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Salinomycin inhibits proliferation and induces apoptosis of human nasopharyngeal carcinoma cell in vitro and suppresses tumor growth in vivo

Danxin Wu, Yu Zhang, Jie Huang, Zirong Fan, Fengrong Shi, Senming Wang *

Department of Oncology, Zhujiang Hospital, Southern Medical University, Guangzhou 510282, China

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ABSTRACT

Salinomycin (Sal) is a polyether ionophore antibiotic that has recently been shown to induce cell death in various human cancer cells. However, whether salinomycin plays a functional role in nasopharyngeal carcinoma (NPC) has not been determined to date. The present study investigated the chemotherapeutic efficacy of salinomycin and its molecular mechanisms of action in NPC cells. Salinomycin efficiently inhibited proliferation and invasion of 3 NPC cell lines (CNE-1, CNE-2, and CNE-2/DDP) and activated a extensive apoptotic process that is accompanied by activation of caspase-3 and caspase-9, and decreased mitochondrial membrane potential. Meanwhile, the protein expression level of the Wnt coreceptor lipoprotein receptor related protein 6 (LRP6) and β-catenin was down-regulated, which showed that the Wnt/β-catenin signaling was involved in salinomycin-induced apoptosis of NPC cells. In a nude mouse NPC xenograft model, the anti-tumor effect of salinomycin was associated with the downregulation of β-catenin expression. The present study demonstrated that salinomycin can effectively inhibit proliferation and invasion, and induce apoptosis of NPC cells in vitro and inhibit tumor growth in vivo, probably via the inhibition of Wnt/β-catenin signaling, suggesting salinomycin as a potential candidate for the chemotherapy of NPC.

1. Introduction

Nasopharyngeal carcinoma is a highly malignant squamous epithelial cancer with a high incidence in Southeast Asia, especially in Southern China and Hong Kong [1–3]. It is increasingly evident that NPC has a multifactorial etiology that includes genetic factors, Epstein-Barr virus infection, alcohol consumption, tobacco smoking, herbal product use and other environmental factors [4,5]. Chemotherapy and radiotherapy have failed to significantly improve the prognosis of patients with metastatic diseases, which show high relapse rates possibly associated with a small number of cancer stem cells that are resistant to therapy [6,7]. Consequently, there is an urgent need to find more effective targeted drugs for this disease.

Salinomycin, a polyether anticoccidial drug that can promote the efflux of K^+ to interfere with the naturalion transport systems of both mitochondria and the cytoplasm, is commonly used to feed poultry to enhance nutrient absorption and feed efficiency [8–10]. Recently, Gupta and his colleagues discovered that compared with the conventional chemotherapeutic drug paclitaxel, only salinomycin could significantly reduce the proportion of breast cancer stem cells (CSCs) in vitro, and that salinomycin inhibited mammary tumor growth and tumorsphere-forming ratio as well as lymphnode metastasis in vivo [11]. Further study indicated that salinomycin could selectively target "CD133" cell subpopulations and decrease the malignant traits in colorectal cancer cells [12]. In addition, salinomycin induced apoptosis in many human cancer cells resistant to apoptosis, but not in normal cells, by multiple mechanisms [13]. In endometrial CSCs, salinomycin showed strong inhibition of proliferation, migration and invasion [14]. Salinomycin was also shown to effectively kill other tumor cells, including osteosarcoma [15], hepatocellular carcinoma [16], cholangiocarcinoma [17], and gastric cancer [18]. However, few studies have focused on the impact of salinomycin treatment on human NPC cells. In the present study, we examined the effectiveness of salinomycin against NPC cells in vitro and in vivo.

2. Materials and methods

2.1. Cell lines and reagents

The human NPC cell lines CNE-1 (a highly differentiated NPC cell line), CNE-2 (a poorly differentiated NPC cell line) were obtained from the Cancer Institute of Southern Medical University (Guangzhou, China) and CNE-2/DDP (a drug resistant NPC cell line)

* Corresponding author.
E-mail address: wsenming@126.com (S. Wang).

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was purchased from the Academy of Military Medical Sciences of China. The three cell lines were maintained in RPMI 1640 (Hyclone, South America) supplemented with 10% fetal bovine serum (Hyclone, South America), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco, Canada) in a humidified incubator at 37 °C and 5% CO_2. Salinomycin (Sigma Aldrich) was dissolved in DMSO (Gibco, Canada) and stored at −20 °C. The primary antibodies used for Western blotting and immunohistochemistry staining were purchased from Cellular Signaling Technology (Bcl-2, Bax, LRP6, β-actin) and Abcam (β-catenin).

2.2. Cell viability

CNE-1, CNE-2, and CNE-2/DDP cells were plated at a density of 2000 cells/well in flat-bottom 96-well plates (100 μl media per well). After 24 h, cells were treated with salinomycin at various concentrations (0, 1, 2, 4, 8 and 16 μmol/l). Cell viability was measured after 24, 48, and 72 h using the Cell Counting Kit 8 (Dojindo, Japan) with a microplate reader at 450 nm. All experimental concentrations were assessed in triplicate.

2.3. Invasion assay

Cell invasion was assessed using a Transwell Boyden chamber (Corning Costar) containing a polycarbonated filter with 8 μM pores coated with Matrigel (Sigma Aldrich). The cells were pretreated with the respective concentrations of salinomycin (0, 8, and 16 μM) for 48 h. The cells (1×10^5) in 0.2 ml culture medium without fetal bovine serum were added to the upper chamber. The lower chamber was filled with 0.6 ml complete medium containing 30% fetal bovine serum. After 36 h of incubation at 37 °C, cells remaining on the upper side of the Transwell membrane were removed using a cotton swab. The membrane was washed with ice-cold PBS twice. Cells that invaded to the under-side were fixed with 4% formaldehyde, stained with crystal violet and counted in five randomly selected fields under a microscope.

2.4. Hoechst 33258 staining

Morphological assessment of apoptotic cells was performed using the Hoechst 33258 staining method. The NPC cells were seeded in 6-well plates (5×10^4 cells/ml). After overnight incubation, the cells were treated with salinomycin (0, 8 and 16 μM) for 48 h and then fixed in 4% formaldehyde for 10 min. After washing twice with PBS, the cells were stained with 0.5 ml Hoechst 33258 (Sigma Aldrich) for 5 min and imaged under an inverted fluorescence microscope.

2.5. Apoptosis analyses by flow cytometry

The NPC cells were plated in 6-well plates and treated with salinomycin (0, 8 and 16 μM). After incubation for 48 h, cells were harvested, washed with PBS twice, resuspended in binding buffer, stained with an annexin-V/PI solution (BD Biosciences) at room temperature and then analyzed using a FACScan system (BD Biosciences).

2.6. Detection of caspase activity

NPC cells exposed to salinomycin (0, 8 and 16 μM) for 48 h were collected, washed and incubated on ice for 30 min in cell lysis buffer (Sigma–Aldrich). A total of 50 μg protein in a volume of 100 μl of cell lysate was mixed with 50 μl of 2× reaction buffer. Then, substrates (BD Biosciences) for caspase-3 (DEVD-pNA), caspase-8 (LETD-pNA) and caspase-9 (LEHD-pNA) were added at a final concentration of 5 μM to each sample for a total volume of 100 μl and incubated at 37 °C for 4 h. The OD values were obtained with a spectrophotometer at a wavelength of 405 nm.

2.7. Detection of mitochondrial membrane potential (MMP) by flow cytometry and fluorescence microscopy

Mitochondrial activity was measured with a JC-1 apoptosis detection kit (BD Biosciences) following the manufacturer's instructions. Briefly, 1×10^6 cells were collected, washed twice with PBS, resuspended in 500 μl JC-1working liquid and then incubated for 20 min at 37 °C in the dark. After washing twice with buffer solution, the NPC cells were analyzed by flow cytometry.

2.8. Western blot analysis

CNE-1, CNE-2 and CNE-2/DDP cells were treated with various concentrations of salinomycin for 48 h, washed twice with PBS solution and resuspended in 0.5 ml lysis buffer. Protein concentrations were determined with a BCA Protein Assay Kit (Thermo Scientific). Samples with equivalent amounts of total protein were electrophoresed using 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane. Immunoblotting was performed using the primary antibodies against Bcl-2, Bax, LRP6, β-catenin and β-actin. Horseradish peroxidase conjugated anti-IgG was used as the secondary antibody and blots were developed using ECL chemiluminescent reagent (Cell Signaling Technology).

2.9. In vivo treatment and immunohistochemistry assay

Four-week-old male nude mice (BALB/c-nu) were purchased from Provincial Animal Center (Guangdong, China). Mice were kept in a standard barrier system and free to food and water. The animal assay was performed according to the Guide for the Care and Use of Laboratory Animals and approved by the Animal Investigation Committee of Sun Yat-sen University (NO. 00054608). CNE-2 cells (5×10^6 cells) suspended in serum-free culture medium were inoculated subcutaneously in the flank region of nude mice. After tumors grew to about 4 mm, mice were randomly divided into a control group and a salinomycin group (10 mg/kg). Drugs were given by intraperitoneal injection daily for 2 weeks. The animals' weight and tumor volumes were monitored every other day. Using vernier calipers, tumor volume was calculated according to the formula $A \times B \times B/2$, where A is the length of the tumor and B is the width. After 2 weeks, nude mice were anesthetized and finally euthanized and xenografts were removed from the animals. Tumor tissues were fixed in 10% formalin solution and paraffin-embedded. Some sample sections were stained with hematoxylin and eosin to confirm the cell origin microscopically and the others with β-catenin for immunohistochemistry. The staining intensity was scored as 0 (negative), 1 (weak), 2 (moderate), and 3 (strong), and the percentage of positive cells was scored as 0 (0%), 1 (<30%), 2 (30–70%) or 3 (>70%). The value for the intensity score time the percentage positive score was used as the final score (0–6). When it was greater than or equal to 2, the specimen was interpreted as positive.

2.10. Statistical analysis

Statistical analyses were performed using SPSS13.0 software and all the data from at least three experiments are presented as the mean ± S.D. The statistical difference between the means was analyzed with a Student's t-test or one-way ANOVA. Compared with the control, $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Salinomycin inhibits cell proliferation and invasion of NPC cells

CNE-1, CNE-2, and CNE-2/DDP cells were treated with 0, 1, 2, 4, 8 and 16 μM salinomycin for 24, 48 and 72 h. Compared with the control groups, salinomycin inhibited the viability of the NPC cells in a dose- and time-dependent manner (Fig. 1A). At a concentration of 8 μM, salinomycin inhibited about 53.0%, 48.0% and 42.3% of the survival of CNE-1, CNE-2 and CNE-2/DDP cells respectively after 48 h treatment. In contrast, when CNE-1 cells were exposed to16 μM salinomycin for 72 h, the inhibition ratio was up to 70%.

The effect of salinomycin on cell invasion was examined using the Transwell migration assay. In the control group, a large fraction of cells migrated to the lower side of the Transwell chamber membrane. While in the treatment group, the invasion of NPC cells was impaired by salinomycin in a dose-dependent manner (Fig. 1B and C). Treatment with 16 μM salinomycin significantly reduced the number of cells that invaded to the under-side of the membrane.

3.2. Salinomycin induces apoptosis in NPC cells

To examine whether salinomycin induces apoptosis in NPC cells, Hoechst 33258 staining and flow cytometry were performed. In Hoechst 33258 staining assay, apoptotic bodies of NPC cells added in response to salinomycin treatment in a concentration-dependent manner. Treatment with 16 μM salinomycin resulted in the appearance of the characteristic features of apoptosis including nuclear condensation and fragmentation (Fig. 2A). The results of the flow cytometry assay showed an increase in the percentage of apoptotic cells compared with the control group. Treatment with 16 μM salinomycin resulted in a percentage of apoptotic cells of 36.2% in CNE-1, 32.1% in CNE-2 and 33.4% in CNE-2/DDP (Fig. 2B).

To further understand whether neither the intrinsic nor the extrinsic apoptosis participated in the apoptosis mediated by salinomycin, we detected the mitochondria function and caspases activity respectively. Mitochondrial membrane potential (MMP) was measured using JC-1 staining and flow cytometry. The MMP is quantified by measuring fluorescence intensity of red (FL2-H) which represents mitochondria under natural conditions over green (FL1-H) which represents damaged mitochondria in each group. As shown in Fig. 2C, salinomycin decreased MMP in a concentration-dependent manner, as reflected by the ratio of red (JC-1 aggregates,) to green (JC-1 monomers) signals, suggesting that salinomycin disturbed the cell mitochondrial activity. Considering the significance of caspases to apoptosis, we measured the activities of initiator caspases (caspase-8 and -9) and effector caspases (caspase-3). Compared with control cells, salinomycin treatment (8 and 16 μM) increased caspase-3 and caspase-9 activities, but had no obvious effect on caspase-8 (Fig. 2D). CNE-1 cells treated with salinomycin at 16 μM had a more than 5-fold higher level of caspase-3 activity than control cells.

To further confirm the induction of apoptosis by salinomycin, we detected the expression of Bcl-2 and Bax by Western blotting. As shown in Fig. 2E, treatment with salinomycin for 48 h significantly suppressed the expression of Bcl-2 and upregulated Bax expression.

3.3. Salinomycin inhibits tumor growth in vivo

To evaluate the anti-tumor effects of salinomycin in vivo, we choosed CNE-2 cell line as a representative for its poorly differentiated character. which can be a good representative for poorly differentiated or undifferentiated nasopharyngeal carcinoma clinically. CNE-2 xenografts were established in nude mice. Assessment of tumor volume showed that the salinomycin treated group showed delayed tumor growth compared to the control group (Fig. 3A). After 2 weeks of treatment, animals were killed and CNE-2 xenografts were dissected and weighed. As shown in Fig. 3B, tumors were smaller in the experimental group than in the control group and the difference was statistically significant (Fig. 3C).

3.4. Salinomycin suppresses Wnt/β-catenin signaling pathway

To determine the mechanism of salinomycin-mediated apoptosis, the key sinaling molecules in the Wnt/β-catenin pathway was examined. By Western blotting, we showed that compared to controls, β-catenin and LRP6 expression were significantly downregulated especially in the high concentration groups (Fig. 4A). In vivo, tissues were exmined by hematoxylin and eosin (H&E) staining (Fig. 4B) and immunohistochemistry, which showed that salinomycin downregulated the expression of β-catenin (Fig. 4C).

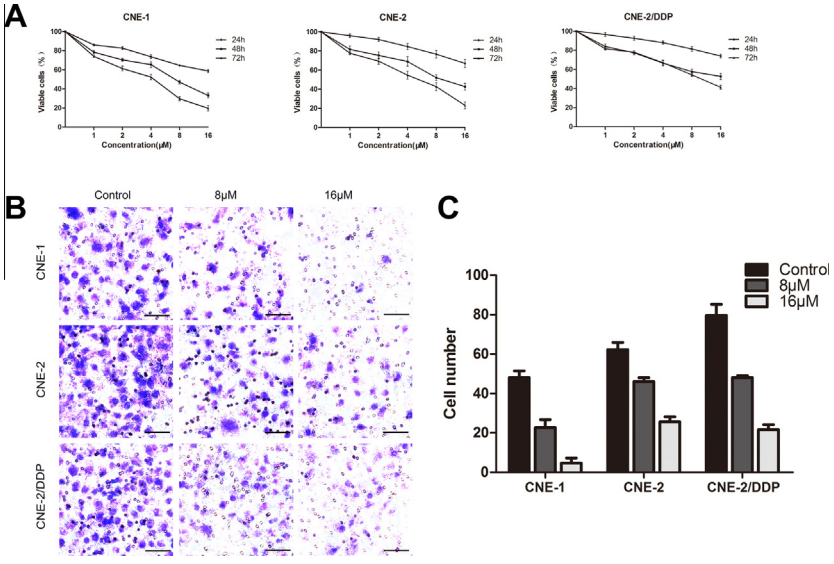

Fig. 1. Salinomycin inhibits proliferation and invasion of NPC cells in vitro. (A) CCK-8 assay: CNE-1, CNE-2, and CNE-2/DDP cells were treated with salinomycin (0, 1, 2, 4, 8 and 16 μM) for 24, 48 and 72 h. As shown in the graphs, salinomycin inhibited cell proliferation in a dose- and time-dependent manner, especially for the CNE-1 cells. (B and C) NPC cells were incubated for 48 h with salinomycin (0, 8 or 16 μM). Cells that invaded into the lower chamber were quantified as described in Section 2. Scale bars, 50 μm. Data are presented as mean ± SD, $P < 0.05$ compared with control.

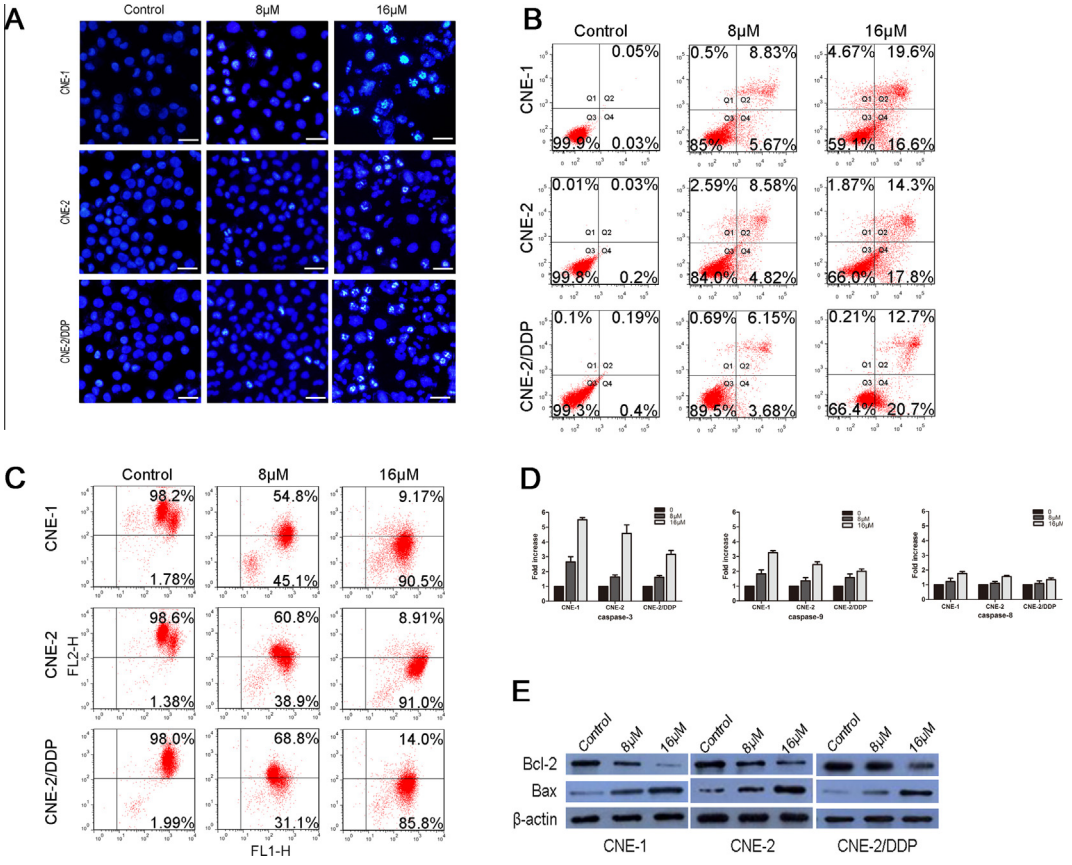

Fig. 2. Salinomycin induces apoptosis of NPC cells treated with salinomycin for 48 h in vitro. (A) Hoechst 33258 staining assay: cells showed nuclear condensation and fragmentation in a dose-dependent manner. Scale bars, 50 μm. (B) Representative dot plots of treated cells labeled with annexin-V FITC and PI by flow cytometry. (C) Flow cytometry analyses of the mitochondrial membrane potential (MMP) after staining with JC-1. Salinomycin decreases the MMP in NPC cells. (D) The caspases activity was also analyzed Salinomycin obviously increases caspase-3 and caspase-9 activity especially for the CNE-1 cells, but has little effect on caspase-8. (E) Salinomycin induces alterations in the expression of Bcl-2 and Bax. Data shown are representative of three independent experiments and presented as mean ± SD, $P < 0.05$.

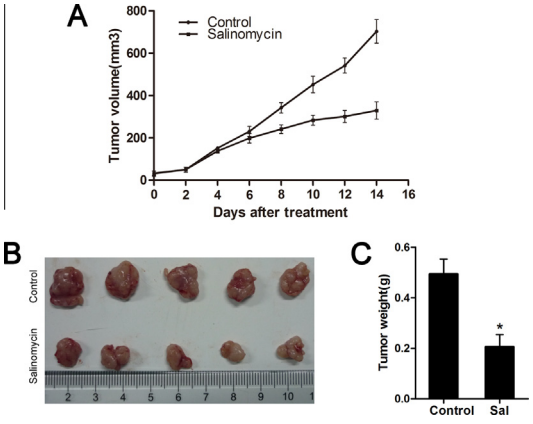

Fig. 3. Salinomycin suppresses tumor growth in vivo. CNE-2 cells (5×10^6) were inoculated subcutaneously into the right flank region of nude mice. The xenografts were excised from the animals 2 weeks after intraperitoneal salinomycin administration. (A) Tumor volume monitored every other day in the two groups. Data represent the means ± S.D. of the control group or Sal treatment group and there was a statistical difference ($P < 0.05$). (B) Images showing the tumors removed from the control and treatment groups and the tumors were smaller in the experimental group. (C) Graph represents the average weight of the tumor between the two groups, $^*P < 0.05$.

According to the Section 2, the tissues were classified as β-catenin negative expression in Sal-treated group, while positive expression in the control group.

4. Discussion

In NPC, multiple environmental factors and genetic abnormalities result in the disruption of various cellular mechanisms, causing alterations of the cell cycle, apoptosis dysregulation, changes in signal transduction pathways, aberrant proliferative signals, and the inhibition of tumor suppressors and other novel pathways [2,19]. Therefore, the development of effective targeted small molecule compounds for the treatment of NPC is necessary. In the present study, we examined the effect of salinomycin on NPC cells.

This study showed that salinomycin obviously inhibited proliferation and invasion of NPC cells. Consistent with previous studies assessing the effect of salinomycin on cell proliferation [16,20–23], our results showed that salinomycin inhibited NPC cell growth, even in the chemotherapy-resistant cell line CNE-2/DDP. Invasion and mobility, which are associated with tumor metastasis, were impaired by salinomycin in NPC cells, similar to the results obtained in other cancers [14,17,22,23].

In vitro, salinomycin blocked the Bcl-2/Bax complex, leading to an increase in the apoptosis rate, activation of caspase-3 and caspase-9 and the reduction of MMP, a characteristic event of early cellular apoptosis. Similar results were reported in murine dorsal root ganglia neurons, in which salinomycin treatment increased caspase-9 and caspase-3 activity [24]. However, in human cholangiocarcinoma cells, salinomycin-induced apoptosis was not associated with caspase-3 activation [17].

Recent studies demonstrated that salinomycin induces apoptosis in human leukemia stem-like cells by overcoming ABC

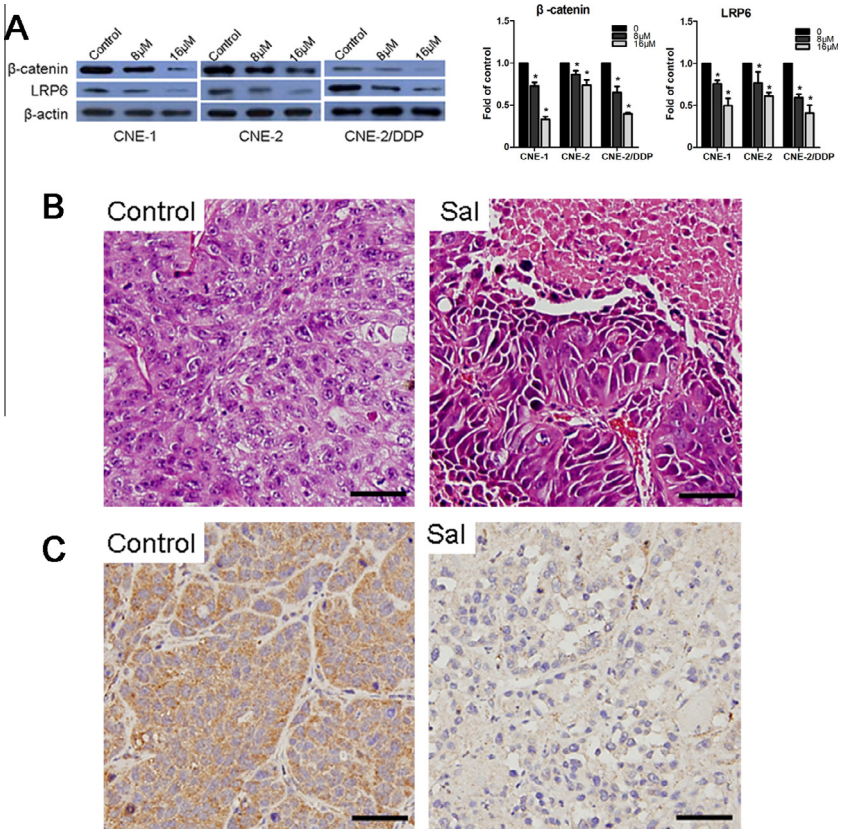

Fig. 4. Salinomycin suppresses Wnt/β-catenin signaling pathway. (A) The NPC cells were treated with salinomycin (0, 8 or 16 μM) for 48 h. By Western blotting, the β-catenin and LRP6 expression was obviously reduced compared with control groups especially in the high concentration group. β-Actin was used as the loading control. (B and C) In vivo, hematoxylin and eosin (H&E) staining and the expression of β-catenin by immunohistochemistry staining (Scale bars, 50 μm). All the staining was independently assessed by two observers who had no knowledge of the study data. After 2-week treatment, the β-catenin expression has been lowered.

transporter-mediated multidrug resistance [25] and in human prostate cancer cells by reactive oxygen species accumulation and mitochondrial membrane depolarization [26]. Furthermore, Wnt signaling also contributes to the anti-cancer effect of salinomycin,such as in hepatocellular carcinoma cells via increased intracellular Ca^{2+} levels [16], in chronic lymphocytic leukemia cells by interfering with LPR6 phosphorylation [27] and in side-population (SP) cells of human endometrial cancer cells by downregulating the expression of Wnt target genes such as LEF1, cyclin D1, and fibronectin [14]. In line with previous studies, we showed by Western blot analysis that salinomycin downregulated the expression of β-catenin and LRP6 in a concentration-dependent manner in vitro.

The Wnt pathway has been examined in relation to many cancers such as leukemia [27], melanoma [28], lung cancer [29], and colorectal cancer [30]. Although the exact involvement of the Wnt pathway in NPC remains to be clarified, there is considerable evidence that aberrant Wnt signaling plays an important role in NPC development [19,31,32]. According to the classical Wnt/β-catenin pathway, in the presence of Wnt, the accumulated β-catenin that escapes from degradation in the axin complex is translocated to the nucleus, stimulating the expression of various target genes [33]. Xu et al. showed that high expression of β-catenin is correlated with poor prognosis of NPC [34]. LRP6, an indispensable co-receptor for the Wnt signaling pathway, is readily expressed in many human cancer cells and malignant tissues [35]. In breast cancer, LRP6 silencing weakens Wnt signaling and inhibits cell proliferation and tumor growth in vivo [36]. Therefore, blockade of the Wnt pathway is expected to be an efficient targeting strategy for the treatment of NPC and salinomycin may be a good candidate.

To facilitate the clinical application of salinomycin in NPC therapy, we examined its anti-tumor effects in vivo. In the presence of salinomycin, tumor growth was delayed and body weight was decreased in xenograft mice. In addition, immunohistochemical staining showed the downregulation of β-catenin expression in response to salinomycin treatment. Our current study is consistent with other reports showing that salinomycin inhibits the tumorigenicity of human endometrial cancer side-population cells [14] and downregulates β-catenin expression in human hepatocellular carcinoma cells [16]. In addition, treatment of mice with salinomycin (5 mg/kg) inhibits mammary tumor growth and induces increased epithelial differentiation of tumor cells [11].

In conclusion, salinomycin treatment had anti-tumor effects on NPC cells in vitro and in vivo by disturbing Wnt/β-catenin signaling. It is well known that comprehensive treatment has played a prominent role in cancer therapy. Kim et al. recently reported that salinomycin sensitizes cells to DNA-damaging compounds [37], radiation [38], and microtubule-targeting drugs [39] via similar mechanisms including preventing G2 arrest or reducing p21 protein levels. Another study revealed that combined treatment with salinomycin and gemcitabine eliminates pancreatic cancer stem cells and differentiated cells in vitro and delays xenograft tumor growth in vivo [40]. Further investigation is needed to determine whether integrating salinomycin with radiotherapy, concurrent chemotherapy and immunotherapy may be an effective strategy for the treatment of NPC.

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