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Sensitization of suberoylanilide hydroxamic acid (SAHA) on chemoradiation for human cervical cancer cells and its mechanism

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Summary

Purpose: To explore the sensitization of suberoylanilide hydroxamic acid (SAHA) on chemoradiation for cervical cancer cells and its mechanism. *Materials and Methods:* After human cervical cancer SiHa cells were treated with SAHA and cisplatin (DDP) of different concentrations, inhibition and apoptosis rates, and cell cycle were detected. SiHa cells underwent radiation of various doses after treated with 20% IC50 of SAHA for 24 hours. The survival fraction of SiHa cells was calculated by colony-forming assay, and related parameters were calculated. mRNA and protein expressions of P21, Bax and Ku70 were detected. *Results:* The inhibition rate was higher in SD (SAHA combined with DDP) group than in D (DDP alone) group ($p < 0.05$). The number of cells in G0/G1 phase was higher, and the number of cells in G2/M+S phase and PI (proliferation index) were lower in S (SAHA), D, and SD groups than in control group, and in SD group than in S and D groups ($p < 0.05$). The apoptosis rate and the expressions of mRNA and protein of Bax and P21 were higher in SD group than in S or D group ($p < 0.05$). The cell survival fraction was lower in SAHA combined with radiotherapy group than in radiotherapy alone group ($p < 0.05$). Do, N, and Dq values were 2.329, 2.761, and 1.721, respectively, in radiotherapy alone group and 1.213, 4.770, and 0.823, respectively, in SAHA combined with radiotherapy group. SER was 1.92. Bax mRNA and protein expressions were higher but Ku70 mRNA and protein expressions were lower in SAHA combined with radiotherapy group than in radiotherapy alone group ($p < 0.05$). *Conclusion:* SAHA promotes SiHa apoptosis in chemotherapy through up-regulation of mRNA and protein of p21 and Bax which leads to cell cycle arrest in G0/G1 phase. Low dose of SAHA promotes SiHa apoptosis and inhibits cell repair in radiotherapy through Bax up-regulation and Ku70 down-regulation.

Key words: SAHA; DDP; SiHa cell; Apoptosis; Sensitization; Bax; P21; Ku70.

Introduction

The incidence of cervical cancer takes the second place in cancer in women [1, 2]. Its morbidity and mortality are growing in recent years [3]. At present, radiotherapy and surgery are mainly used in the treatment of cervical cancer, supplemented by chemotherapy. Radiotherapy is one of main methods for the treatment of cervical cancer. However, except killing tumor cells, radiotherapy also produces damage on normal tissues such as the bladder and intestinal canal, therefore the radiologic dose is limited. In recent years, more and more attention has been paid to chemotherapy for cervical cancer. Drug resistance is common in the chemotherapy of tumor, and chemotherapeutics not only kill tumor cells, but also produce damage on normal cells [4-6]. The key to improve the therapeutic effects of chemoradiotherapy is to increase the sensitivity of tumor tissue to radiation and chemotherapeutics under the condition that the doses of radiation and chemotherapeutics are not increased.

Histone deacetylase inhibitor (HDACi), a kind of antitumor drug, causes serious concern due to its characteristics of high efficiency and low toxicity [7]. It does not only inhibit tumor cell migration, invasion and metastasis, and the generation of tumor blood and lymph vessels, but also sensitizes chemoradiotherapy [8]. Suberoylanilide hydroxamic acid (SAHA), a kind of HDACi, has proved to be effective for many tumors and has been applied in phase II clinical trial [9, 10]. It has strong specificity and selectivity with fewer side effects. Little research has been done in the application of SAHA combined with radiotherapy or chemotherapy in the treatment of cervical cancer. In this study, the authors explored the inhibitory effects of SAHA combined with cisplatin (DDP) or radiotherapy on human cervical cancer SiHa cells and its potential mechanisms, providing new ideas for the chemoradiotherapy of cervical cancer.

Materials and Methods

Cell culture

Cells were incubated in high glucose-DMEM medium containing 100 U/ml of penicillin, 100 U/ml of streptomycin, and 10% of fetal bovine serum in an atmosphere of 5% CO_2 at 37°C. The cells in log phase growth were used for future experiments.

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Effects of SAHA, DDP or SAHA combined with DDP on SiHa cell proliferation detected with MMT assay

The cells (5×10^4/ml, 100 µl/per well) were seeded in 96-well plate for incubation in an atmosphere of 5% CO_2 at 37°C. When cells were adherent, different-concentration DDP (1, 2, 4, 6, 8, and 10 µg/ml) and SAHA (0.25, 0.5, 1, 2, 4, and 6 µmo l/l) were respectively added for 12, 24, and 36 hours, respectively. In combination group, SAHA (1, 2, and 4 µmo l/l) and DDP (1, 2, and 4 µg/ml) were respectively added for 24 hours. There were four wells for each dose. Control group were not administered any drugs. Blank control group had only culture solution. Optical density (OD) value for each well was measured with a microplate reader. The inhibition rate of cell proliferation (%) = (OD value of control group-OD value of experimental group) / OD value of control group×100%. The effects of DDP combined with SAHA were evaluated with the method described by Cao [7].

Cell cycle measured with flow cytometer

There were four groups including control group without any drugs; S groups in which cells were treated with 1, 2 and 4 µmo l/l of SAHA, respectively, for 24 hours; D group in which cells were treated with 1, 2, and 4 mg /ml of DDP, respectively, for 24 hours; and SD group in which cells were treated with 1, 2, and 4 µmo l/l of SAHA for 24 hours, and then 1, 2, and 4 mg /ml of DDP were respectively added. Cells were centrifuged at 800-1,000 r/min for five minutes to get rid of supernatant. After washed three times with PBS, about 1×10^6 cells were prepared into suspensions with one ml of PBS. Cells were fixed with 70% ice alcohol, and then 50µl of RNase (one mg/ml) and 50µl of propidium iodide (400µg /ml) were added for 30 minutes in the dark at 4°C. Cells were filtered followed by analysis with ModFitLTV3.0 (PMac) software. Cell cycles (G0, S and G2/M) were calculated, and then cell proliferation index (PI) in each group was evaluated by the formula PI = (G2/M+S) / (G1/G0+S+G2/M).

Apoptosis rates determined with flow cytometry

There were four groups including group A in which cells were respectively treated with 1, 2, and 4 mol/l of SAHA for 24 hours; group B in which cells were respectively treated with 1, 2, and 4 µg /ml of DDP for 24 hours; group C in which cells were first treated with 1, 2, and 4 mol/l of SAHA for 24 hours, respectively, and then with 2 µg /ml of DDP for 24 hours; group D in which cells were first treated with 2 mol/l of SAHA for 24 hours, and then with 1, 2, and 4 µg /ml of DDP, respectively, for 24 hours. Apoptosis rates were determined with PI/AnnexinV-FITC double labeling method. FITC-/PI- was regarded as living cells, FITC+/PI- as apoptotic cells, FITC+/PI+ as necrotic cells and FITC-/PI+ as mechanical injured cells. Testing was performed in triplicate for each group.

SiHa cells treated with SAHA combined with radiation

There were four groups including control group in which nothing was done, SAHA group in which cells were treated with SAHA of different concentrations (0.5, 1, 2, 4, 6, and 8 umol/l) for 24 hours, irradiation group in which cells underwent radiation of various doses (2, 4, 6, and 8 Gy), combination group in which cells underwent radiation of various doses (2, 4, 6, and 8 Gy) after treated with 20% IC50 of SAHA (0.96 µmol /l) for 24 hours. After irradiation, cells were digested with 0.25% of trypsin, and then counted after trypan blue staining. Cells were seeded in 60 mm-culture dish. After culture, cells were washed with PBS twice, fixed with 100% of methanol for 30 minutes, stained with crystal violet for 15 minutes, washed with double distilled water three times, followed by counting the number of colonies containing more than 50 cells. The survival fraction (SF) was calculated based on the following formulas: colony forming efficiency = the number of colony formation/ the number of seeded cells ×100%, SF = the number of colonies in each group/(the number of seeded cells in this group × colony forming efficiency). Based on the single-hit multi-target (SHMT): $S = 1 - (1-eDo/D)^n$, $Dq = Do\cdot\log N$, the radiological dose-survival curve was drawn. And then the extrapolation number (N), mean lethal dose (D_0), quasi-threshold (D_q) and sensitization enhancement ratio (SER) was calculated (SER = Do value in radiation alone group/Do value in combination group).

mRNA expression of P21, Bax and Ku70 in SiHa cells

Total mRNA was extracted with Trizol from SiHa cells. cDNA was obtained by reverse transcription with 2 µl of total RNA and M-MLv inverse transcriptase. PCR was performed with GAPDH as internal control. PCR conditions were as follows: pre-denaturing at 95°C for two minutes, denaturing at 95°C for 15 seconds, reannealing and elongation at 60-68°C for 20-60 seconds, 15 cycles. Obtained GAPDH fragment was 288 bp, P21 123 bp, Bax 126 bp, and Ku70 478 bp. The primer sequences were as follows:

GAPDH: 5'- GGCATCGTGATGGACTCCG-3'
5'- GCTGGAAGGTGGACAGCGA-3'
p2l: 5'- AAGACCATGTGGACCTGTCACTGT-3'
5'- GAAGATCAGCCGGCGTTTG-3'
Bax: 5'-GCGAGTGTCTCAAGCGCATC-3'
5'-CCAGTTGAAGTTGCCGTCAGAA-3'
Ku70: 5'- CTGTGCCAACCTCTTTAGTGATG -3'
5'- TGGTTCATTTGTTTCCCGATA -3'

Protein expression of P21, Bax and Ku70 in SiHa cells

RIPA (200 u1) was added in Siha cells for 30 minutes followed by centrifugation at 12,000 r/min for 30 minutes at 4°C. The supernatant underwent SDS-PAGE. Membrane blocking was performed, and then antibodies were incubated and stained.

Statistical analysis

Statistical treatment was performed with SPSS13.0 software. Measurement data were expressed as $\bar{x}+s$. Normal distribution test was performed in all measurement data. Variance analysis and corresponding *t* test were used in the measurement data which were in line with normal distribution and Wilcoxon nonparametric test was used in the measurement data which were no consistent with normal distribution. Statistical significance was established at $p < 0.05$.

Results

Effects of SAHA combined with DDP on SiHa cell proliferation

With the increase in the dose of SAHA, the inhibition rate for SiHa cells was increased. Based on Q = E(AB)/(EA+EB-EA×EB) [7], the Q values of 1 µg/ml DDP combined with 1, 2, and 4 µmol/l SAHA were 1.63, 1.54, and 1.46, respectively; and the Q values of 2 µg/ml DDP combined with 1, 2, and 4 µmol/l SAHA were 1.31, 1.28, and 1.19, respectively. SAHA combined with moderate or low dose of DDP (1 µg/ml and 2 µg/ml) exhibited synergistic effects, but it combined with higher dose (4 µg/ml) of DDP additive effects. The IC50 of SAHA was 4.80 µmol/l. The dose of 20% IC50 of SAHA (0.96 µmol /l) was used in radiosensitization test.

Table 1. — *Effects of DDP combined with SAHA on SiHa Cell Cycle ($\bar{x} \pm s$, n =4).*

Group	Cell cycle (%)			
	G0/G1	G2/M	S	PI
Control	36.05±1.44	27.97±3.21	35.98±1.22	63.95±1.72
S1	47.69±1.14	26.24±2.80	26.07±1.98	52.31±1.38#
S2	50.57±2.53	16.51±0.53	32.92±0.44	49.43±3.57#
S4	54.45±1.20	14.78±3.21	30.77±1.42	45.55±1.28#
D1	55.49±2.87	24.26±2.09	20.25±0.77	44.51±1.54#
D2	58.20±0.77	2.00±1.89	41.80±0.52	42.94±0.87#
D4	58.44±2.43	13.85±1.80	27.72±3.11	41.57±1.39#
S1+D1	57.46±2.00	2.38±0.59	40.16±1.29	42.54±0.69#△
S1+D2	61.67±0.97	10.56±3.34	27.76±2.13	38.32±1.22#△▲
S1+D4	63.78±0.41	7.85±1.46	28.37±0.92	36.22±0.99#△▲
S2+D1	61.44±0.16	5.13±2.33	33.42±1.02	38.55±0.75#△▲
S2+D2	63.51±0.25	15.13±1.12	21.36±2.44	36.49±1.23#△▲
S2+D4	64.43±3.22	9.65±3.78	25.92±1.37	35.57±0.98#△▲
S4+D1	62.74±2.66	7.88±2.55	29.38±0.71	37.26±1.22#△▲
S4+D2	63.89±1.63	10.94±1.91	25.17±0.85	36.11±0.98#△▲
S4+D4	65.24±1.17	10.25±2.11	24.50±1.13	34.75±1.77#△▲

#: vs control group, $p < 0.05$; △ : vs S group, $p < 0.05$; ▲ : vs D group, $p < 0.05$;

Effects of DDP combined with SAHA on SiHa cell cycle

Flow cytometry indicated that compared with control group, the number of cells in G0/G1 phase was increased, and the number of cells in G2/M+S phase was reduced and PI was decreased in S, D, and SD groups ($p < 0.05$). Compared with S and D groups, the number of cells in G0/G1 phase was increased, and the number of cells in G2/M+S phase was reduced and PI was decreased in SD group ($p < 0.05$, Table 1).

Effects of DDP combined with SAHA on SiHa cell apoptosis

Compared with control group, apoptosis was increased in 4 μmol/l SAHA group ($p < 0.05$). The apoptosis rates were higher in SD groups than in S or D groups ($p < 0.05$). After SiHa cells were treated with 2 μg/ml of DDP, apoptosis was not marked, but these cells were also treated with SAHA, apoptosis was significantly increased and with the increase in the dose of SAHA, the apoptosis rate was also increased ($p < 0.05$, Figure 1).

Table 2. — *Cell survival fraction in each group ($\bar{x} \pm s$).*

Radiological dose	SF of radiation group	SF of combination group
0 Gy	1.00	1.00
2 Gy	0.750±0.044▲☆	0.645±0.114▲☆
4 Gy	0.480±0.022▲☆	0.160±0.021▲☆
6 Gy	0.160±0.011▲☆	0.043±0.008▲☆
8 Gy	0.065±0.004▲☆	0.009±0.002▲☆
F	463.942	77.705
p	0.000	0.000

▲: indicates $P<0.05$, compared with SF of different doses in the same group.
☆: indicates $P<0.05$, compared with SF of different groups in the same dose.
SF: survival fraction; Combination group: SAHA combined with radiation

Effects of radiation combined with SAHA on the clone formation of SiHa cells

The number of colonies in radiation groups including 0, 2, 4, 6, and 8 Gy were 120, 225, 576, 480, and 778, and in combination groups were 95, 152, 152, 102, and 163, respectively. SF in each group is shown in Table 2. There were significant differences in SF between different radiological doses ($p < 0.05$). With the increase in radiological doses, SF was gradually decreased. In the same radiological dose, SF was significantly lower in combination group than in radiation alone group ($p < 0.05$). SHMT models was drawn using SigmaPlot 2000 Demo software with radiological doses as X axis and with SF as Y axis (Figure 2). Compared with radiation group, the curve of combination group moved left and was relatively flat without marked "shoulder area". Do, N, and Dq values were 2.329, 2.761, and 1.721 in radiation group and 1.213, 4.770, and 0.823 in combination group. SER was 1.92.

Effects of SAHA combined with DDP or radiation on mRNA expressions of P21, Bax and Ku70 in SiHa cells

Compared with control group, there was no statistical significance in Bax mRNA expression in groups S and D ($p > 0.05$). However, Bax mRNA expression was higher in SD group than in other groups ($p < 0.05$). P21 mRNA expression was up-regulated in all groups, and was higher in SD group than in other groups ($p < 0.05$, Table 3).

Compared with control group, there was no statistical significance in Bax mRNA expression of SAHA group ($p >$

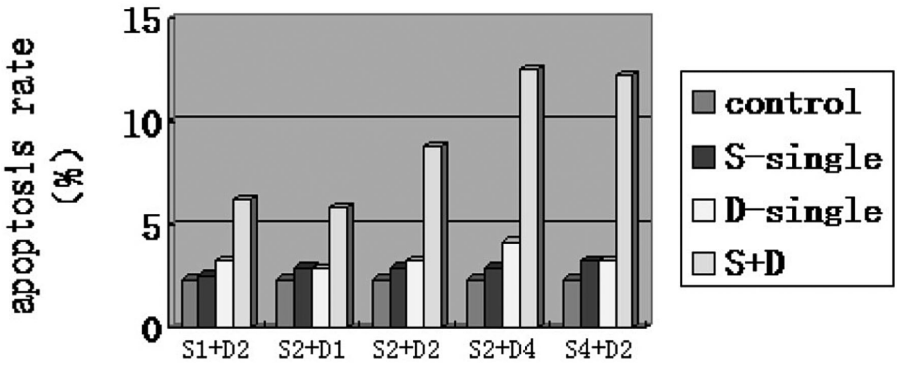

Figure 1. — Effects of DDP, SAHA, and DDP combined with SAHA on apoptosis rates of SiHa cells.

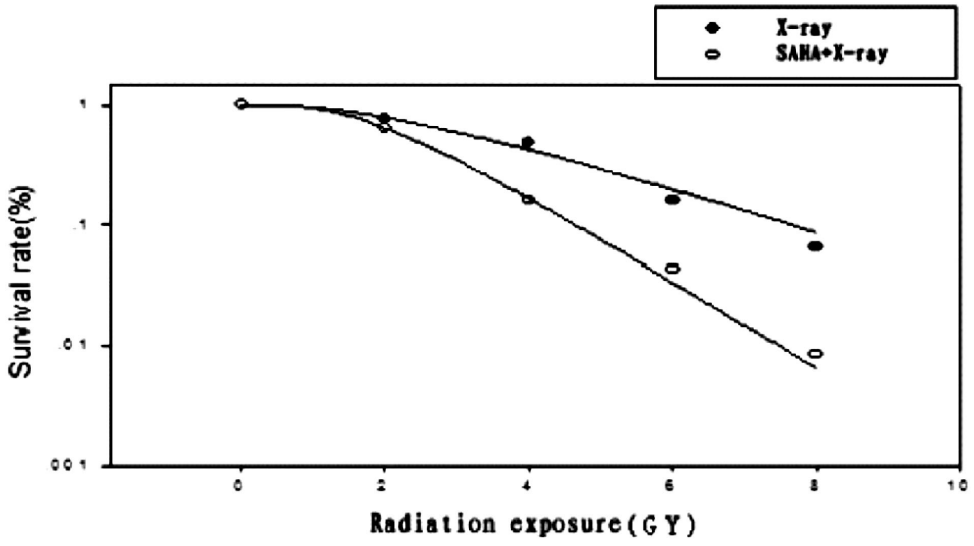

Figure 2. — Cell survival curves of radiation alone groups and SAHA combined with radiation groups.

Table 3 — *mRNA and protein expressions of Bax and P21 in S, D, and SD Groups ($\bar{x} \pm s$, n=3).*

Groups	Bax expressions		P21 expressions	
	$mRNA2^{-\triangle\triangle Ct}$	Protein	$mRNA2^{-\triangle\triangle Ct}$	Protein
Control	1.00±0.00	1.00±0.00	1.00±0.00	1.00±0.00
S	1.16±0.22	1.37±0.25	4.78±0.86#	2.69±0.16#
D	1.41±0.54	2.07±0.14#	10.77±1.34#	2.31±0.21#
SD	4.46±0.48#△	2.63±0.18#△	15.54±1.12#△	4.18±0.12#△

# indicates P <0.05, compared with control group.
△ indicates P <0.05, compared with other groups.
S group: SAHA group; D group: DDP group;
SD group: SAHA combined with DDP group.

Table 4. — *mRNA and protein expressions of Bax and Ku70 in SAHA, radiation, and combination group (±s, n=3).*

Groups	Bax expressions		Ku70 expressions	
	$mRNA2^{-\triangle\triangle Ct}$	Protein gray value	$mRNA2^{-\triangle\triangle Ct}$	Protein gray value
Control	1.14±0.17	7631.19.±547.12	1.17±0.09	7486.256±648.72
SAHA	1.78±0.74	8122.88±482.40	0.26±0.34#	3991.12±241.35#
Radiation	5.77±1.12#	16541.69±600.08#	6.38±1.25#	18752.12±929.60#
Combination	10.25±1.38△	24345.01±859.13#△	2.33±0.86☆	9559.90±845.58☆

#: indicates P<0.05, compared with control group.
△: indicates P<0.05, compared with other groups.
☆: indicates P<0.05, compared with radiation group.
Combination group: SAHA combined with radiation.

0.05). However, Bax mRNA expression was higher in radiation group than in control group and in combination groups than in other groups ($p < 0.05$). Ku70 mRNA expression was lower in SAHA group than in control group, and in combination group than in radiation group; but was higher in radiation group than in control group ($p < 0.05$, Table 4).

Effects of SAHA combined with DDP or radiation on protein expressions of P21, Bax and Ku70 in SiHa cells

Western blot indicated that compared with control group, there was no statistical significance in Bax protein in S group ($p > 0.05$), but Bax protein expression was higher in D group than in control group, and in SD groups than in S, D, or control groups ($p < 0.05$); P21 protein expression was higher in S, D, and SD groups than in control group, and in SD group than in other groups ($p < 0.05$). The results of Western blot were consistent with that of PCR (Table 3 and Figure 3). In SAHA combined with radiation, there was no statistical significance in Bax protein expression in SAHA group ($p > 0.05$), but Bax protein expression was higher in radiation group than in control group, and in SAHA combined with radiation group than in other groups ($p < 0.05$). Ku 70 protein expression was lower in SAHA group than in control group, and in SAHA combined with radiation group than in radiation group ($p < 0.05$), but higher in radiation group than in control group ($p < 0.05$, Table 4 and Figure 4).

Discussion

The incidence of cervical cancer takes the second place in women cancer [11]. About 78% of patients with cervical cancer are in developing countries where cervical cancer takes the second place in the leading cause of female cancer death. In recent years, its incidence is growing, and the age at onset is younger than ever [7]. Since young patients require high postoperative life quality, treatment methods for cervical cancer remain to be further improved. At present, radiotherapy and surgery are mainly used in treatment of cervical cancer. However, the recurrence rate of cervical cancer is as high as 35%. For the patients with moderate, advanced, recurrent or metastatic cervical cancer, radiotherapy is one of main treatment methods, but chemotherapy is also necessary, so it attracts more and more attention [12].

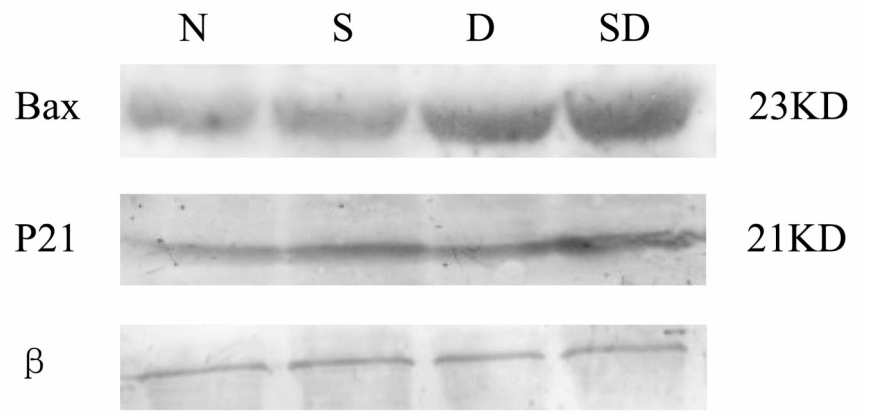

Figure 3. — Protein expressions of Bax and P21 in control, SAHA, DDP, and SAHA combined with DDP groups. N: control group; S: SAHA group; D: DDP group; SD: SAHA combined with DDP group.

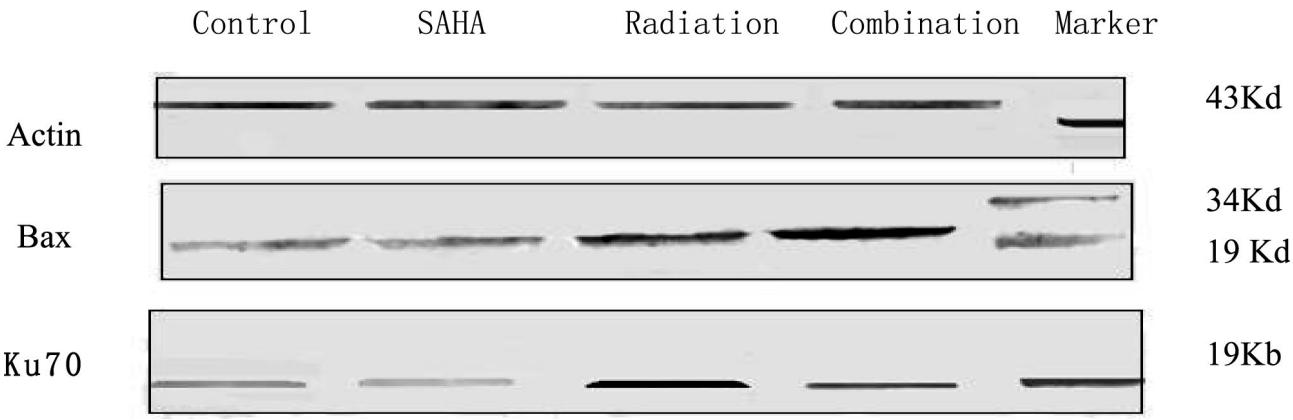

Figure 4. — Protein expressions of Bax and Ku70 in control, SAHA, radiation and SAHA combined with radiation groups. Combination: SAHA combined with radiation group.

Sodium phenylbutyrate has good effects on cervical cancer cells in vitro [13]. It can inhibit the growth of cervical cancer cells and induce the apoptosis of cervical cancer cells. It is reported that HDACi combined with chemotherapeutics can restore the sensitivity of tumor cells to chemotherapeutics, obtaining synergistic anti-tumor effects [14]. SAHA, a kind of HDACi, has proved to be effective for many tumors and has been applied in phase II clinical trial [15]. SAHA re-activates the expression-blocked genes through histone acetylation, inducing tumor cell apoptosis.

Platinum-based chemotherapeutics play an important role in preoperative adjunctive treatment for bulky cervical cancer, radiosensitization, and improvement of prognosis of the patients with advanced or recurrent cervical cancer. Although the target of DDP is different from that of SAHA, the changes in DNA are closely associated with that in chromatin during tumorigenesis, therefore DDP combined with SAHA play stronger anti-tumor effects probably through DNA and chromatin. Therefore, in this study, SAHA combined with DDP was used in cervical cancer SiHa cells in order to obtain synergetic effects.

In this study, the authors used 20% IC50 of SAHA in radiosensitization experiments. Do value of SAHA-pretreated cells was decreased, suggesting that cellular sublethal damage repair was inhibited.

In this study, SAHA combined with moderate or low dose of DDP exhibited synergistic effects, but it combined with higher dose of DDP additive effects. The number of SiHa cells in G0/G1 phase was higher in SAHA combined with DDP group than in DDP alone ($p < 0.05$), suggesting that SAHA and DDP had synergistic effects.

This study indicated that SAHA combined with DDP could up-regulate the expressions of P21 and Bax genes, and SAHA combined with radiation could up-regulate Bax expression and down-regulate Ku70 expression. P21 is an inhibiting factor of cyclin dependent protein kinase, Bax an apoptotic factor and Ku70 a DNA repair gene. SAHA can inhibit HDAC activity, increasing the expression levels of P21 and Bax genes. P21 and Bax can induce tumor cell differentiation and (or) apoptosis, inhibiting tumor cell proliferation.

DNA is a main target molecular of radiotherapy. DNA damage includes double-strand break, single-strand break,

base damage and protein crosslink. Double-strand break is strongly associated with radiosensitivity. DNA repair system plays a crucial role in radiation resistance. Ku70 is a main repair protein for DNA double-strand break. The radiosensitizing effect of HDACi is closely related to that it can inhibit radiation-sublethal damage repair gene. Munshi *et al.* [16] have found that NaB can significantly decrease the expressions of repair-related factors such as Ku70, Ku80, and DNA-dependent protein kinase catalytic subunit, exhibiting a radiosensitizing effect. Subramanian *et al.* [17] have reported that HDAC6-specific inhibitor may induce Ku70 acetylation, Bax release, and neuroblastoma cell death. Cheng *et al.* [18] described that HDAC inhibitors, HDAC42, MS-275, and TSA can induce Ku70 protein acetylation, inhibiting the repair of DNA double-strand break. In this study, Ku70 expression was higher in radiation alone group than in control group ($p < 0.05$); was lower in SAHA alone group than in control group ($p < 0.05$), and in SAHA combined with DDP group than in radiation alone group ($p < 0.05$). Based on above data, it can be seen that the radiosensitizing effect of SAHA is achieved by down-regulating Ku70 expression, a radiation sub-lethal damage repair gene.

In chemotherapy, all chemotherapeutics have the function to induce tumor cell apoptosis or tumor necrosis. Cell cycle arrest is closely associated with cell apoptosis and differentiation [19]. The present authors found that the cytotoxicity against tumor cells was stronger in SAHA combined with DDP than in either of both alone. The mechanism may be that although the low dose of SAHA fails to markedly kill tumor cells, can quickly increase the level of histone acetylation, which can cause the DNA in chromatin to be fully exposed, promoting the cross-linking of DDP with DNA and enhancing DDP cytotoxicity against tumor cells.

The limitations in this study were that the authors only studied the effects of SAHA combined with chemotherapeutics or radiation on SiHa cells in vitro, hence, in vivo studies on cell line SiHa and other cervical cancer cell lines remain to be further carried out.

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