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Salinomycin enhances radiotherapy sensitivity and reduces expressions of BIRC5 and NEIL2 in nasopharyngeal carcinoma

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Abstract. – OBJECTIVE: The aim of this study was to investigate the effects of salinomycin (Sal) on expressions of baculoviral IAP repeat-containing 5 (BIRC5) and Nei endonuclease VIII-like 2 (NEIL2) and radiotherapy sensitivity of nasopharyngeal carcinoma (NPC).

MATERIALS AND METHODS: Human NPC CNE-2 cell lines were used as research objects in this study. Subsequently, the cells received intervention with Sal at different concentrations, radioactive rays at different doses and Sal combined with radioactive rays. The growth inhibition rate of CNE-2 cells was detected via methylthiazolyl tetrazolium (MTT) assay. The dose-effect relations of Sal, radioactive rays and combination therapy with the inhibitory effect on CNE-2 cells were obtained. CNE-2 cells receiving intervention with Sal at an appropriate concentration or radioactive rays at appropriate dose alone and Sal combined with radioactive rays were used as intervention groups (Control group, Radiation group and Combination group). However, those added with an equal amount of DMSO were set as Control group. Next, the cell cycle, apoptosis and apoptotic morphology of CNE-2 cells were observed *via* flow cytometry and Hoechst assay, respectively. Moreover, the expressions of apoptosis-related proteins Caspase-3, B-cell lymphoma-2 (Bcl-2) and Bcl-2 associated X protein (Bax), as well as BIRC5 and NEIL2 proteins in CNE-2 cells were determined using Western blotting.

RESULTS: Under the intervention with Sal or radioactive rays alone, the growth inhibition rate of CNE-2 cells in a concentration/dose-dependent manner. With the increase in Sal concentration in combination therapy, the growth inhibition rate of CNE-2 cells significantly increased ($p<0.05$). Compared with Control group, Sal group, Radiation group, and Combination group exhibited remarkably lower colony formation rate, higher proportion of CNE-2 cells in the G2/M phase, enhanced apoptosis of CNE-2 cells with nuclear fragmentation, increased expressions of pro-apoptotic proteins Caspase-3 and Bax, decreased expression of anti-apoptotic protein Bcl-2, and lower protein expressions of BIRC5 and NEIL2 cells ($p<0.05$). Compared with Radiation group, Combination group had significantly decreased colony formation rate, increased proportion of CNE-2 cells in the G2/M phase, enhanced apoptosis of CNE-2 cells with more nuclear fragmentation and other apoptosis characteristics, increased expressions of pro-apoptotic proteins Caspase-3 and Bax, decreased expression of anti-apoptotic protein Bcl-2, and decreased protein expressions of BIRC5 and NEIL2 in cells ($p<0.05$).

CONCLUSIONS: Sal enhances the radiotherapy sensitivity of NPC and reduces the protein expressions of BIRC5 and NEIL2 in cells.

Key Words:
Salinomycin, Nasopharyngeal carcinoma (NPC), CNE-2, BIRC5, NEIL2.

Introduction

Nasopharyngeal carcinoma (NPC) is a highly invasive and metastatic malignant tumor of nasopharynx, whose morbidity rate is high in southern China and Southeast Asia[1,2]. NPC patients are usually sensitive to radiotherapy. Therefore, radiotherapy is considered as the major treatment for NPC[3]. In recent years, the survival rate of NPC patients has been greatly prolonged with the development of radiotherapy and radiotherapy combined with chemotherapy. The latest statistical data have shown that the 5-year overall survival is nearly 85%. However, high recurrence rate and proneness to distant metastasis greatly limit the application of radiotherapy in NPC treatment. Meanwhile, the prognosis of patients with advanced NPC is still far from satisfactory[4-6]. Radiotherapy resistance is a major cause

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of treatment failure, which is also a major predisposing factor for recurrence and metastasis. Therefore, reducing radiotherapy resistance in effective ways and improving radiotherapy sensitivity are of great importance for the treatment and prognosis of NPC patients[7].

In the research on therapeutic drugs for breast cancer, it has been found that the anti-tumor effect of small molecule compound salinomycin (Sal) is nearly a hundred times stronger than that of commonly-used drugs[8]. Sal exerts an effective anti-cancer effect in lung cancer, ovarian cancer, gastric cancer and liver cancer, which can reduce tumor growth and metastasis[9-11]. In addition, Sal enhances the sensitivity of tumor cells to chemotherapeutic drugs and radiotherapy by arresting cell cycle in the G_2 phase and destroying deoxyribonucleic acid (DNA). However, the efficacy of Sal combined with radiotherapy in NPC has not been clarified yet[12]. Baculoviral IAP repeat-containing 5 (BIRC5) is an oncogene that can inhibit apoptosis and promote cell proliferation, which is regulated by P53. Nei endonuclease VIII-like 2 (NEIL2) is the coding gene of DNA repair enzyme. Mandal et al[13] have demonstrated that it promotes DNA repair, reduces the sensitivity to chemotherapeutic drugs and produces tolerance of tumor cells. The expressions of BIRC5 and NEIL2 in NPC tissues are significantly higher than those in nasopharyngitis tissues. However, they are prominently lower in patients sensitive to radiotherapy than those in patients insensitive to radiotherapy. These findings indicate that BIRC5 and NEIL2 are key factors of NPC recurrence and radiotherapy resistance. Therefore, the associations of BIRC5 with radiotherapy sensitivity and expression of BIRC5 and NEIL2 in NPC cells were preliminarily explored in this study. All our findings might help provide theoretical and practical basis for the treatment of NPC.

Materials and Methods

Materials

Cell lines: Human NPC CNE-2 cell lines were purchased from Shanghai Institutes for Life Sciences (Shanghai, China). The reagents were: Roswell Park Memorial Institute (RPMI)-1640 medium, fetal bovine serum (FBS), penicillin and streptomycin purchased from Gibco (Rockville, MD, USA), Sal, methyl thiazolyl tetrazolium (MTT) and dimethylsulfoxide (DMSO) from Sigma-Aldrich (St. Louis, MO, USA), cell cycle assay kit and Annexin V- fluorescein isothiocyanate (FITC) apoptosis assay kit from Becton Dickinson (Franklin Lakes, NJ, USA), Hoechst 33342 staining solution from Beyotime Biotechnology (Shanghai, China), Caspase-3, B-cell lymphoma-2 (Bcl-2), Bcl-2 associated X protein (Bax), BIRC5, NEIL2 and β-actin antibodies from Abcam (Cambridge, MA, USA), and horseradish peroxidase (HRP)-labeled goat anti-rabbit/mouse secondary antibodies from Applygen (Beijing, China).

Cell Culture and Drug Intervention

Human NPC CNE-2 cells were cultured in RPMI-1640 medium containing 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin in an incubator with 5% CO_2 and saturated humidity at 37°C. When the cells covered 95% of the bottle bottom, they were digested with 0.25% trypsin. Subsequently, the cells were added with fresh medium and pipetted into single suspended cells, followed by cell passage in a new culture flask. The cells in the logarithmic growth phase were selected for the following experiments. CNE-2 cells received intervention with Sal at different concentrations, radioactive rays at different doses and Sal combined with radioactive rays for 48 h. The growth inhibition rate of CNE-2 cells was detected. The effects of Sal at an appropriate concentration or radioactive rays at an appropriate dose alone and Sal combined with radioactive rays on cycle and apoptosis of CNE-2 cells, as well as the expressions of BIRC5 and NEIL2 were determined. Radiation method: at room temperature, the linear accelerator (SI-MENS PM) emitted 6 MV X-rays at 100 cm away from the skin (radiation field: about 10 cm × 10 cm, dose rate: 200 cGy/min).

Detection of Cell Proliferation Via MTT Assay

CNE-2 cells in the logarithmic growth phase were inoculated into 96-well plates at a density of 5×10^3/well. After adherence for 24 h, the cells were added with Sal at different concentrations (0, 1, 2, 4, 8 and 16 μM), irradiated with radioactive rays at different doses (0, 2, 4, 6 and 8 Gy), and cultured for 48 h. Later, the cells were added with 90 μL of fresh medium and 10 μL of MTT solution, followed by incubation in an incubator with 5% CO_2 and saturated humidity at 37°C for 4 h. After discarding the supernatant, 150 μL of DMSO was added into each well to fully dissolve the crystals under low-speed oscillation. Absor-

bance (A) of each well at 570 nm was measured using a micro-plate reader. Cell growth inhibition rate = (1 - $A_{\text{experimental well}}$/$A_{\text{control well}}$) × 100%.

Detection of Cell Cycle Using Flow Cytometry

CNE-2 cells in the logarithmic growth phase were inoculated into 6-well plates at a density of 3×10^5/well. When covering 70% of the bottle bottom, the cells were divided into Control group, Sal group (4 μM), Radiation group (6 Gy) and Combination group for intervention. After 48 h, the cells were collected, fixed with 70% pre-cooled ethanol overnight and washed with pre-cooled phosphate-buffered saline (PBS). Next, the cells were stained with 300 μL of staining solution containing 100 μg/mL RNase A, 50 μg/mL propidium iodide (PI) and 0.2% Triton X-100 at room temperature for 30 min in the dark. Finally, cell cycle was detected using a flow cytometer.

Detection of Apoptosis Via Flow Cytometry

CNE-2 cells in the logarithmic growth phase were inoculated into 6-well plates at a density of3×10^5/well. When covering 70% of the bottle bottom, they were divided into Control group, Sal group (4 μM), Radiation group (6 Gy), and Combination group for intervention. After 48 h, the cells were collected, washed with pre-cooled PBS and suspended in 300 μL of buffer. Next, the cells were reacted with 5 μL of Annexin V-FITC and 5 μL of PI for 15 min in the dark. Cell apoptosis was finally detected using via flow cytometry.

Hoechst Apoptosis Assay

CNE-2 cells in the logarithmic growth phase were first seeded into 6-well plates (3×10^5/well). When 70% of the bottle bottom was covered, the cells were divided into three groups for intervention, including: Control group, Sal group (4 μM), Radiation group (6 Gy) and Combination group. 48 h later, cells were collected, washed with pre-cooled PBS and fixed with 4% paraformaldehyde solution for 15 min. After staining with 5 mg/mL Hoechst 33342 dye for 5 min in dark, the cells were washed twice with PBS. Morphological changes in apoptotic nuclei were finally observed under a fluorescence microscope.

Western Blotting Assay

CNE-2 cells in each group were collected and lysed with RIPA lysis buffer, from which total protein was extracted. The concentration of extracted protein was measured *via* the bicinchoninic acid (BCA) colorimetry (Beyotime, Shanghai, China). Prepared protein samples were mixed with sodium dodecyl sulphate (SDS) loading buffer and boiled at 95°C for 3 min. After separation *via* 8-10% polyacrylamide gel electrophoresis (Applygen, Beijing, China), the total proteins were transferred onto polyvinylidene difluoride (PVDF) membranes. Next, the membranes were sealed with 10% skimmed milk and incubated with primary antibodies on a shaking table at 4°C overnight. On the next day, the membranes were washed with Tris-buffered saline and Tween-20 (TBST) for 3 times and incubated with corresponding secondary antibodies on a shaking table at room temperature for 1 h. After that, the membranes were washed again with TBST for 3 times. Immuno-reactive bands were exposed by the enhanced chemiluminescence (ECL) method. The relative expression levels of target proteins were finally analyzed using Image J software (NIH, Bethesda, MD, USA).

Colony Formation Assay

CNE-2 cells in the logarithmic growth phase were inoculated into 6-well plates at a density of 3×10^5/well. Subsequently, they were divided into Control group, Sal group (4 μM), Radiation group (6 Gy), and Combination group for intervention when covering 70% of the bottle bottom. After about 2 weeks, the visible colonies were washed with PBS for 3 times and fixed with 100% ethanol for 10 min. After discarding the ethanol, formed colonies were stained with 2 mL of 1× crystal violet for 20 min, washed with natural water, and dried at room temperature. The colonies were then analyzed using cytotoxic T lymphocytes (CTL), and one colony was defined as ≥50 cells. Colony formation rate = (number of colonies/number of cells inoculated) × 100%.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 software (IBM Corp., Armonk, NY, USA) was used for all statistical analysis. Measurement data were expressed as mean ± standard deviation. Differences between the two groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ was considered statistically significant.

Table I. Growth inhibition rate of CNE-2 cells under different treatment conditions (%, $\bar{x} \pm s$).

Dose (Gy)	Sal (μM)					
	0	1	2	4	8	
0	0.0 ± 0.0	9.5 ± 1.5	12.5 ± 3.5	22.4 ± 2.6	36.2 ± 1.7	.7 ± 7.2
2	13.6 ± 2.2	14.4 ± 3.2	15.6 ± 2.4	23.5 ± 1.6	33.5 ± 3.	43.8 ± 4.8
4	18.1 ± 1.6	19.2 ± 2.6	27.4 ± 3.6	37.7 ± 2.8	41.1 ± 5.	9 ± 1.9
6	29.4 ± 2.5	32.2 ± 2.5	34.5 ± 1.5	50.1 ± 3.8	55. ± 4.1	± 3.8
8	51.2 ± 3.1	52.5 ± 3.5	58.3 ± 1.8	66.6 ± 2.9	3.9	

Results

Cell Growth Inhibition In Each Group

After the cells were reacted with Sal at different concentrations (0, 1, 2, 4, 8 and 16 μM) and irradiated with radioactive rays at different doses (0, 2, 4, 6 and 8 Gy), the growth inhibition of CNE-2 cells was detected *via* MTT assay (Table I). The results indicated that under the intervention with Sal at different concentrations or radioactive rays at different doses alone, the growth inhibition rate of CNE-2 cells rose in a concentration/dose-dependent manner. In other word[illegible] growth inhibition of CNE-2 cells becam[illegible] evident with the increase in concentratio[illegible]se (p<0.05). With the increase in Sal concentra[illegible] in combination therapy, the growth inhibition r[illegible] of CNE-2 cells increased sig[illegible]y as we[illegible] (p<0.05).

Changes In Colony F[illegible]ation Each Group

CNE-2 cells in[illegible]ntrol g[illegible] Sal group (4 μM), Radiati[illegible]roup (6 Gy[illegible]d Combination group[illegible]ected to [illegible]vention. After 2 week[illegible] the c[illegible] formation rate was determin[illegible]he results [illegible]d that Sal group, Radiati[illegible]group and Com[illegible]ion group had signi[illegible]tly lo[illegible] colony formation rate than Co[illegible] gro[illegible]p<0.05). Furthermore, Combinatio[illegible]ad re[illegible]ably decreased colony [illegible]atio[illegible] co[illegible]ed with Radiation group ([illegible]05) (F[illegible]

C[illegible]n Cell Cycle In Each Group

[illegible]s in Control group, Sal group (4 [illegible]M), Radiation group (6 Gy) and Combination [illegible]p received intervention, and cell cycle was de[illegible]d using flow cytometry after 48 h. The results demonstrated that Sal group, Radiation group and Combination group exhibited significantly higher proportion of CNE-2 cells in the G_2/M phase than C[illegible]nt[illegible]p<0.05). Moreover, Combinati[illegible]oup [illegible]sted si[illegible]icantly increased pro[illegible]on of CNE[illegible]ll[illegible]he G_2/M phase com[illegible]h Radiatio[illegible]p (p<0.05) (Figure 2).

Cell[illegible]tosis In Ea[illegible]Group

[illegible]er intervention in C[illegible]E-2 cells in Control [illegible]p, Sal group [illegible]μM), Radiation group (6 Gy) [illegible] Combinatio[illegible]oup for 48 h, cell apoptosis w[illegible]amined v[illegible]ow cytometry. The apoptosis rate[illegible]l g[illegible] Radiation group and Combination g[illegible]as (48.35±2.52)%, (44.26±4.63)% [illegible]nd (83.52±6.23)%, respectively, which was [illegible]antly elevated in comparison with Con[illegible]p [(9.45±0.94)%] (p<0.05). Meanwhile, it was also remarkably higher in Combination group than that in Radiation group (p<0.05) (Figure 3). Besides, the apoptotic morphology was observed through Hoechst assay. As shown in Figure 4, the nuclei were regular and uniform

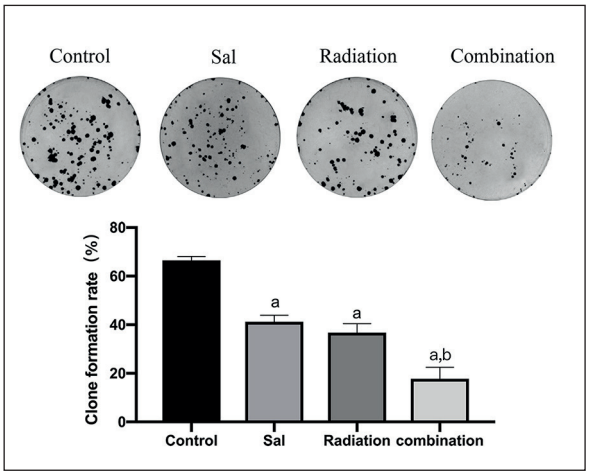

Figure 1. Colony formation rate of CNE-2 cells under different treatment conditions analyzed *via* CTL, (magnification: 40×) Note: [a]p<0.05 *vs.* Control group, [b]p<0.05 *vs.* Radiation group.

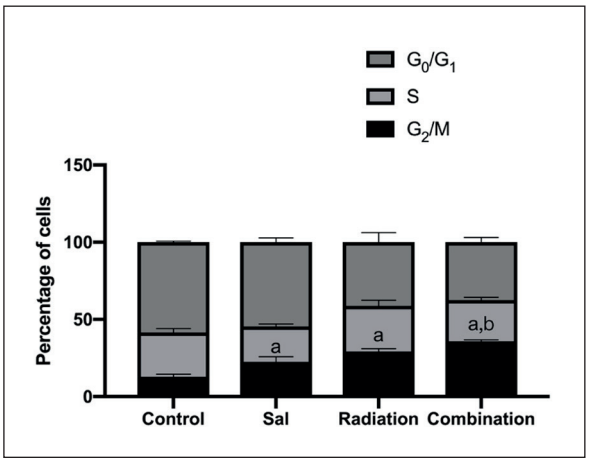

Figure 2. CNE-2 cell cycle under different treatment conditions detected *via* flow cytometry. Note: [a]$p<0.05$ *vs.* Control group, [b]$p<0.05$ *vs.* Radiation group.

in Control group. Sal group, Radiation group and Combination group had nuclear fragmentation and other apoptosis characteristics, more evidently in Combination group.

Changes In expressions of Apoptosis-Related Proteins Each Group

After intervention in CNE-2 cells control group, Sal group (4 μM), Radiation group and Combination group 48 h, the expressions of apoptosis-related proteins were determined using Western blotting. As shown in Figure 5, compared with Control group, Sal group, Radiation group and Combination group displayed significantly up-regulated expressions of Caspase-3 and Bax, and decreased expression of Bcl-2 ($p<0.05$). Compared with Radiation group, Combination group exhibited

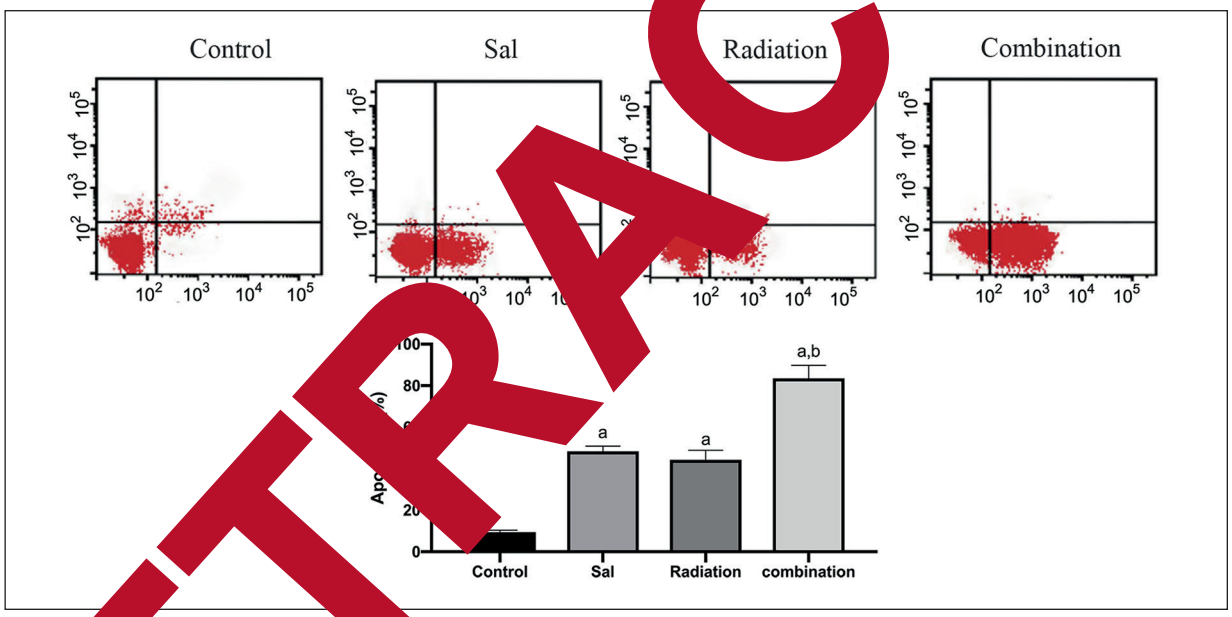

Figure Apoptosis of CNE-2 cells under different treatment conditions detected *via* flow cytometry. Note: [a]$p<0.05$ *vs.* Control group, [b] *vs.* Radiation group.

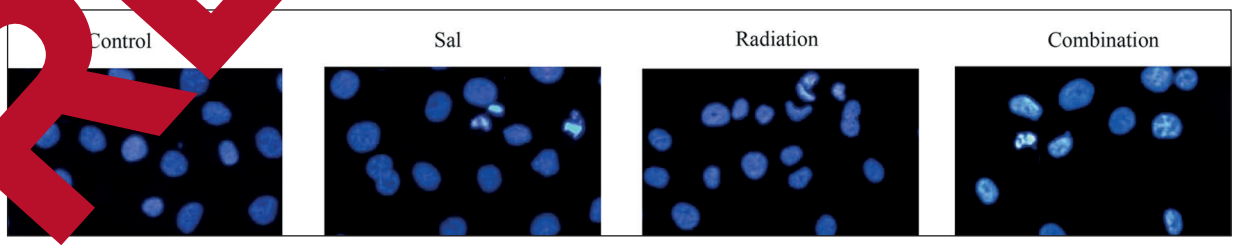

Figure 4. Apoptotic morphology of CNE-2 cells under different treatment conditions detected *via* Hoechst assay (magnification: 200×).

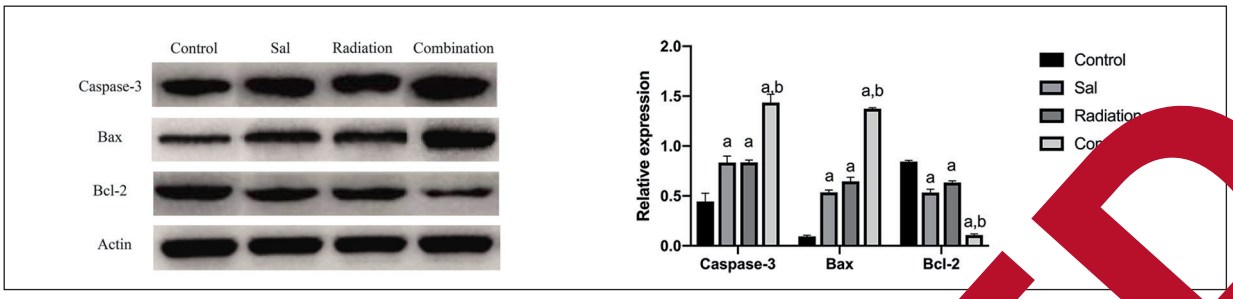

Figure 5. Expressions of apoptosis-related proteins in CNE-2 cells under different treatment c[illegible]ons det[illegible]ted *via* W[illegible] blotting. Note: [a]$p<0.05$ *vs.* Control group, [b]$p<0.05$ *vs.* Radiation group.

obviously increased expressions of Caspase-3 and Bax, and decreased expression of Bcl-2 ($p<0.05$).

Expressions of BIRC5 and NEIL2 In Each Group

CNE-2 cells in Control group, Sal group (4 μM), Radiation group (6 Gy) and Combination group received intervention, and the expressions of BIRC5 and NEIL2 were measured using Western blotting 48 h later. The results revealed that the protein expressions of BIRC5 and N[illegible]2 were significantly lower in Sal group, [illegible]tion group and Combination group than th[illegible]in Control group ($p<0.05$). Moreover, the pr[illegible] expressions of BIRC5 and NEIL2 in Combinat[illegible] group were evidently lower tha[illegible]in Radi[illegible]tion group ($p<0.05$) (Figure [illegible]

Dis[illegible]ss[illegible]

NPC is a mali[illegible]t tumor tha[illegible]urs in nasopharyngeal [illegible]ells, whose [illegible]evalence is high in so[illegible]east A[illegible] particular, its morbidity an[illegible]ortality rate[illegible] 60/100,000 and 34/100,000, r[illegible]ctively, in [illegible]China in 2015[14,15]. Di[illegible]tors and E[illegible]-Barr virus infection [illegible]cons[illegible]d as major causes of NPC. Currently, the main [illegible]ent means for NPC is radi[illegible]y, including [illegible]dimensional radiotherapy, three-dimensional radiotherapy and in[illegible]ty-modulated [illegible]adiotherapy. These treatment [illegible]sures greatly [illegible]prove the 5-year survival rate o[illegible]C patients. However, 20% of NPC patien[illegible]for f[illegible]ocal recurrence and metastasis even a[illegible]ment. Radiotherapy resistance is a major factor leading to treatment failure, which [illegible]a major precipitating factor for recurrence [illegible]d [illegible]astasis. Therefore, reducing radiotherapy resistance in effective ways and improving radiotherapy sensitivity are of great importance for the treatment and prognosis of NPC patients[7].

Sal, a small molecule compound, can suppress the proliferation and promote the apoptosis of various cancer cells *in vitro*, such as lung cancer, ovarian cancer, gastric cancer, and liver cancer. Preclinical studies[10,18,19] in 2010 suggest that Sal can reduce the growth and metastasis of ovarian cancer and breast cancer. However, the effect of Sal on NPC cells and whether it can enhance the

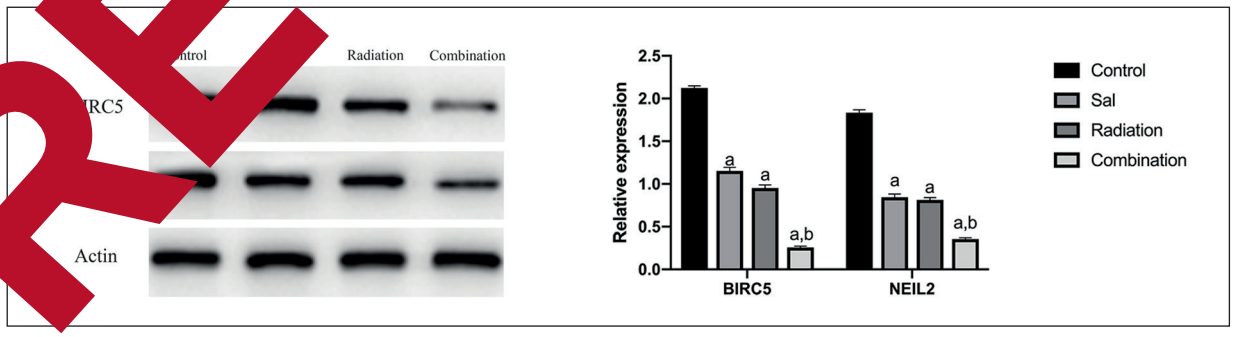

Figure 6. Protein expressions of BIRC5 and NEIL2 in CNE-2 cells under different treatment conditions detected *via* Western blotting. Note: [a]$p<0.05$ *vs.* Control group, [b]$p<0.05$ *vs.* Radiation group.

sensitivity of NPC cells to radiotherapy has not been fully elucidated. Therefore, the association between Sal and radiotherapy sensitivity of NPC cells was preliminarily explored in this study. Our findings might help to provide theoretical and practical basis for reducing radiotherapy resistance of NPC. Human NPC CNE-2 cell lines were used as research objects in this study. The cells received intervention with Sal at different concentrations, radioactive rays at different doses and Sal combined with radioactive rays. The growth inhibition rate of CNE-2 cells was then detected. Under the intervention with different concentrations of Sal or different doses of radioactive rays alone, the growth inhibition rate of CNE-2 cells rose in a concentration/dose-dependent manner. As Sal concentration was increased in combination therapy, the growth inhibition rate of CNE-2 cells also rose significantly. After intervention with Sal or radioactive rays alone and combined intervention, the CNE-2 cell cycle was mostly arrested in the G_2/M phase. Meanwhile, cell apoptosis significantly increased, and is more evident in Combination group than Radiation group. These results are consistent with previous experimental result[ ]at Sal can increase the sensitivity of cancer [ ] radiotherapy/chemotherapy drugs by arresti[ ]ll cycle in the G_2 phase[20]. In addition, BIRC5 [ ] oncogene regulated by P53, whose abnormal [ ] pression will lead to weakened a[ ] NEIL2 [ ] the main protein causing tole[ ] o[ ]er cells. Both BIRC5 and NEIL2 h[ ]been c[ ]rmed to be remarkably upregulat[ ] NPC[ ] more remarkably in p[ ]nts[ ]e to r[ ]- therapy. Therefore, [ ]ther Sa[ ]eased the radiotherapy sensiti[ ]f CNE-2 c[ ]s through these two pro[ ]explored i[ ]s study. The results m[ ]ifested [ ]Sal group, Radiation group, a[ ]ombination [ ]had significantly lower e[ ]ssions of BIRC5 a[ ]EIL2 than Control [ ]. More[ ], combination group exhibited re[ ]bly d[ ]ased expressions of BIRC5 and NEIL[ ]ed wit[ ]diation group. Sal can [ ]roy [ ] thus [ ]ancing the sensitivity of [ ] cells [ ]herapy/chemotherapy drugs, wh[ ]NEIL2 [ ]epair damaged DNA. Besides, Sa[ ]further inhibit the expression of NEIL2. [ ] that Sal can not only destroy DNA [ ]t also suppress the effect of DNA repair enzyme, [ ]by increasing the sensitivity of cancer cells t[ ]otherapy/chemotherapy drugs. This is the reason for the strong anti-cancer effect of Sal. Many incidences of toxicity have been reported[21] and showed that SAL was accidentally fed or ingested in higher doses in different animals. Thus, more relative clinical experiments are still needed in future.

Conclusions

Shortly, Sal can reduce the [ ]ions of BIRC5 and NEIL2, enhan[ ]the se[ ]ty of human NPC CNE-2 cells [ ]diotherapy, [ ] crease the apoptosis of [ ]-2 cell[ ]. Our fin[ ] provide theoretical a[ ]practic[ ]ases for the treatment of NPC.

Conflict of

The Author[ ]hare [ ]y have no co[ ]flict of interests.

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