 knockdown efficiency. Our results showed that the SIRT1 expression was significantly downregulated in both HeLa/PTX cells and SiHa/PTX cells compared to the cor-

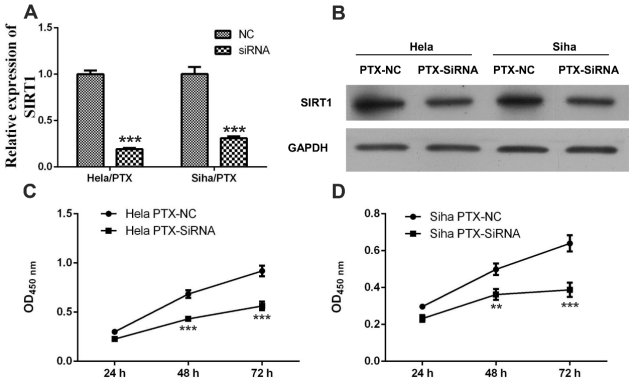

Figure 4. Knockdown efficiency of SIRT1-siRNA and the effects of SIRT1 knockdown on cell proliferation. The efficiency of SIRT1 knockdown after siRNA transfection in SiHa/PTX cells and HeLa/PTX cells was confirmed by qRT-PCR assay (A) and Western blot analysis (B). Cell proliferation was monitored with the CCK-8 assay at different time points (24 h, 48 h, and 72 h) in HeLa/PTX cells transfected with either NC or siRNA (C), or SiHa/PTX cells treated with either NC or siRNA (D).

responding HeLa cells and SiHa cells (Figure 4A and 4B). These results demonstrated the high efficiency and specificity of SIRT1 siRNA. We then used the CCK-8 assay to determine if SIRT1 plays a role in the cell proliferation of PTX-resistant CC cell lines. We found that cell proliferation was significantly inhibited in HeLa/PTX and SiHa/PTX cells transfected with SIRT1 siRNA compared with those cells transfected with negative control (NC) at 24 h and 48 h after transfection (Figure 4C and 4D). These results show that silencing SIRT1 inhibits proliferation of PTX-resistant cell lines.

Knockdown of SIRT1 promotes cell apoptosis of PTX-resistant CC cell lines

To understand the influence of SIRT1 on cell cycle in CC cells, we used flow cytometry to determine the cell cycle distribution of HeLa/PTX and SiHa/PTX cells. Results showed that SIRT1 knockdown decreased the percentage of cells in the S phase and increased the percentage of cells in the G1 phase (Figure 5A). To further investigate whether SIRT1 participates in cell apoptosis of PTX-resistant CC cells, we performed Annexin V/PI staining assay and flow cytometry analysis in HeLa/PTX and SiHa/PTX cells transfected with either SIRT1 siRNA or NC. The data showed that the apoptotic rate significantly increased after SIRT1 knockdown in both HeLa/PTX cells and SiHa/PTX cells (Figure 5B). In addition, we measured the expression levels of two apoptosis-related proteins, Bcl-2, and Bax. Western blots revealed that Bcl-2 expression was significantly downregulated, and Bax expression was significantly upregulated in SiHa/PTX or HeLa/PTX cells after transfection with SIRT1 siRNA (Figure 5C). These data indicate that silencing SIRT1 promotes cell apoptosis of PTX-resistant cell lines.

Knockdown of SIRT1 decreases the MRP expression in PTX-resistant cell lines

Multidrug-resistance-associated proteins (MRPs) are considered to be major contributors of multi-drug resistance (MDR). Immunofluorescence was performed to explore any potential correlation between SIRT1 and

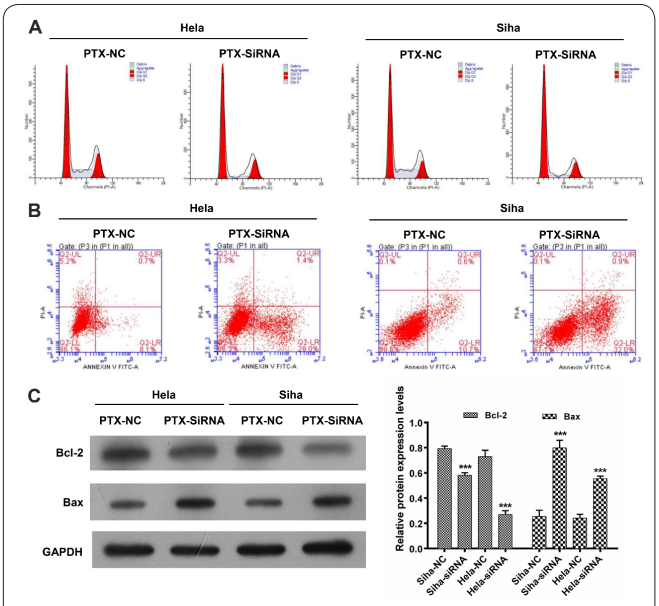

Figure 5. Effects of SIRT1 knockdown on cell apoptosis and the expression profiles of apoptosis-related proteins in PTX-resistant cell lines. (A and B) flow cytometry analysis was performed to examine the apoptotic ratio and cell cycle of HeLa/PTX cells or SiHa/PTX cells after transfection with either SIRT1 siRNA or NC. (C and D) Apoptosis-related proteins (Bcl-2 and Bax) were measured by Western blot and qRT-PCR in HeLa/PTX cells and SiHa/PTX cells transfected with either SIRT1 siRNA or NC.

MRPs. Our results indicated that the fluorescence intensity was significantly decreased in the SIRT1 siRNA transfected HeLa/PTX and SiHa/PTX cells compared to the NC siRNA transfected cells (Figure 6A and 6B). These results suggest that MRPs are involved in the oncogenic functions of SIRT1 in PTX-resistant CC cell lines.

Discussion

Cervical cancer is one of the most common cancer affecting women in the world, accounting for approximately 12-14% of all cancers globally (15). The societal and economical burdens of CC are enormous, and costs an estimated billions of dollars every year in developing countries, including China and India (16).Due to lack of effective preventive, diagnostic, and therapeutic measures, the prognosis of CC has not improved over the past few decades (17, 18). Currently, chemotherapy is the main treatment modality, and PTX, a critical chemotherapeutic agent, is the most widely used front-line treatment for CC (19-21). However, the effects of PTX on CC treatment are limited by PTX-resistance. PTX

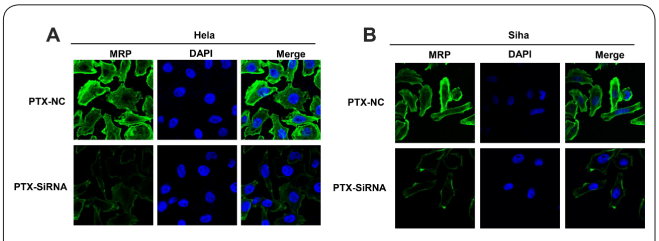

Figure 6. Effects of SIRT1 knockdown on MRP expression in PTX-resistant CC cell lines. (A and B) Immunofluorescence was used to examine the expression levels of MRP in two PTX-resistant CC cell lines (HeLa/PTX and SiHa/PTX) treated with either SIRT1 siRNA or NC.

influences the composition of microtubule, spindle assembly, and cell division by interfering with tubulin, and leads to cancer cell death without genome damage (19, 22). Resistance to PTX is extremely complicated, and studies demonstrate that it may be caused by numerous mechanisms: aberrant expression of P-glycoprotein, tumor suppressor genes (TSGs), and microtubule-associated proteins; alteration in cell cycle and cell survival pathways; induction of treatment-related autophagy (23-25). However, the exact molecular mechanisms of PTX-resistance in the progression of CC are not fully illustrated and warrants further work.

Sirtuin proteins, originally discovered in yeast, consist of seven members (SIRT1-7) with distinct molecular structures and biological functions (26). Among them, SIRT1 is the most characterized family member and is involved in histone deacetylation and tumor progression, DNA damage repair, autophagy and other biological processes (27-29). Studies have also demonstrated its involvement in cell proliferation, invasion, apoptosis, metastasis, and chemo-resistance in various tumors, such as gastric cancer, breast cancer, and colorectal cancer (30-32). Evidence suggests that the expression of SIRT1 is significantly higher in chemo-resistant ovarian cancer, breast cancer, and leukemia, than their drug-sensitive counterparts. Chu et al reported that aberrant overexpression of SIRT1 induces the expression of P-glycoprotein, resulting in the chemo-resistance of cancer cells to doxorubicin (an anti-tumor drug) (33). Although increasing evidence indicates that SIRT1 participates in the progression and development of CC via various pathways, little work has been conducted to explore its role in PTX-resistant CC (34-36).

In this study, we first compared SIRT1 expression in normal, PTX-sensitive, and PTX-resistant CC tissues by qRT-PCR assay and Western blot analysis. Our results showed that the expression levels of SIRT1 were higher in PTX-sensitive CC tissues compared to the corresponding normal tissues. In addition, SIRT1 expression was significantly upregulated in PTX-resistant CC tissues compared to PTX-sensitive CC tissues. Next, we established two PTX-resistant CC cell lines by treating the HeLa and SiHa cells with increasing concentrations of PTX. We found that SIRT1 expression significantly increased in the PTX-resistant cell lines (HeLa/PTX and SiHa/PTX cells), compared to the PTX-sensitive cell lines (HeLa and SiHa cells). These results suggest that SIRT1 plays an indispensable role in the regulation of PTX-resistance of CC. Therefore, to further decipher the exact functions of SIRT1 in PTX-resistant CC, we silenced SIRT1 expression by transfecting cells with either SIRT1 siRNA or siRNA NC. Data from the CCK-8 assay showed that cell proliferation was significantly inhibited when SIRT1 expression was silenced in HeLa/PTX and SiHa/PTX cells. Results from flow cytometry analysis and Western blot assays indicated that the apoptotic rate increased in SIRT1 siRNA-treated PTX-resistant CC cell lines compared to siRNA NC-treated cell lines. These data suggest that knockdown of SIRT1 in HeLa/PTX and SiHa/PTX cells inhibit cell proliferation and promote cell apoptosis.

Multidrug resistance-associated proteins (MRPs), consist of nine members each with different structures and functions. These proteins belong to subfamily C of the ATP-binding cassette (ABC) transporter super family. Previous work suggested that aberrant expression of MRPs contributes to the downregulation of drug sensitivity to various chemotherapeutic agents, including cisplatin, doxorubicin, and PTX, in cancer patients (37, 38). In this study, we found that MRP expression was significantly decreased in SIRT1 blocked HeLa/PTX and SiHa/PTX cells, suggesting that MRP is involved in the PTX-resistance of CC.

In conclusion, our results suggest that SIRT1 expression is significantly increased in the PTX-resistant CC tissues and cell lines, and blocking SIRT1 expression inhibits cell proliferation and promotes cell apoptosis of HeLa/PTX and SiHa/PTX cells. In addition, knockdown of SIRT1 decreases the expression of MRP in PTX-resistant CC cell lines.

Acknowledgements

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Conflict of Interest

All authors declared no conflict of interest.

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