Introduction

Esophageal cancer is a kind of malignant digestive tract tumor with a potent invasiveness (1). Currently, surgical resection is a common therapeutic strategy for early-stage esophageal cancer. However, with the progression of the esophageal cancer, the recurrence rate after surgery increases gradually, and the outcome is not satisfactory (2). Therefore, for advanced or recrudescent esophageal cancer patients, chemotherapy combined with surgery is essential. However, the clinical effect of chemotherapy targeted with esophageal cancer is not satisfactory. Due to individual difference, the sensitivity of patients to chemotherapy varies. Low sensitivity even chemo-resistance would lead to a poor outcome along with elevating economic burden to patients. Esophageal cancer chemo-resistance to cisplatin, a widely used chemotherapeutic agent nowadays, has already been reported (3). Yet, the underlying mechanism of reduced sensitivity of cisplatin is not clear.

Sirtuin type 1 (SIRT1), a kind of nicotinamide adenine dinucleotide (NAD+)—dependent histone deacetylase,

Original Article

Higher expression of SIRT1 induced resistance of esophageal squamous cell carcinoma cells to cisplatin

Bin Cao, Qintong Shi, Wengong Wang

Department of cardiothoracic surgery, Nanjing Drum Tower Hospital, Nanjing 210008, China

Correspondence to: Bin Cao. Department of cardiothoracic surgery, Nanjing Drum Tower Hospital, Nanjing 210008, China. Email: caobdth@163.com.

Background: High expression of Sirtuin type 1 (SIRT1) exists in some cancer cells. However, it is still unclear whether SIRT1 affects the sensitivity of esophageal cancer cells to cisplatin. This study was designed to explore the relationship between SIRT1 expression and resistance of esophageal squamous cell carcinoma (ESCC) cells to cisplatin and reveal the underlying mechanism.

Methods: The tissue samples of 68 ESCC patients were collected from Nanjing Drum Tower Hospital, China. All the patients had undergone cisplatin based combination chemotherapy. The expression of SIRT1 and Noxa in tissue samples were analyzed by quantitative real-time reverse PCR (qRT-PCR) and Western blot. Human ESCC cell line (ECa9706 cells) was cultured and a cisplatin-resistant subline (ECa9706-CisR cells) was established by continuous exposure to cisplatin at different concentrations. The expression of SIRT1 and Noxa in both cell lines was analyzed by qRT-PCR and Western blot. siRNA technology was utilized to down-regulate the SIRT1 expression in ECa9706-CisR cells. The influence of SIRT1 silence on sensitivity of ECa9706-CisR cells to cisplatin was confirmed using CCK-8 assay and flow cytometry. Furthermore, the level change of Noxa after SIRT1 silence in ECa9706-CisR cells was determined by qRT-PCR and Western blot.

Result: SIRT1 and Noxa expression in chemo-resistant patients was significantly increased and decreased respectively, compared with chemo-sensitive patients. SIRT1 expression in ECa9706-CisR cells was significantly increased with a lower Noxa level, compared with normal ECa9706 cells. Cisplatin 5 μM could cause proliferation inhibition, G2/M phase arrest and apoptosis in ECa9706-CisR cells and these effects could be enhanced dramatically by SIRT1 silencing. Moreover, Noxa expression was increased after treated with SIRT1 siRNA.

Conclusions: Over-expression of SIRT1 may cause resistance of ESCC cells to cisplatin through the mechanism involved with Noxa expression.

Keywords: Sirtuin type 1 (SIRT1); esophageal squamous cell carcinoma (ESCC); Noxa; cisplatin; drug resistance

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is one of the seven members of the sirtuin family. SIRT1 involves in DNA damage repair, cell cycle, apoptosis and oxidative stress in normal cells. While for tumor cells, SIRT1 has an anti-apoptotic effect and thus promoting carcinogenesis (4). Overexpression of SIRT1 has been found in a variety of solid tumors (5). Recently, some studies focus on the relationship between SIRT1 expression and efficacy of chemotherapy. Studies have shown that, possible mechanisms of SIRT1 and chemoresistance involve Mdr-1, P-pg, FOXO3 and other signaling pathways (6).

Noxa is one member of the Bcl-2 family with pro-apoptotic effect, has been found overexpressed in 3,4,5, 4'-tetramethoxystilbene (DMU-212)-treated colon cancer cells (7). Another study showed that Noxa might be related with sensitivity of Bcl-2 inhibitor ABT-737 in small cell lung cancer, as low Noxa expression could inhibit the apoptotic effect of ABT-737 (8).

Previous study has shown that high SIRT 1 expression had a significantly higher chance to be resistant to platinum-based chemotherapy (9). Mutation or expression changes of P53 induced cisplatin resistance and SIRT1 can regulate the deacetylation P53 and change its activation (10,11). As a downstream gene of P53, we suspect that Noxa may be also involved with cisplatin resistance and be regulated by SIRT1. However, whether SIRT 1 and Noxa play a role in cisplatin resistance of esophagus cancer should be analyzed.

**Materials and methods**

Sixty-eight patients (30 men and 38 women with the median age of 67 years) with histopathologically proven ESCC were included in this study, the clinical characteristics of patients was shown in Table 1. All patients underwent surgical treatment but developed recurrence. In order to control the recurrence, the patients received cisplatin based combination chemotherapy between May 2012 and February 2014 in Nanjing Drum Tower Hospital, China. To evaluate the effect of chemotherapy, we followed the RECIST (Response Evaluation Criteria in Solid Tumors) guideline. By calculating the diameter of tumor, patients were assessed as complete response (CR), partial response (PR), stable disease (SD) and progressive disease (PD). We defined CR + PR patients as chemo-sensitive patients and the others as chemo-resistant patients. In brief, 40 patients were assessed as chemo-sensitive patients while 28 patients were chemo-resistant. Excepting the curative effect of chemotherapy, the information about demographic data such as age, sex, stage of disease had no statistical difference between the chemo-sensitive group and the chemo-resistant group.

**Table 1 Clinical features of 68 esophageal squamous cell carcinoma (ESCC) patients**

<table>
<thead>
<tr>
<th></th>
<th>Chemo-sensitive patients</th>
<th>Chemo-resistant patients</th>
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</tr>
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<tr>
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<tr>
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Human esophageal squamous cell carcinoma (ESCC) cell line (ECa9706 cells) was purchased from Beijing Zhongyuan Ltd (Beijing, China). ECa9706 cells were maintained in PRMI1640 medium (Thermo Fisher Scientific, USA) supplemented with 10% fetal bovine serum (Teaching Biological, Hangzhou, China) at 37 °C in the 5% CO₂ incubator (Hera cell 150i, Thermo Fisher Scientific, USA). According to Wang’s methods (9), a cisplatin-resistant subline named ECa9706-CisR cell line was obtain from parental ECa9706 cells through a continuous exposure to increasing cisplatin (from 1.5 to 12 μM) over 12 months.
Quantitative real-time reverse PCR (qRT-PCR) for SIRT1 and Noxa mRNA expression

Total RNA extraction of tissues and cells were performed using Trozol reagent (Thermo Fisher Scientific, USA). Qualified RNA was used for cDNA synthesis with PrimeScript® RT reagent (Takara Biotechnology, Dalian, China) according to manufacturer's instruction. cDNA was amplified with SYBR® Premix Ex Taq™ II kit(Takara Biotechnology, Dalian, China) on a 7900HT fast real-time PCR system (Applied Biosystems, USA). The cycling conditions were set by manufacturer's protocol. Primers of targeted genes and β-actin were as follows: SIRT1, 5’GCC TCA TCT GCA TTT TGA TG’3(sense), 5’TCT GGC ATG TCC CAC TAT CA’3(antisense). Noxa, 5’TTC GTG TTC AGC TCG CGT CC’3(sense), 5’CTC GGT GTA GCC TTC TTG CC’3(antisense). β-actin, 5’ CTC CAT CCT GGC CTC GCT GT’3(sense), 5’GCT GTC ACC TTC ACC GTT CC’3(antisense). Using ΔΔ cycle threshold (2−ΔΔCt) method determined the fold change of SIRT1 and expression data were normalized by β-actin.

Western blot analysis for SIRT1 and Noxa expression

Total protein in tissue samples, ECa9706 cells and ECa9706-CisR cells were extracted using the RIPA reagent containing 1% protease inhibitors cocktail (Applygen, Beijing, China). The protein concentration in sample was determined by BCA kit(Applygen, Beijing, China) and pre-treated with 6× Loading Buffer at 100℃ for 3-5 min. Protein 45 μg was separated on 10% SDS-polyacrylamide gel and then transferred polyvinylidene difluoride (PVDF) membranes. Blots were blocked with 5% skim milk in PBS for 1 h at room temperature and incubated at 4℃ for 12 h with the primary antibody anti-SIRT1 (1:500, Santa Cruz Biotechnology, USA), anti-Noxa (1:1,000, Santa Cruz Biotechnology, USA) and anti-β-actin (1:3,000, Santa Cruz Biotechnology, USA). Membranes were washed by PBST buffer and followed an incubation with horseradish peroxidase-conjugated secondary antibodies. ECL kit (Applygen, Beijing, China) was used to detected the band signals.

Transfection of siRNA targeted to SIRT1

Inhibition of SIRT1 expression in ECa9706-CisR cells was induced using specific siRNA(Sequence-sense: GCA AUU GGC CUC UUA AUU Att; antisense: UAA UUA AGG CCU AUU GCt). Target siRNA and control siRNA were all designed and synthesized by Shanghai GenePharma Company (China). ECa9706-CisR cells were planted into 24 well-plate with a density of 1x10^5 and transfected with 60 nM siRNA using Lipofectamine® 2000 reagent (Life Technologies Corporation, USA) according manufacture's protocol, while silencer negative control siRNA was used. To confirm the down-regulation effect of siRNA, qRT-PCR and western blot was utilized to determine the level of SIRT1 at 24 and 48 h respectively.

Cell proliferation analysis

CCK-8 assay was performed to analyze the resistance of ECa9706-CisR cells to cisplatin. Non-transfected or SIRT1 silenced ECa9706-CisR cells were planted into 96 well-plate with a density of 1x10^4 μL and treated with 5 μM cisplatin for 24 h. As control, non-transfected ECa9706-CisR cells were cultured without any treatment. After 24 h, cells were incubated with CCK-8 counting reagent for 4 h at 37℃. The optical density (at 450 nm) in each well was measured by enzyme-linked immunosorbent assay plate reader and viability rate was calculated.

Apoptosis and cell cycle analysis

For cell apoptosis analysis, ECa9706-CisR cells were collected and Annexin V-FITC assay kit was used according to the manufacturer's protocol after treated with 5 μM cisplatin for 24 h. For cell cycle analysis, treated cells was fixed in 70% cold ethanol and were stained with 50μg/mL propidium iodide (Sigma-Aldrich, USA) and incubated for 30 min. Analysis was performed using a BD FACSCalibur flow cytometer (Beckman Coulter, USA) within 15 min. Using CellQuest software analyzed cell cycle and apoptosis rate.

Noxa expression analysis

Total mRNA and protein in different ECa9706-CisR cells were extracted after treated with 5 μM cisplatin for 24 h. The expression of Noxa after SIRT1 inhibition was determined using qRT-PCR and Western blot with the protocol described above.

Statistical analysis

Statistical analysis of the data took advantage of SPSS
Results

Expression of SIRT1 and Noxa in tumor tissue specimens

We evaluated the expression of SIRT1 in patients with ESCC. qRT-PCR analysis demonstrated higher expression of SIRT1 and lower expression of Noxa in chemo-resistant patients compared to chemo-sensitive patients (P=0.008 and 0.000, respectively) (shown in Figures 1, 2). The western blot also confirmed the higher level of SIRT1 and lower level of Noxa in chemo-resistant patients (Figure 3). Correlation analysis showed a negative correlation between SIRT1 and Noxa in mRNA level (r=-0.803 P=0.000) (Figure 4), indicating that SIRT1 is associated with sensitivity of patients to chemotherapy and Noxa expression is also possibly involved.

Expression of SIRT1 and Noxa in ECa9706 cells and ECa9706-CisR cells

The resistance to cisplatin of ECa9706-CisR cells was proven in preliminary experiment (Figure 5). We evaluated the expression of SIRT1 and Noxa in ECa9706 cells and ECa9706-CisR cells. qRT-PCR analysis demonstrated higher level of mRNA and lower level of Noxa mRNA in ECa9706-CisR cell compared to the ECa9706 cells (P=0.000 and 0.003 respectively). The western blot also confirmed the results of qRT-PCR analysis (Figures 6-8).

SIRT1 Inhibition improves the proliferation inhibitory effect of cisplatin

As expected, 5 μM cisplatin could inhibit proliferation of ECa9706-CisR cells significantly (Figure 9). After 5 μM cisplatin treatment, the viability rate of non-transfected ECa9706-CisR cells was (73.3±6.9)%. However, the
Figure 4 Correlation between Sirtuin type 1 (SIRT1) and Noxa in chemo-sensitive and chemo-resistant patients.

Figure 5 Inhibition effect of 5 μM cisplatin on ECa9706 cells and ECa9706-CisR cells. *, compared with ECa9706 cells group, P<0.05.

Figure 6 Relative mRNA expression of SIRT1 in ECa9706 cells and ECa9706-CisR cells. *, compared with ECa9706 cells group, P<0.05.

Figure 7 Relative mRNA expression of Noxa in ECa9706 cells and ECa9706-CisR cells. *, compared with ECa9706 cells group, P<0.05.

Figure 8 Protein expression of Sirtuin type 1 (SIRT1) and Noxa in ECa9706 cells and ECa9706-CisR cells.
viability rate of SIRT1 silenced ECa9706-CisR cells was further lower (55.4%±4.8%) and this difference was statistically significant (P=0.000).

**SIRT1 inhibition enhances the apoptosis caused by cisplatin**

As shown in Figure 10, flow cytometry demonstrated that SIRT1 inhibition enhances the apoptosis rate from (23.5±1.9)% in 5 μM cisplatin treated non-transfected ECa9706-CisR cells to (35.6±2.8)% in 5 μM cisplatin treated SIRT1 silenced ECa9706-CisR cells, and the difference between them was statistically significant (P=0.000).

**SIRT1 Inhibition aggravates the G2/M phase arrest induced by cisplatin**

As shown in Figure 11, we tested the impact of SIRT1 inhibition on cell cycle arrest induced by cisplatin. In the control (non-transfected ECa9706-CisR cells without cisplatin treatment) the percentages of G0/G1 phase and G2/M phase were (70.3±4.1)% and (4.5±0.7)%, respectively. With the 5 μM cisplatin treatment, in control cells, the proportion of G0/G1 phase fell to (56.3±5.2)%, and the proportion of G2/M phase increased to (35.5±4.1)%, suggesting G2/M phase arrest occurred. Meanwhile, in SIRT1 silenced ECa9706-CisR cells, the proportion of G0/G1 phase was lower (35.5±6.5)% and the percentage of cells in the G2/M phase was further increased (45.3±5.9)%, compared with control and SIRT1 silenced ECa9706-CisR cells without cisplatin treatment.

**SIRT1 inhibition increases the expression of Noxa**

As shown in Figures 12 and 13, 5 μM cisplatin induced slight higher expression of Noxa in ECa9706-CisR cells compared with control. More excitedly, obvious higher expression was observed after SIRT1 targeted siRNA transfection. It suggested that SIRT1 can improve sensitivity of ESCC cells to cisplatin by regulating Noxa.

**Discussion**

The present treatments of esophageal cancer are surgery, chemotherapy, radiotherapy and immunotherapy, among
which surgery is the preferred strategy of early cancer (12). But for the advanced cancer patients, the effect of surgical treatment is less effective compared with the early cancer, adjuvant radiotherapy, and chemotherapy are always necessary for them (13,14). Especially in China, most of the patients with esophageal cancer are at the advanced stage when diagnosed with cancer, and lose the opportunity of surgery (15,16). For these patients, chemotherapy is very important. Cisplatin is an effective broad spectrum anticancer drug; however, extensively published studies have reported the cisplatin resistance in human cancer cells both in vivo and in vitro (17,18). We have to admit that there are complexities of cisplatin sensitivity and resistance. Changes can occur in almost every mechanism influencing cell growth, developmental pathways, apoptosis, DNA repair, drug metabolism, drug transporters (19). In this study, we focused on abnormal expression of SIRT1. High expression of SIRT1 existed in both chemo-resistant patients and ECa9706-CisR cells resistance to cisplatin. More importantly, after further using siRNA to silence SIRT1, ECa9706-CisR cells sensitivity to cisplatin improved, which showed cell viability decreased, G2/M arrest proportion increased, and apoptosis rate increased. Actually, previous studies have showed that SIRT1 may influence the sensitivity of tumor cells to chemotherapeutical agents, such as Chen et al. found that overexpression of SIRT1 promoted tumor genesis and resistance to chemotherapeutical agent and sorafenib (20). Kojima et al. found that up-regulation of SIRT1 expression may play an important role in promoting cell growth and chemo-resistance in androgen-refractory prostate cancer PC3 and DU145 cells (21).

In the aspect of tumor drug resistance, some studies found that after using RNA interfering technology to reduce SIRT1 expression, P-glycoprotein (P-gp) and multidrug resistance (MDR) proteins expression also decreased accordingly, and the latter two overexpression were the important reason for the tumor drug resistance (22,23). Transient transfection experiments showed that in human embryo kidney cells, SIRT1 can directly induce gene expression of MDR1 which resulted in the decrease of

Figure 11 Cisplatin 5 μM caused ECa9706-CisR cells cycle arrest in G2/M phase and SIRT1 inhibition reinforces this effect. *, compared with control, P<0.05; #, compared with cisplatin treated ECa9706-CisR cells, P<0.05 (only show the difference of G2/M phase).

Figure 12 Sirtuin type 1 (SIRT1) inhibition increased relative mRNA expression of Noxa in ECa9706-CisR cells. *, compared with control, P<0.05; #, compared with cisplatin treated ECa9706-CisR cells, P<0.05.

Figure 13 Sirtuin type 1 (SIRT1) inhibition increased protein expression of Noxa in ECa9706-CisR cells. *, compared with control, P<0.05; #, compared with cisplatin treated ECa9706-CisR cells, P<0.05.
cellular sensitivity to drug (24). These studies indicated that SIRT1 inhibiting can partly improve sensitivity of tumor to chemotherapy drugs. Our studies found that, SIRT1 affected the resistance of cells to chemotherapeutical agents such as cisplatin by adjusting Noxa. Noxa's effect of promoting apoptosis has been relatively clear, such as Baou et al. found that Noxa high expression played a key role in the process during which bortezomib inhibit chronic lymphocyte leukemia cells (25). It is generally acknowledged that Noxa play a role of promoting apoptosis mainly through mitochondrial cytochrome C-way, after combing with mitochondria, Noxa can affect mitochondrial permeability, the outer membrane potential, which lead to the release of cytochrome c, to activate Caspase family and induce apoptosis (26). Most importantly, Noxa expression is regulated by P53, P53 binding sites exist in its upstream startup sequence, when cells get damaged by chemotherapy drugs or oxidative stress, P53 will combine with Noxa startup sequence and up regulate its expression (27,28). Based on this, whether or not there are other pathways independent of P53 which can activate Noxa awaits further research.

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References


