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Original article

Salinomycin can effectively kill ALDH^{high} stem-like cells on gastric cancer

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ABSTRACT

Salinomycin is a novel identified cancer stem cells (CSCs) killer. Higher ALDH activity represents CSCs characterization. Here, we screened ALDH activities on several gastric cancer cell lines and divided them into ALDH^{high} and ALDH^{low} gastric cancer groups. ALDH^{high} cancer cells (NCI-N87 and SNU-1) disclosed more CSCs characteristics, such as higher levels of Sox2, Nanog and Nestin, more floating spheroid bodies, more colony formation and more resistance to conventional chemotherapeutic drugs 5-Fu and CDDP, compared to these parameters observed in ALDH^{low} cancer cells ($P < 0.01$). Importantly, ALDH^{high} cancer cells are relatively sensitive to salinomycin when compared to ALDH^{low} cancer cells ($P < 0.01$). Our results confirmed ALDH as functional marker of CSCs population on gastric cancer. Salinomycin might be selective therapy for CSCs fraction, which is resistant to conventional anticancer drugs 5-Fu and CDDP.

1. Introduction

Solid tumors are composed of complex populations of tumorigenic and non-tumorigenic cancer cells. The subpopulation of tumorigenic cells or cancer stem cells (CSCs) has exclusive ability to self-renew and undergo differentiation to phenotypically diverse cell populations [1,2]. Much effort has been made to sort CSCs from cancer by means of flow cytometry or other immunoselection procedure [3,4]. CSCs may play a decisive role in cancer relapse and metastasis, because dormant CSCs in G_0 stage activate and enter into cell cycle after a long interval [5]. Tumor relapse and metastasis cause a large number of human fatalities ever year. Recent evidences demonstrate that chemoresistance of cancer also involves in CSCs subpopulations with enhanced capability of DNA repair, decreased entry into apoptosis and increased expression of ABC transporter [6,7].

In 2009, American scientists published their infusive finding that salinomycin compound can effectively kill CSCs on breast cancer [8]. Salinomycin is a 751 Da monocarboxylic polyether antibiotic that constitutes a large pentacyclic molecule with a unique tricyclic spiroketal ring system. It is isolated from *Streptomyces albus* strain that acts as a potassium ionophore. It is reported that salinomycin could selectively deplete breast cancer CSCs, compared to conventional anticancer drugs [8–11].

Aldehyde dehydrogenase (ALDH) is a detoxifying enzyme, which is responsible for the oxidation of intracellular aldehydes. It catalyzes the irreversible oxidation of a range of aliphatic and aromatic aldehydes to corresponding carboxylic acids. In recent studies, cell population with high ALDH activity (ALDH^{high}) has been indicated to define CSCs in many types of cancer by Aldefluor® assay, including breast cancer [12,13], liver cancer [14], lung cancer [15,16], pancreas cancer [17], prostate cancer [18] and osteosarcoma [19]. So, ALDH may serve as useful biomarker for CSCs [20]. Hitherto, there is no publication about ALDH analysis on gastric cancer. In this study, we examined enzyme activities and mRNA expression of ALDH on gastric cancer cell lines. Because ALDH^{high} gastric cancer cell lines may comprise more CSCs, we evaluate several embryonic stem cell markers (Sox2, Nanog and Nestin) simultaneously. Salinomycin was used for therapeutic experiment, compared to conventional anticancer drugs 5-Fu and cisplatin. This paper firstly revealed that salinomycin significantly inhibits ALDH^{high} cancer cells on gastric cancer.

2. Materials and methods

2.1. Cell culture

Gastric cancer cell lines of SGC-7901, NCI-N87, BGC-823, MKN-28, MKN-45, AGS were originally obtained from Institute of Biochemistry and Cell Biology, Shanghai Chinese Academe of Science. KATO-III, SNU-1 and SNU-16 were purchased from American Type Culture Collection. The cells were cultured in

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RPMI-1640 medium with 10% fetal bovine serum (Gibco, Invitrogen) in 5% CO_2 cell culture incubator at 37 °C.

2.2. Examination of ALDH activity by Aldeflour® reagent

Aldeflour® assay kit was purchased from Stemcell Technologies, Vancouver, Canada. In this examination, once ALDH substrate (BODIPY-aminoacetaldehyde, BAAA) is taken up by living cells, it is converted into negatively-charged BODIPY-aminoacetate (BAA^-) via intracellular ALDH. BAA^- is retained in cells, and caused highly fluorescent. Only cells with an intact cell membrane can retain BAA^-. Briefly, 2×10^6/ml cancer cells were placed in Aldeflour® assay buffer, and incubated with the BAAA substrate for 45 min at 37 °C. As a negative control, a 1.5-mM diethylaminobenzaldehyde (DEAB), an ALDH specific inhibitor was used in each experiment. FACS Calibur flow cytometer was used for cell analysis.

2.3. Examination of ALDH1A1 mRNA and other stem cell markers by qRT-PCR

Total RNA was extracted using Trizol solution (Invitrogen). Reverse transcription (RT) was performed in a 20-μl reaction system according to the manufacturer's recommendation (Promega). qRT-PCR primers for ALDH1A1 were 5'-AGCCTTCACAGGATCAACAGA-3' and 5'-GTCGGCATCAGCTAACACAA-3'. Primers for Sox2 were 5'-GCCGAGTGGAAACTTTTGTCG-3' and 5'- GCAGCGTGTACTTATCCTTCTT-3'. Primers for Nanog were 5'-TAACCTTGGCTGCCGTCTCTGG-3' and 5'- AAGCCTCCCAATCCCAAACAATACG-3'. Primers for Nestin were 5'- GAAACAGCCATAGAGGGCAAA-3' and 5'-TGGTTTTCCAGAGTCTTCAGTGA-3'. Primers for GAPDH were 5'-GGACCTGACCTGCCGTCTAG-3' and 5'-GTAGCCCAGGATGCCCTTGA-3'. Real-time PCR was performed with the follow program: initial denature at 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. The data were analyzed by the comparative Ct method.

2.4. Analysis of gastrosphere bodies

Gastric cancer cells (1×10^4/ml) were seeded into serum-free media supplemented with epidermal growth factor (EGF) 20 μg/L, fibroblast growth factor-basic (bFGF) 20 μg/L, 100 U/ml penicillin G sodium and 100 μg/ml streptomycin sulfate. After 7-day incubation at 37 °C, the growth state of the cells in 6-well plates was observed under microscope (Olympus IX71) according to previous reports [21,22]. The number of floating gastrosphere bodies were photographed and counted under 20-x magnification in five random fields.

2.5. Colony formation assay in soft agar media

Briefly, 1×10^4 cells/ml were resuspended in 2 ml growth media containing 0.3% agar (Sigma, USA), and then paved on a 2 ml base layer containing 0.6% agar in six-well plates. The plates were incubated for 21 days at 37 °C, 5% CO_2 condition, then stained with 0.02% crystal violet for 1 h (Sigma, USA). The colonies were counted in 5 random fields under an inverted phase-contrast microscope (Olympus IX71). Colony cells more than 50 cells were counted as 1 positive colony.

2.6. Drugs sensitivity analysis by cell counting kit-8

Gastric cancer cells (5×10^3/well) were seeded in 96 well plates in serum-containing medium and treated with different chemotherapeutics (5-fluorouracil or 5-Fu, cis-diamminedichloroplatinum or CDDP and salinomycin, Sigma, USA) in different concentrations for 72 h. A 20 μl cell counting kit-8 (Dojindo,

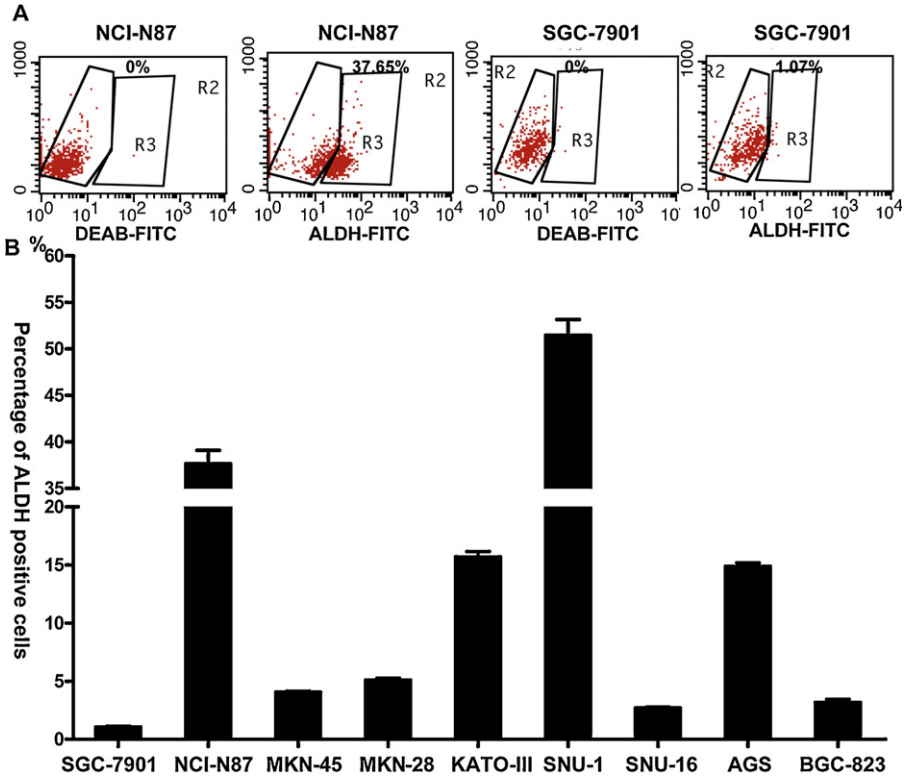

Fig. 1. Aldeflour® assay by flow cytometry. A. Cells gated in R3 represent the subpopulation of gastric carcinoma cells with ALDH activity. Whereas, parallel control sample with DEAB, an ALDH specific inhibitor was negative for ALDH activity. NCI-N87 is a representative of $ALDH^{high}$ cell line. SGC-7901 is a representative of $ALDH^{low}$ cell line. B. The bar chart is an overview of percentage of ALDH activity in different gastric cancer cell lines. The data represent the mean ± SD of three independent experiments.

Kumamoto, Japan) was added in each well. After 4 h incubation at 37 °C, the coloring reaction was quantified by an automatic plate reader (Tecan, Swiss) at 450 nm. Growth inhibitory effects were expressed as cell viability curve (XLfit 5.2 software, IDBS, UK). The drug sensitivity was evaluated by IC50 parameter (inhibitory concentration of 50% cells).

2.7. Statistical analysis

All data were expressed as mean ± SD. Statistically significant differences were determined using one-way analysis of variance (ANOVA) followed by Student *t*-test. Differences were considered significant at $P < 0.05$. All computations were performed using SPSS 16.0 software.

3. Results

3.1. ALDH activities on gastric cancer cell lines

We detected ALDH activity in gastric cancer cell lines by Aldeflour® assay kit. Representative images of ALDH activity for NCI-N87, SNU-1, SGC-7901 and BGC-823 cell lines were shown in Fig. 1A. The percentage of ALDH positive cells in NCI-N87 and SNU-1 was 37.65 ± 1.45% and 51.46 ± 1.71%, respectively, which are significantly higher than that in SGC-7901 (1.07 ± 0.08%) and BGC-823 (3.18 ± 0.28%) ($P < 0.01$). The overview of enzyme activity of ALDH on gastric cancer cell lines was summarized in Fig. 1B.

3.2. mRNA expressions of ALDH1A1 and other stem cell markers on gastric cancer cell lines

As shown in Fig. 2A, the ALDH1A1 mRNA level of NCI-N87 and SNU-1 was higher than that in other cell lines. It is compatible with the results by Aldeflour® assay. So, we defined NCI-N87 and SNU-1 as ALDH^{high} cell lines, and SGC-7901 and BGC-823 as ALDH^{low} cell lines for subsequent analysis. We compared mRNA expressing levels of Sox2, Nanog and Nestin with ALDH1A1 mRNA level by qRT-PCR. As shown in Fig. 2B, the mRNA expressing levels of Sox2 in ALDH^{high} cells are14.67 ± 1.21 (NCI-N87) and 6.66 ± 0.68 (SNU-1). They are significantly higher than that in ALDH^{low} cells (1.99 ± 0.21 in SGC-7901 and 2.01 ± 0.12 in BGC-823, $P < 0.01$). The mRNA expressing levels of Nanog between ALDH^{high} and ALDH^{low} cells are 14.62 ± 2.21 (NCI-N87) and 83.07 ± 6.32 (SNU-1) vs. 4.51 ± 0.56 (SGC-7901) and 1.65 ± 0.23 (BGC-823) ($P < 0.01$). The mRNA expressing levels of Nestin between ALDH^{high} and ALDH^{low} cells are 10.03 ± 0.65 (NCI-N87) and 10.27 ± 1.09 (SNU-1) vs. 6.63 ± 0.43 (SGC-7901) and 3.33 ± 0.43 (BGC-823) ($P < 0.01$).

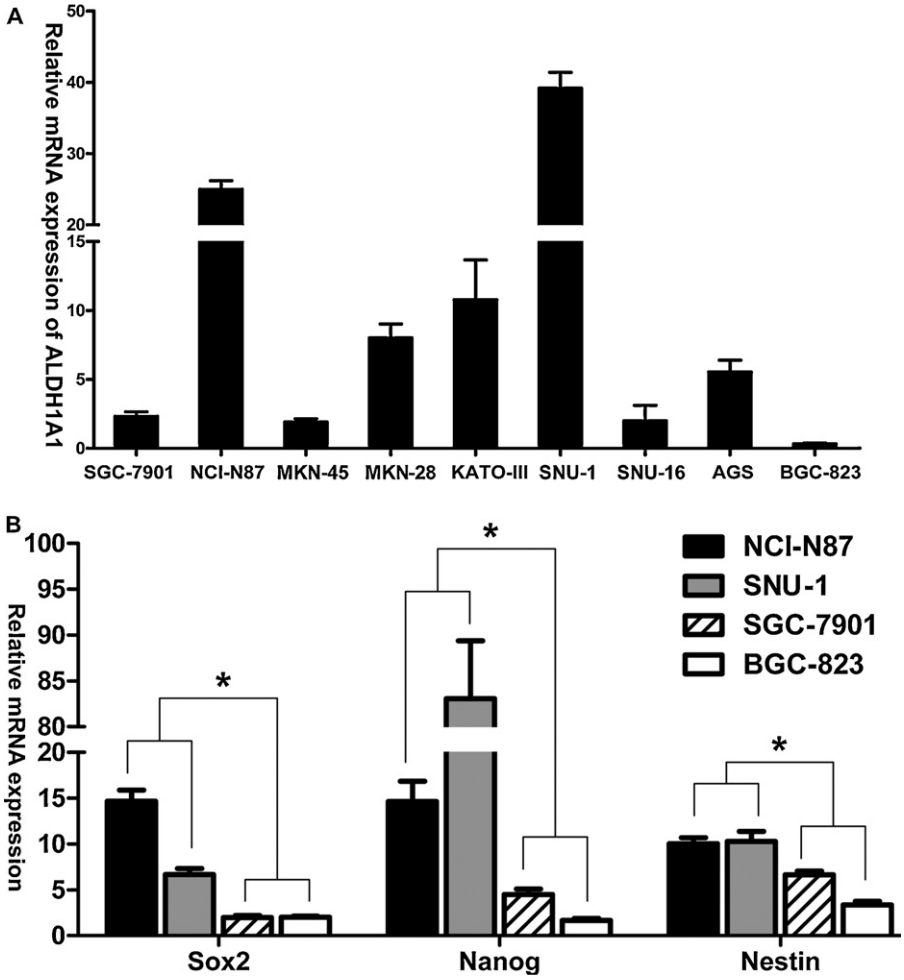

Fig. 2. Examination of ALDH1A1 mRNA and other stem cell markers by qRT-PCR. A. The bar chart is an overview of ALDH1A1 mRNA expression in different gastric cancer cell lines. From this analysis combined with Aldeflour® assay, we selected NCI-N87 and SNU-1 as ALDH^{high} cells, and SGC-7901 and BGC-823 as ALDH^{low} cells. B. The bar chart of mRNA expression of Sox2, Nanog and Nestin. The expressing levels of Sox2, Nanog and Nestin are significantly higher in ALDH^{high} cells (NCI-N87 and SNU-1), compared to that in ALDH^{low} cells (SGC-7901 and BGC-823). The asterisk indicates the statistical significance between ALDH^{high} cells and ALDH^{low} cells ($P < 0.01$). The data represent the mean ± SD of three independent experiments.

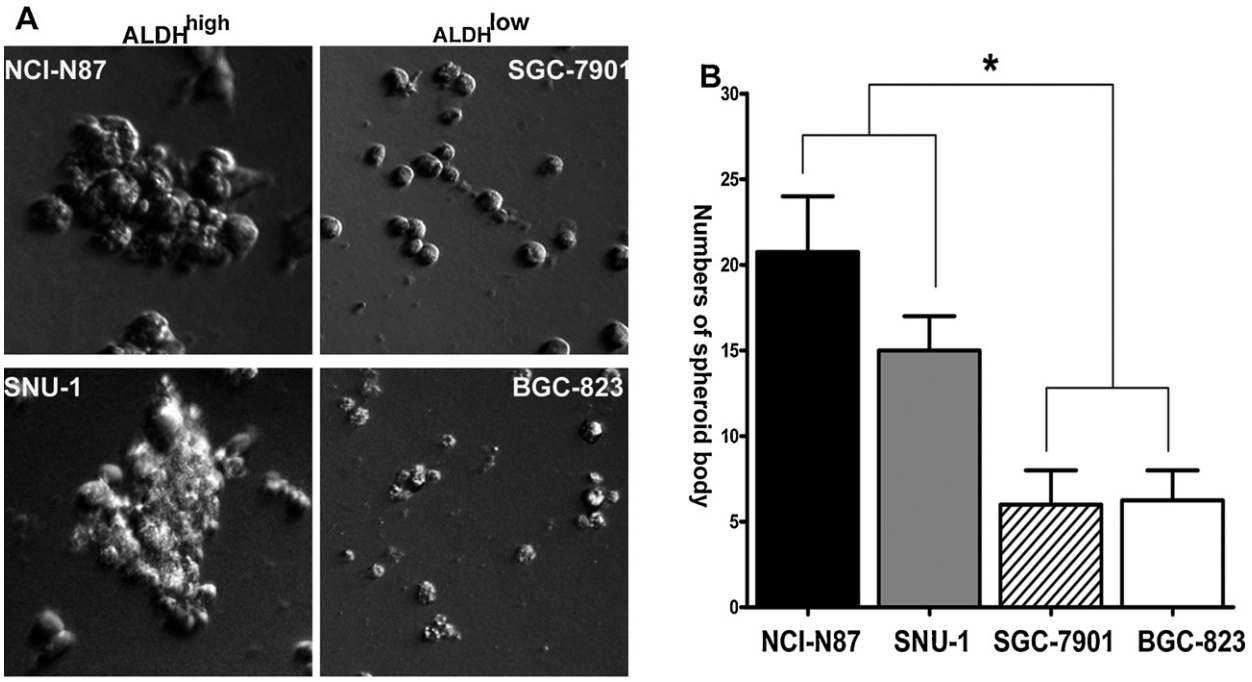

Fig. 3. Analysis of gastrosphere bodies in serum-free media. A. Representative image of floating spheroid bodies. Obvious gastrosphere formation is observed on ALDH^{high} cells (NCI-N87 and SNU-1), compared to ALDH^{low} cells (SGC-7901 and BGC-823). B. The bar chart is a comparison of the numbers of floating spheroid body between different cell lines (*$P < 0.01$).

3.3. Gastrosphere bodies assay on ALDH^{high} and ALDH^{low} cell lines

As shown in Fig. 3A, under the serum-free culture medium with EGF and bFGF after 7-day incubation, the ability to form floating spheroid bodies in ALDH^{high} cells of NCI-N87 and SNU-1 cells are 20.75 ± 3.30 and 15 ± 1.83, respectively. They are significantly higher than that in ALDH^{low} cells of SGC-7901 (6.00 ± 1.41) and BGC-823 (6.25 ± 1.26) (Fig. 1B, $P < 0.01$).

3.4. Colony formation on ALDH^{high} and ALDH^{low} cell lines

ALDH^{high} cells (1×10^5) from NCI-N87 or SNU-1, as well as ALDH^{low} cells (1×10^5) from SGC-7901 or BGC-823 are seeded into soft agar media for three weeks incubation at 37 °C, 5% CO_2 condition. As shown in Fig. 4A, the colony numbers of NCI-N87 and SNU-1 are higher than those observed in SGC-7901 and BGC-823 cells. The colony numbers of NCI-N87 and SNU-1 are 12.3 ± 1.64

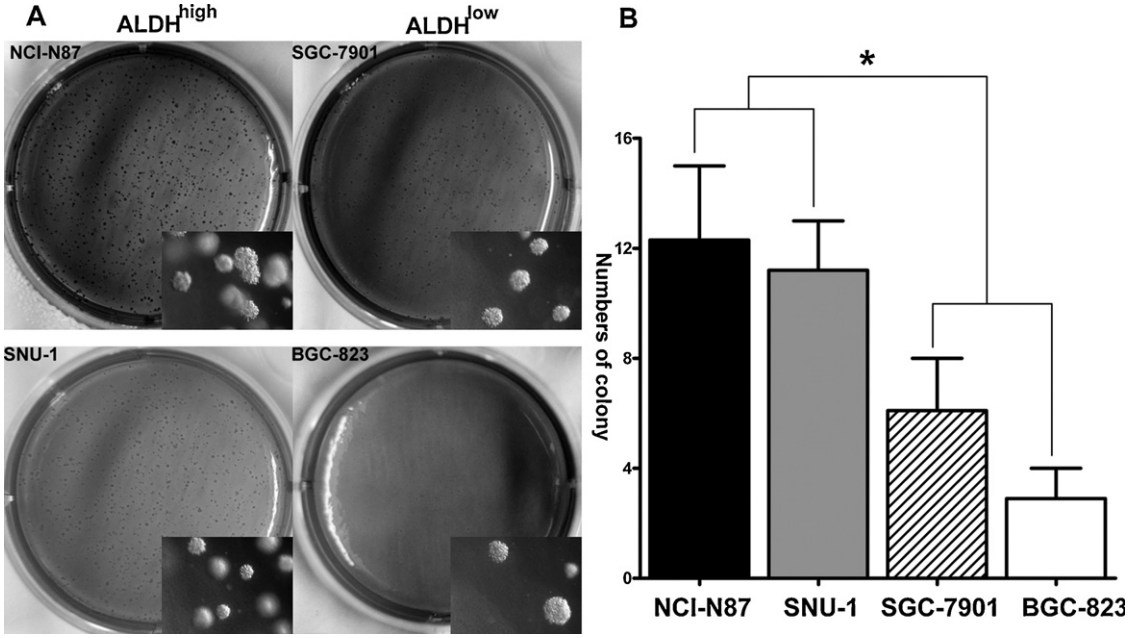

Fig. 4. Colony formation assay in soft agar media. A. Representative images of colony formation. The small pictures in right-low corner are high power magnification. Obvious colony formation is observed on ALDH^{high} cells (NCI-N87 and SNU-1), compared to ALDH^{low} cells (SGC-7901 and BGC-823). B. The bar chart is a comparison of numbers of colony between different cell lines (*$P < 0.01$).

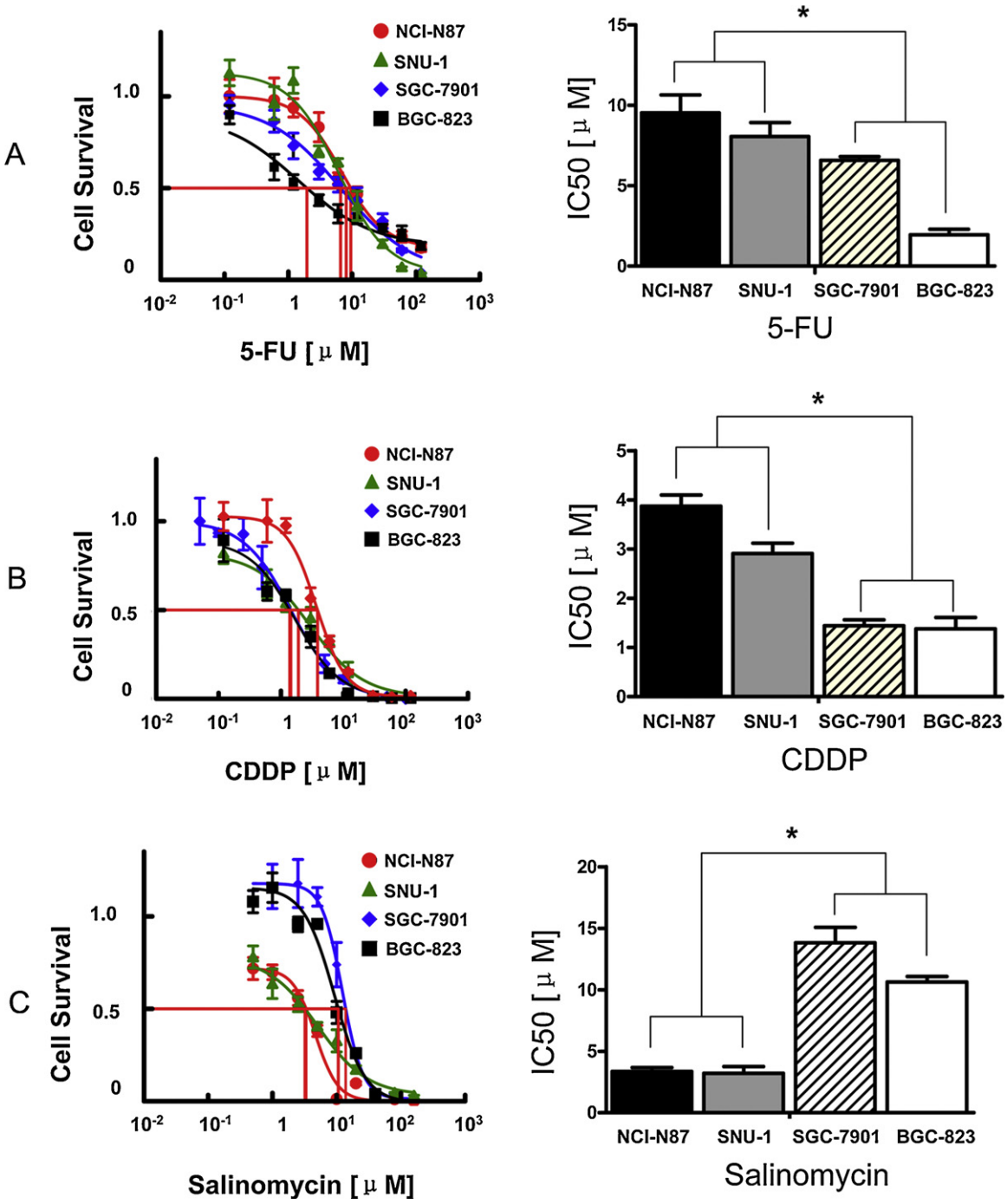

Fig. 5. Drugs sensitivity analysis by cell counting kit-8. A. Dose-response curves and drug concentration of IC 50 for 5-Fu between ALDH^{high} and ALDH^{low} gastric cancer cell lines. B. Dose-response curves and drug concentration of IC 50 for CDDP between ALDH^{high} and ALDH^{low} gastric cancer cell lines. C. Dose-response curves and drug concentration of IC 50 for salinomycin between ALDH^{high} and ALDH^{low} gastric cancer cell lines. The ALDH^{high} cells of NCI-N87 and SNU-1 are more sensitive to salinomycin than that in ALDH^{low} cells of SGC-7901 and BGC-823 (*$P < 0.01$). But ALDH^{high} cells of NCI-N87 and SNU-1 are not sensitive to 5-Fu and CDDP, compared to that in ALDH^{low} cells of SGC-7901 and BGC-823 (*$P < 0.01$).

and 11.2 ± 1.14, respectively. While the numbers of colony in SGC-7901 and BGC-823 are 6.1 ± 1.29 and 2.9 ± 0.99, respectively ($P < 0.01$) (Fig. 4B).

3.5. Analysis of chemosensitivity on ALDH^{high} and ALDH^{low} cell lines

We assayed chemosensitivity for 5-Fu, CDDP and salinomycin between ALDH^{high} and ALDH^{low} gastric cancer cell lines. We found that the IC50 of NCI-N87 and SNU-1 for 5-Fu was 9.53 ± 1.12 μM and 8.06 ± 0.87 μM, respectively, while, the IC50 of SGC-7901 and BGC-823 cells was 6.58 ± 0.23 μM and 1.95 ± 0.34 μM (Fig. 5A, $P < 0.05$). The IC50 of NCI-N87 and SNU-1 for CDDP was 3.87 ± 0.23 μM and 2.91 ± 0.21 μM, while, it was 1.44 ± 0.12 μM and 1.38 ± 0.23 μM in SGC-7901 and BGC-823 cells, respectively (Fig. 5B, $P < 0.01$). The IC50 of NCI-N87 and SNU-1 for salinomycin was 3.35 ± 0.32 μM and 3.21 ± 0.53 μM, while, it was 13.83 ± 1.24 μM and 10.63 ± 0.45 μM in SGC-7901 and BGC-823 cells, respectively (Fig. 5C, $P < 0.01$). It means that sensitivity of salinomycin for ALDH^{high} cell lines NCI-N87 and SNU-1 was 4.13 to 4.29-fold higher than that for ALDH^{low} cell lines SGC-7901,

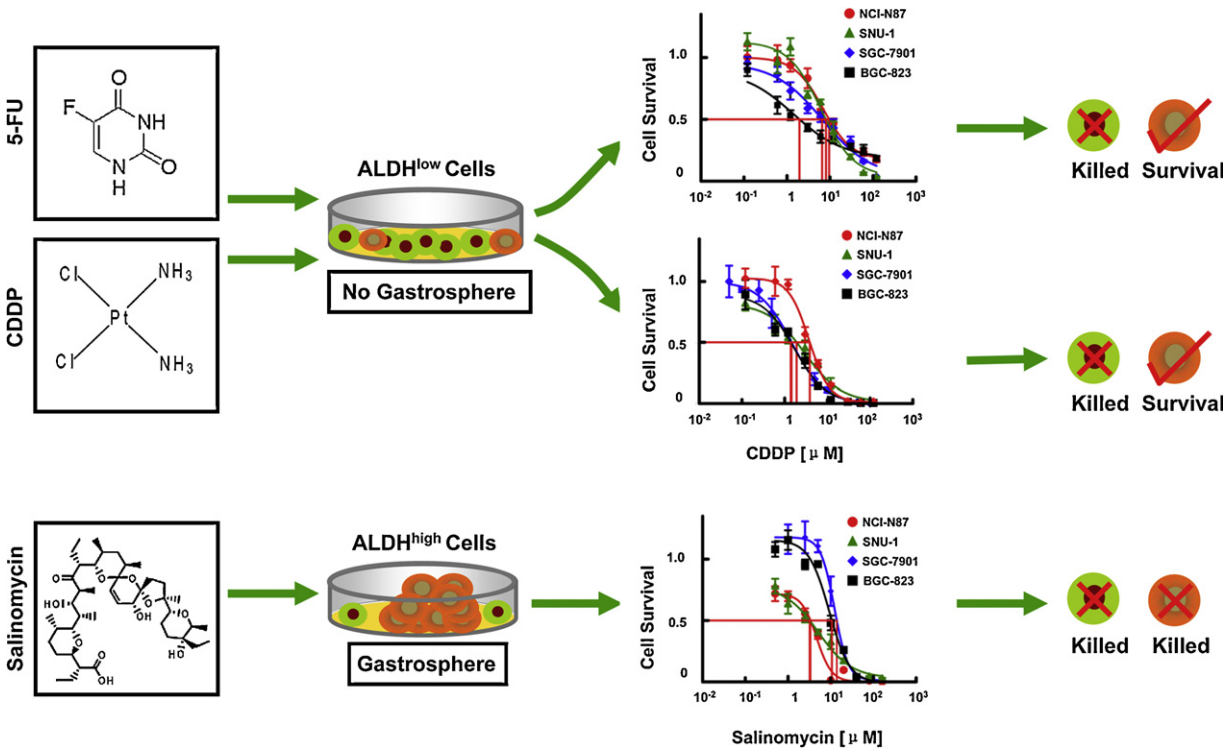

Fig. 6. Schematic summary of salinomycin on ALDH^{high} and ALDH^{low} gastric cancer cell lines. Salinomycin can effectively kill ALDH^{high} cancer cells on gastric cancer. In our study, conventional anticancer drugs 5-Fu and CDDP are used for controls.

respectively. While, the sensitivity of salinomycin for ALDH^{high} cell lines NCI-N87 and SNU-1 was 3.17 or 3.31-fold higher than that for ALDH^{low} cell lines BGC-823, respectively.

4. Discussion

Gastric cancer remains a leading cause of morbidity and mortality in Asia, mainly due to metastasize to peritoneal cavity and distant sites. Tumor recurrence can take place after decades of apparent disease-free survival. The mechanisms underlying remain poorly understood. Current evidence suggests that CSCs contribute to survive from current cancer therapies and to initiate relapse, long-term recurrence and metastasis. The Aldeflour® assay was originally developed to detect ALDH activity in hematopoietic tissues. The fluorescent Aldeflour® reaction product accumulates in stem cells and correlates with ALDH activity [20]. Since higher ALDH activity represents one of CSCs features [12,20,23–26], we screened stem cell fractions in several gastric cancer cell lines by aldehyde dehydrogenase assay combined with stem cell marker analysis. We demonstrated that gastric cancer cells with higher ALDH activity may express high levels of embryonic stem cell markers, including Sox2, Nanog and Nestin. Gastric cancer cell lines NCI-N87 and SNU-1 expressed higher levels of ALDH activities (defined as ALDH^{high}), compared to others. It is suggested that different cell lines express different levels of ALDH. The ALDH^{high} cancer cells indeed retained more stem cell-like properties like other's reports. Moreover, ALDH^{high} cells can form more spheroid bodies and more colony formation, which are characteristics of CSCs [27–30]. By IC 50 analysis, we found that ALDH^{high} cancer cells of NCI-N87 and SNU-1 are more resistance to conventional anticancer drugs 5-Fu and CDDP, but sensitive to salinomycin compound. Salinomycin was recently proposed as CSCs killer by American scientists. It can inhibit breast cancer CSCs in mice [8]. Salinomycin is a polyether antibiotic isolated from *S. albus* that acts in different biological membranes as an ionophore with a strong selectivity for potassium. This is the first report about efficacy of salinomycin on gastric cancer. Our experiments reveal that although ALDH^{high} gastric cancer cell lines were resistant to conventional chemotherapy drugs, but still sensitive to CSCs killer salinomycin. The sensitivity of salinomycin for ALDH^{high} gastric cancer cell lines is over 4-fold higher than that for ALDH^{low} cancer cells. Our result is consistent to other's finding [8,10,31]. Salinomycin as a new CSCs killer should be regarded as an effective agent for eliminating CSCs and other tumor cells exhibiting ABC transporter-mediated multidrug resistance [11,31].

In order to improve overall survival of cancer patient, much effort has been made. Some therapies are targeting growth factor receptors and others focused on signaling pathways [32–34]. These strategies can shrink the malignant mass and achieve clinical response. However, too often these responses are followed by eventual regrowth of the tumor. The bottleneck could be explained by existence of CSCs in tumor mass. One ideal anticancer strategy would be to look for agents that target both the CSCs and non-CSCs within tumors. Alternatively, it may be preferable to develop combination therapies that apply agents with specific toxicity for CSCs together with agents that specifically target non-CSC populations within tumors. Therefore, the finding of targeting CSCs subpopulation should be improved the current treatments against highly aggressive, metastatic, recurrent, and lethal CSCs subpopulation (Fig. 6).

In summary, our study firstly demonstrated that ALDH^{high} gastric cancer cell lines have more characteristics of CSCs than ALDH^{low} cells have. Detection of ALDH activity may take as one of CSCs parameters. Most importantly, the sensitivity for salinomycin at ALDH^{high} gastric cancer cells was over 4-fold superior to that in ALDH^{low} cancer cells. These findings will provide pivotal clue for selective chemotherapy on gastric carcinoma.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

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