Salinomycin may inhibit the cancer stem-like populations with increased chemoradioresistance that nasopharyngeal cancer tumorspheres contain

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Abstract. Tumor recurrence and metastasis of nasopharyngeal cancer (NPC) often result in the failure of treatment due to chemoradioresistance. Cancer stem cells (CSCs) have been observed to drive tumor initiation and tumor chemoradioresistance. Therefore, the poor prognosis of advanced NPC is likely to result from the failure to kill CSCs. Sphere formation may be used as an experimental method to enrich potential CSC subpopulations. At present, there are few reports on NPC tumorspheres. The present study focused on examining the cancer stem-like properties of NPC tumorspheres from NPC cell lines. Western blot analysis revealed that NPC tumorspheres had a higher expression of stem cell markers Nanog homeobox and SRY-box 2, compared with parental cells. It was additionally verified that NPC tumorspheres contained a high aldehyde dehydrogenase (ALDH) enzymatic activity compared with parental cells. ALDH+ cells were amplified by 9- to 10-fold in tumorspheres, compared with parental cells (1.8 vs. 16.9%). The tumorsphere cells exhibited an increased half maximal inhibitory concentration value of >10-fold with cisplatin compared with the control parental cells. Compared with the parental cells, the percentage of side population cells in the tumorsphere cell population increased significantly (10.3 vs. 2.3%; $P<0.05$). NPC tumorsphere cells demonstrated enhanced resistance to radiation. Further investigation verified that salinomycin inhibited NPC CSCs by selectively targeting its stem cells. Altogether, the data revealed that NPC tumorspheres contain cancer stem-like populations with increased chemoradioresistance. It was suggested that the serum-free culture of NPC cells may provide an appropriate model for researching the sensitivity of CSCs to therapeutic agents. It was additionally revealed that salinomycin is an efficient inhibitor of NPC CSCs, supporting the hypothesis that salinomycin may eliminate CSCs and imply a need for further clinical evaluation.

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Introduction

Nasopharyngeal cancer (NPC) is a rare cancer globally, but is prevalent in Southeast Asia (1). Due to its radiosensitivity, radiotherapy is the primary treatment of NPC. In locally advanced stages, combined radiotherapy and chemotherapy have been considered to be an effective treatment in order to improve survival, preferred to radiotherapy alone (2). Local control rate of NPC has improved markedly in the past decade (3). However, local recurrence and metastasis remain the primary causes of mortality from this cancer, particularly in advanced stages (4), and management of local treatment failure remains a challenge in NPC treatment.

Emerging evidence supports the notion that cancer stem cells (CSCs) contribute to NPCs resistance to chemoradiation, which results in a poor prognosis for numerous different types of human cancer (5). CSCs possess the ability to recreate the complete phenotypic heterogeneity of the parental tumor cells. These cells possess distinct surface markers allowing for self-renewal (6). Multiple stem cell markers, including nanog homeobox (Nanog), SRY-box 2 (Sox-2) and aldehyde dehydrogenase (ALDH) have been used successfully to identify CSCs in normal and tumor tissue (7,8). Furthermore, side population (SP) cells exhibit CSC characteristics in NPC (9).

The anchorage-independent serum-free culture of stem cells was instrumental in the research of CSCs (10,11). Sphere formation may be specifically used to enrich the potential CSC subpopulation as a functional method (12,13). Therefore, the suspension culture system may maintain CSCs in their undifferentiated condition, facilitating their enrichment. However, few reports exist at present regarding tumorspheres in NPC. Therefore, the present study evaluated NPC cell subsets with CSC properties.

Enhanced chemoradioresistance to therapy is another characteristic of CSCs and has been identified in numerous different types of cancer cells (14,15). The NPC tumorsphere may be a valuable model for the further research of CSCs and

chemoradioresistance. In the present study, it was therefore evaluated as to whether NPC tumorsphere cells acquired the chemoradiation-resistant characteristics of CSCs.

Although NPC CSCs may be experimentally identified, drugs or compounds that selectively target NPC CSCs have not yet emerged. Salinomycin is a carboxylic polyether ionophore extracted from *Streptomyces albus* (16). Salinomycin has been identified as a selective inhibitor of breast and lung CSCs (17,18), however its function in the inhibition of NPC CSCs remains to be revealed. In the present study, a tumorsphere was successfully used to enrich NPC CSCs, and the results demonstrated that salinomycin was able to kill NPC CSCs.

Materials and methods

Cells and culture conditions. SUNE-1 and 5-8F human nasopharyngeal cancer cell lines were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). SUNE-1 and 5-8F cells were cultured in DMEM medium (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone; GE Healthcare Life Sciences) and 100 U/ml penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Cells were cultured in a humidified air with 5% CO_2 at 37˚C.

Tumorsphere culture and selection. SUNE-1 and 5-8F cells (1,000 cells/ml) were cultivated in serum-free Ham's F-12 medium (Gibco; Thermo Fisher Scientific, Inc.), supplemented with B27 (1:50; Gibco; Thermo Fisher Scientific, Inc.), 20 ng/ml epidermal growth factor (Invitrogen; Thermo Fisher Scientific, Inc.) and 20 ng/ml fibroblast growth factor (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C. To expand spheres *in vitro*, spheres were harvested by centrifugation at 125 x g for 5 min at 24˚C, separated to single cells and then cultured for 72 h at 37˚C to produce tumorspheres of the next generation. The tumorspheres were filtered using a cell strainer (BD Biosciences, San Jose, CA, USA). Spheroids with a diameter >40 μm were selected to perform the experiment.

Western blot analysis. The tumorspheres and parental cells were washed three times with 5 ml phosphate-buffered saline. Total protein was extracted from cells using a cell lysis buffer (20 mmol/l Tris-HCl, 150 mmol/l NaCl, 1% NP40, 5 mmol/l EDTA, 1 mmol/l Na3VO4; pH 7.5) supplemented with a protease inhibitor cocktail and a phosphatase inhibitor (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), and was incubated on ice for 30 min. Protein concentration was determined using a bicinchoninic acid protein assay kit (Pierce; Thermo Fisher Scientific, Inc.). Protein (50 μg/lane) were loaded and separated on a 10% gel using SDS-PAGE and then transferred to polyvinylidene membranes. Following blocking in 50 g/l non-fat milk in tris-buffered saline with Tween 20 (20 mmol/l Tris-HCl, 137 mmol/l NaCl and 1 g/l Tween 20; pH 7.6) for 2 h at room temperature, the membranes were incubated at 4˚C overnight with the following primary antibodies: Mouse anti-Nanog (dilution, 1:1,000; cat. no. sc-374001; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), Sox-2 (dilution, 1:1,000; cat. no. sc-365823, Santa Cruz Biotechnology) and GAPDH (dilution, 1:1,000; cat. no. sc-51907; Santa Cruz Biotechnology). The membranes were then incubated for 1 h at 24˚C with horseradish peroxidase-conjugated anti-mouse immunoglobulin secondary antibodies (dilution, 1:1,000; cat. no. A32729, Invitrogen; Thermo Fisher Scientific, Inc.). Finally, the membranes were visualized using the Image lab 3.0.1 software (Bio-Rad Laboratories, Inc., Hercules, CA, USA) following analysis using an enhanced chemiluminescence-Plus detection system (Bio-Rad Laboratories, Inc.).

ALDEFLUOR assay by fluorescence-activated cell sorting (FACS). The identification of ALDH activity using the ALDEFLUOR assay (Stem Cell Technologies, Inc., Vancouver, BC, Canada) was followed by FACS analysis. Cells were suspended in ALDEFLUOR assay buffer, which contains ALDH substrate, and were incubated for 40 min at 37˚C. As a negative control, for each sample of cells an aliquot was treated with 50 mM diethylaminobenzaldehyde (DEAB), a specific ALDH inhibitor. FACS analysis was performed using a FACSAria flow cytometer (BD Biosciences). The results were analyzed using FlowJo 7.6.3 software (FlowJo LLC, Ashland, OR, USA).

Hoechst staining and SP cell assay. The parental or spheroid cells were suspended in DMEM/2% FBS at a density of $1x10^6$ cells/ml. The cells were then dispersed into single cells and incubated with Hoechst 33342 dye (5 μg/ml; Sigma-Aldrich; Merck KGaA) either alone or in combination with verapamil (50 mmol/ml; Sigma-Aldrich; Merck KGaA) for 90 min at 37˚C. Following incubation, cells were washed with PBS and stained with propidium iodide (1 μg/ml; Sigma-Aldrich; Merck KGaA) for 30 min at 4˚C. Finally, the cells were maintained at 4˚C for the flow cytometric analysis and for the sorting of the SP fraction using a FACSAria flow cytometer. The results were analyzed using the FlowJo 7.6.3 software.

Drug sensitivity assay. Parental or spheroid cells were collected in 96-well microplates at a density of 3,000 cells per well. The cells were then treated with increasing concentrations of cisplatin (10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} M; Sigma-Aldrich, St. Louis, MO, USA). Subsequent to incubation for 72 h at 37˚C, an MTT assay was used to evaluate the cell viability. The number of living cells was calculated according to the absorbance at 490 nm. Each experiment was repeated three times.

Clone formation assay. Parental or spheroid cells were irradiated at indicated doses (0, 2, 4, 6, 8 and 10 Gy). Irradiation of cells was performed using 250 kV orthovoltage X-rays by a linear accelerator (Elekta Instrument AB, Stockholm, Sweden). Following irradiation, the cells were collected and subsequently replated in a 30-mm culture dish at a density of 200-5,000 cells per dish. Subsequent to culturing for 14 days at 37˚C, cells were fixed with 10% formalin and stained with 0.1% crystal violet for 15 min at 24˚C; clones consisting of >50 cells were selected. The survival fraction was calculated by dividing the number of colonies formed by the number of cells plated. The data were entered into single hit multi-target formula, as follows: $S=1-(1-e^{-D/D^{\circ}})^N$ (where D, quasi-threshold

dose; D^0, mean lethal dose; N, extrapolation number; and S, survival fraction). Graphpad Prism 5.0 (Graphpad Software, Inc., La Jolla, CA, USA) was used to draw the survival fraction curve. Experiments were repeated three times.

Sphere formation efficiency assay. Parental or spheroid cells were collected in 96-well microplates at a density of 3,000 cells per well. The cells were pretreated with dimethyl sulfoxide (DMSO; Sigma-Aldrich; Merck KGaA), 2 μM cisplatin (Sigma-Aldrich; Merck KGaA) and 2 μM salinomycin (Sigma-Aldrich; Merck KGaA) for 72 h at 37˚C. Subsequently, the cells were transferred into serum-free Ham's F-12 medium in 24-well microplates at a density of 100 cells/well. After 48 h, the tumorspheres were counted under a light microscope (magnification, x200; Nikon Corporation, Tokyo, Japan). Each experiments was repeated for three times.

Statistical analyses. P<0.05 was considered to indicate a statistically significant difference. Data were analyzed using the SPSS 19.0 statistical software package (IBM Corp., Armonk, NY, USA) and were presented as the mean ± the standard deviation. Differences between the groups were determined using a one-way analysis of variance and least significant difference method for multiple comparisons.

Results

NPC tumorspheres contain cells with cancer stem-like properties. It has been reported that breast CSC populations may be generated *in vitro* as mammospheres under serum-free culture conditions (19). In the present study, the NPC CSC population was enriched from the SUNE-1 cell line. Parental cells were cultivated in serum-free culture. After culturing for 72 h, floating tumorspheres were formed (Fig. 1A). SUNE-1 spheroids with a diameter of >40 μm, which were filtered out using a cell strainer, were selected. Two typical CSC markers, Nanog and Sox2, were detected using immunoblotting. As revealed in Fig. 1B, a marked increase in the expression of Nanog and Sox2 were observed in the SUNE-1 spheroids, compared with the parental cells. ALDH has been identified as a potential marker for NPC CSCs (20). ALDH is a detoxifying enzyme responsible for the oxidation of intracellular aldehydes. To further confirm this finding, an ALDEFLUOR assay was used to assess ALDH enzymatic activity in the SUNE-1 spheroids. ALDEFLUOR-positive cells were increased 9-10-fold in tumorsphere cells, compared with the parental cells (1.8 vs. 16.9%; Fig. 1C). The results indicated that NPC tumorspheres possessed increased stem-like cancer cells.

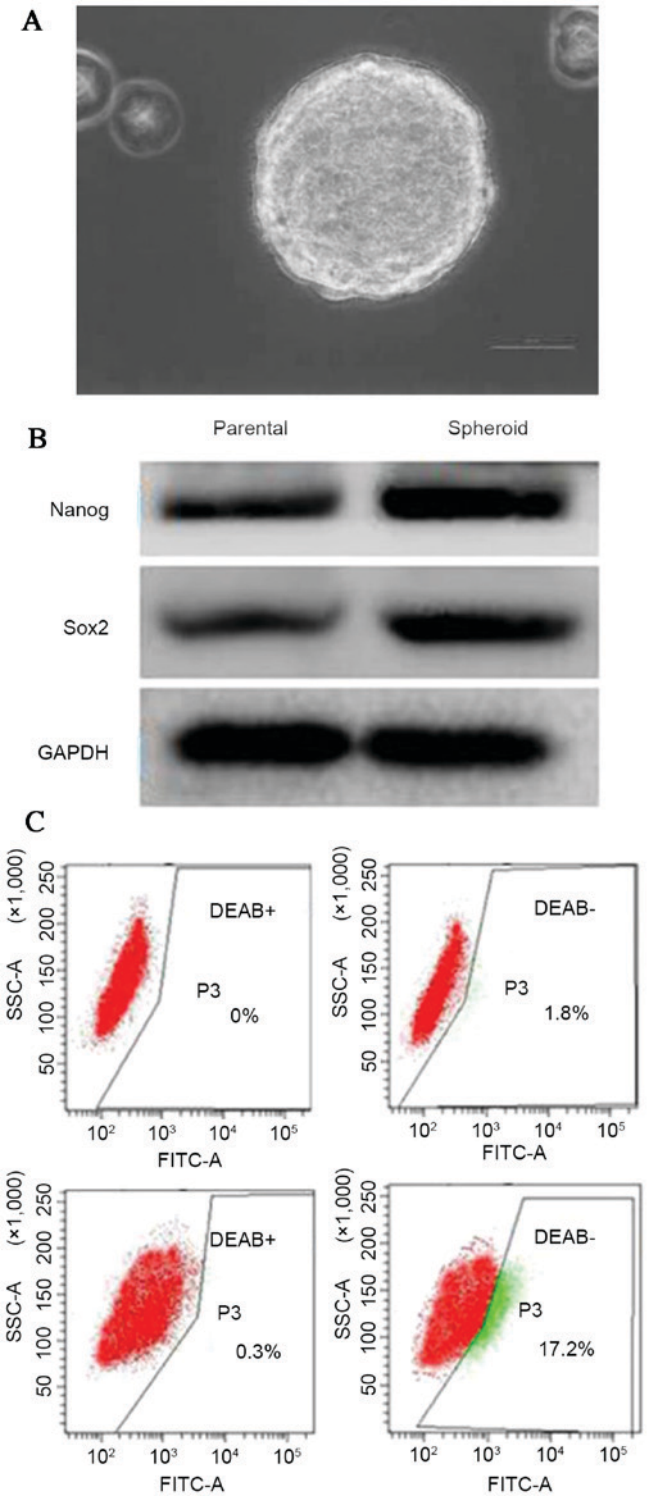

Figure 1. NPC tumorsphere formation and detection of CSC markers. (A) A light microscopic-derived image of SUNE-1 NPC spheroid cultivated in serum-free culture for 72 h. Scale bars, 100 μm (magnification, x200). (B) Western blot analysis of Nanog and Sox-2 expression between the parental and spheroid SUNE-1 cells. (C) ALDEFLUOR assay of Aldehyde dehydrogenase-positive cells of parental (upper, 1.8%) and spheroid (lower, 16.9%) SUNE-1 cells. NPC, nasopharyngeal cancer; CSC, cancer stem cell; Nanog, nanog homeobox; Sox-2, SRY-box 2.

NPC tumorspheres exhibit increased chemoresistance. Tumor cells resistant to chemotherapy occur partly due to the overexpression of the ATP-binding cassette sub-family (21). This characteristic is associated with the ability to expel dyes, identified by flow cytometry to be a SP (22). SP cells have been reported to possess NPC CSC properties (23). In the present study, NPC tumorsphere cells cultured in serum-free cultures were detected to possess a 4.5-fold increase in the proportion of SP cells compared with the parental cells (10.3 vs. 2.3%; Fig. 2A). Furthermore, the sensitivity of NPC tumorsphere cells and parental cells to cisplatin, which is usually used for chemotherapy against NPC, was examined. The tumorsphere cells from the spheroids demonstrated an increased half maximal inhibitory concentration value of >10-fold with cisplatin compared with the control parental cells (Fig. 2B). These

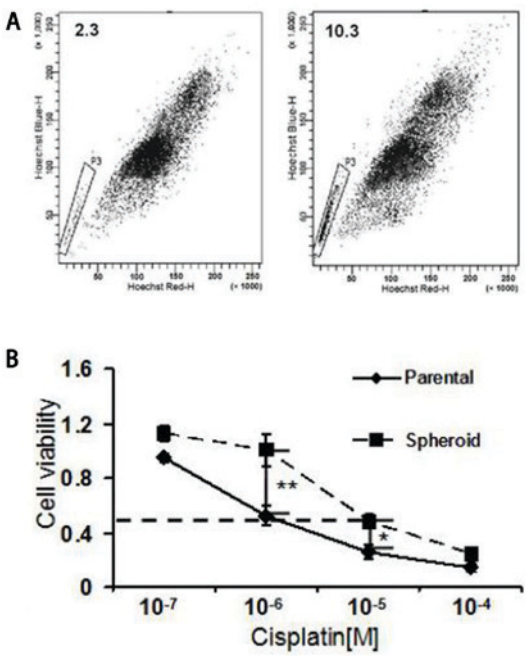

Figure 2. Proportion of SP cells and resistance to cisplatin between nasopharyngeal cancer tumorsphere cells and parental cells. (A) FACS analysis of the proportion of SP cells in parental (left panel, 2.3%) and spheroid (right panel, 10.3%) SUNE-1 cell groups. (B) Dose-response curves of parental and spheroid SUNE-1 cells following 72 h of treatment with cisplatin. Transverse line corresponds to the half maximal inhibitory concentration value. Bars represent the mean ± standard deviation. *P<0.05 (10^{-5} M cisplatin in parental cells vs. spheroid cells), **P<0.01 (10^{-6} M cisplatin in parental cells vs. spheroid cells). SP, side population; FACS, fluorescence-activated cell sorting.

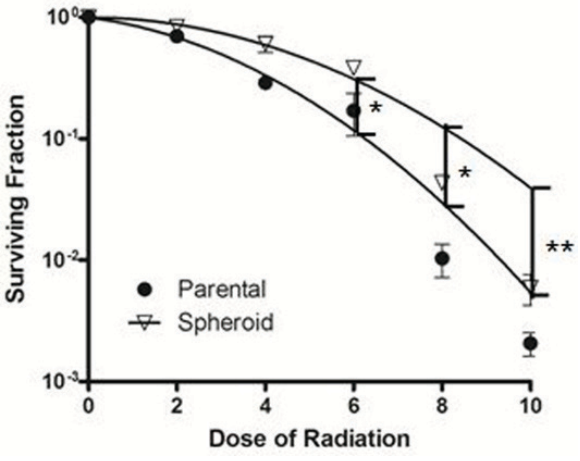

Figure 3. Survival curves of parental and spheroid SUNE-1 cells that underwent radiotherapeutic treatment. Bars represent the mean ± standard deviation. *P<0.05 (6 and 8 Gy radiation in parental cells vs. spheroid cells), **P<0.01 (10 Gy radiation in parental cells vs. spheroid cells).

results indicate that NPC tumorspheres possess increased chemoresistant properties of CSCs.

NPC tumorsphere cells demonstrate enhanced resistance to radiation. Radiotherapy is the primary treatment of NPC due to its radiosensitivity (24). To assess whether self-renewing cells from spheroids possessed a radiotherapy resistant phenotype, the radiosensitivity of parental and tumorsphere cells was analyzed using a clone formation assay. Following

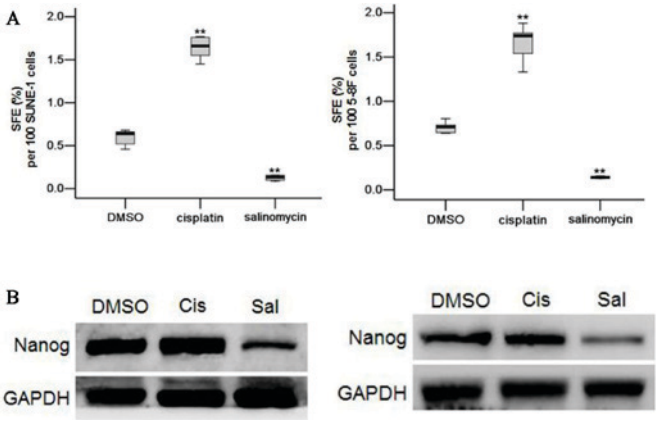

Figure 4. Effect of salinomycin on the SFE and Nanog expression of SUNE-1 and 5-8F cells. (A) SFE of SUNE-1 (left panel) and 5-8F (right panel) cells in serum-free medium, which were pre-treated with DMSO, cisplatin and salinomycin for 72 h in serum-contained medium. Results were presented as box plots. **P<0.01 vs. DMSO. (B) Immunoblotting of Nanog in SUNE-1 (left panel) and 5-8F (right panel) spheroid cells treated with DMSO, cisplatin and salinomycin for 72 h. SFE, sphere formation efficiency; Nanog, nanog homeobox; DMSO, dimethyl sulfoxide.

radiotherapeutic treatment, the survival fraction (SF) of the cells cultured as spheroids was significantly decreased compared with that of the parental cells [spheroid cells, mean SF at 6 Gy (SF6 Gy)=0.383±0.064 vs. parental cells SF6 Gy=0.171±0.113; P<0.05; Fig. 3]. These results supported radioresistance characteristics of NPC CSC-like cells.

Salinomycin selectively kills NPC CSCs. Salinomycin has been reported to possess potent anti-CSC activity (25). As a functional measure of CSC frequency, the ability of SUNE-1 and 5-8F cells to form tumorspheres following treatment for 72 h with salinomycin, cisplatin or a DMSO control when cultured in suspension cultures, was tested as an *in vitro* measure of CSC activity. Parental SUNE-1 and 5-8F cells were treated by DMSO, cisplatin (2 μM) or salinomycin (2 μM) for 72 h. Following treatment, tumor cells were cultivated in suspension cultures. Sphere formation efficiency (SFE) of SUNE-1 and 5-8F cells with the salinomycin treatment demonstrated a significant 4.7-fold and 5.0-fold decrease relative to DMSO treatment (0.592 spheres vs. 0.126 spheres per 100 SUNE-1 cells, P<0.01; 0.706 spheres vs. 0.142 spheres per 100 5-8F cells, P<0.05; Fig. 4A). In contrast, cisplatin treatment demonstrated a significant increase in the SFE of SUNE-1 and 5-8F cells compared with DMSO treatment (P<0.01; Fig. 4A). Nanog, a CSC marker, of SUNE-1 and 5-8F tumorsphere cells treated for 72 h with DMSO, cisplatin (2 μM) and salinomycin (2 μM) was also directly assayed. SUNE-1 and 5-8F tumorsphere cells treated with salinomycin presented a decrease in Nanog expression, compared with the DMSO control. The expression of Nanog did not decrease in the cisplatin-treated SUNE-1 and 5-8F cells (Fig. 4B). These results suggested that salinomycin may inhibit NPC CSC properties.

Discussion

Radiotherapy is the initial treatment mode of NPC and using radiotherapy in combination with chemotherapy is recommended for the treatment of locally advanced tumors (26).

Tumor recurrence and metastasis often result in the failure of treatment due to chemoradioresistance (27).

A previous study reported the application of serum-free culture to enrich and isolate potential CSC subpopulations in multiple different types of tumor (28). In general, as with all stem cells, the tumorsphere forming cells are capable of proliferation, self-renewal and exhibit increased tumorigenicity (29). In the present study, a comprehensive investigation of tumorsphere cells that are derived from the SUNE-1 cell line was provided. It was revealed that SUNE-1 tumorsphere cells acquire the characteristics of CSCs, with the increased expression of stem cell markers (Nanog and Sox-2), compared with the parental cells. It has been demonstrated that in SUNE-1 spheroids, a comparatively large subpopulation of cells had elevated the enzymatic activity of ALDH. These results suggest that NPC tumorsphere cells are associated with cancer stem-like populations.

Enhanced chemoresistance to therapy is another characteristic of CSCs that has been identified in numerous different types of cancer cells (30). In the present study, the tumorsphere cells demonstrated increased resistance, compared with that in the parental cells, to cisplatin treatment. NPC tumorspheres also exhibited an increased prevalence of SP cells. Therefore, it was suggested that the non-adherent tumorspheres cultured in serum-free conditions possessed NPC CSC properties. Suspension culture may be used to enrich drug-resistant NPC cells.

Radioresistance has been implied to be associated with CSCs in multiple types of cancer (31,32). Radiotherapy is the most important method in the treatment of NPC. NPC cells are more sensitive to radiation than other cancer cells (33). In the present study, tumorsphere cells displayed enhanced resistance to radiation compared with that displayed by its radiosensitive SUNE-1 parental cells. Therefore, eradicating radiotherapy-resistant cells is critical for successful anti-NPC therapy.

Salinomycin is a polyether anticoccidial drug produced by an *S. albus* strain. Previously, salinomycin had been reported to possess potent anti-CSC activity (34). The present study revealed that a decrease of SFE was observed following salinomycin treatment *in vitro*, implying that salinomycin may kill NPC CSCs selectively. NPC CSCs are more sensitive to salinomycin compared with the parental cells. In contrast, an increase in SFE was observed following cisplatin treatment *in vitro*; it was theorized that the increased SFE was due to the already increased proportion of CSCs present in the NPC cells treated with cisplatin. This may be due to the fact that cisplatin may only kill common tumor cells rather than CSCs (35).

To conclude, the present study demonstrated that chemoresistant NPC tumorsphere cells are rich in 'stem-cell-like' tumor cells and may be inhibited by salinomycin selectively. An effective method for the enrichment of CSCs was provided, which is beneficial for the research of the characteristics of NPC stem-like cells in terms of their biology and their unique cell surface markers. Finding novel therapies to overcome chemoresistance in NPC CSCs is key to improving long-term survival rates for patients with NPC.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

GZ performed the FACS analysis, and SZ performed the irradiation. JR performed the tumorsphere culture and selection. CY and ZZ conducted the western blot analysis. XQ and XZ conducted the clone formation assay. SW was responsible for the drug sensitivity assay. GZ and LL performed the statistical analysis. GZ designed the study and wrote the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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