Salinomycin induces apoptosis in cisplatin-resistant colorectal cancer cells by accumulation of reactive oxygen species☆

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HIGHLIGHTS

- Cisp-resistant SW620 cells maintained as a relative quiescent state (G0/G1 arrest).
- Cisp-resistant SW620 cells displayed more stem-like signatures.
- Cisp-resistant SW620 cells were sensitive to salinomycin.
- Salinomycin induced more apoptosis in Cisp-resistant SW620 cells.

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ABSTRACT

Postoperative chemotherapy for Colorectal cancer (CRC) patients is not all effective and the main reason might lie in cancer stem cells (CSCs). Emerging studies showed that CSCs overexpress some drug-resistance related proteins, which efficiently transport the chemotherapeutics out of cancer cells. Salinomycin, which considered as a novel and an effective anticancer drug, is found to have the ability to kill both CSCs and therapy-resistant cancer cells. To explore the potential mechanisms that salinomycin could specifically target on therapy-resistant cancer cells in colorectal cancers, we firstly obtained cisplatin-resistant (Cisp-resistant) SW620 cells by repeated exposure to 5 μmol/l of cisplatin from an original colorectal cancer cell line. These Cisp-resistant SW620 cells, which maintained a relative quiescent state (G0/G1 arrest) and displayed stem-like signatures (up-regulations of Sox2, Oct4, Nanog, Klf4, Hes1, CD24, CD26, CD44, CD133, CD166, Lgr5, ALDH1A1 and ALDH1A3 mRNA expressions) ($p < 0.05$), were sensitive to salinomycin ($p < 0.05$). Salinomycin did not show the influence on the cell cycle of Cisp-resistant SW620 cells ($p > 0.05$), but could induce cell death process ($p < 0.05$), with increased levels of LDH release and MDA contents as well as down-regulations of SOD and GSH-PX activities ($p < 0.05$). Our data also showed that the pro-apoptotic genes (Caspase-3, Caspase-8, Caspase-9 and Bax) were up-regulated and the anti-apoptotic gene Bcl-2 were down-regulated in Cisp-resistant SW620 cells ($p < 0.05$). Accumulated reactive oxygen species and dysregulation of some apoptosis-related genes might ultimately lead to apoptosis in Cisp-resistant SW620 cells. These findings will provide new clues for novel and selective chemotherapy on cisplatin-resistant colorectal cancer cells.

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1. Introduction

Colorectal cancer (CRC) is the third most common malignancy worldwide with increasing incidence over the past years (Sung et al., 2005). Surgical resection remains the most effective treatment for CRC. However, after the radical surgery, the overall five-year survival rate still remains poor. Most of the CRC patients die from recurrence and metastasis (Stillwell et al., 2011). Furthermore, postoperative chemotherapy for CRC patients is not all effective. A small proportion of patients are naturally tolerant to

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some traditional chemotherapeutics, and the main reason might lie in cancer stem cells (CSCs) (Morrison et al., 2011).

CSCs are a small proportion of cancer cells that exist in the cancer cell population, that have the ability to self-renew and undergo differentiation. And CSCs are found to be resistant to conventional cancer treatments, including chemotherapeutic drugs and radiation therapy (Dalerba et al., 2007; Santin, 2009; Tan et al., 2006). CSCs highly express ATP-binding drug transporters. Standard chemotherapy or radiation therapy is effective in killing the bulk of the tumor but not the CSCs. The mortality of cancer remains high, because conventional therapies often fail to eradicate the CSC population, allowing relapse to occur (Lu et al., 2011). Alternatively, tumor drug-resistant cells induced by traditional chemotherapeutics also present cancer stem-like characteristics (Gopalan et al., 2013). So novel therapeutic strategies targeting specifically on CSCs are urgently needed.

In 2009, Gupta et al. identified salinomycin by a high throughput screening that could potentially be used to target breast CSCs, and it killed breast CSCs at least 100 times more effectively than paclitaxel in mice (Gupta et al., 2009). Emerging evidences showed that salinomycin could effectively kill various types of cancer stem-like cells, including gastric cancer (Zhi et al., 2011), pancreatic cancer (He et al., 2013; Zhang et al., 2011), hepatocellular carcinoma (Wang et al., 2012), ovarian cancer (Zhang et al., 2012), prostate cancer(Ketola et al., 2012; Kim et al., 2011) and some other types of malignancy (Kuo et al., 2012; Scherzed et al., 2013). However, the mechanism underlying salinomycin and drug-resistant cells in CRC has not been studied yet.

In our study, we continuously treated the coloretal cancer cells by 5 μmol/l of cisplatin and successfully obtained the Cisp-resistant SW620 cells. Then we detected some specific stem cell markers to confirm whether Cisplatin-resistant SW620 cells displayed a stem-like signature. In order to discuss the killing effect of salinomycin on Cisplatin-resistant SW620 cells, we treated Cisplatin-resistant SW620 cells and orginal SW620 cells with a certain concentration of salinomycin, and observed the change of cell cycle and apoptosis. Meanwhile, we also evaluated the levels of oxidative stress markers (LDH release, MDA contents, SOD and GSH-PX activities) and apoptosis-related genes (Caspase-3, Caspase-8, Caspase-9, Bcl-2 and Bax) to explore the possible mechanism of salinomycin on Cisp-resistant SW620 cells.

2. Materials and methods

2.1. Chemicals and cell culture

Cisplatin and salinomycin were obtained from Sigma-Aldrich (Sigma, USA). Human colorectal cancer cell line SW620 was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (Gibco, Invitrogen) in a humidified atmosphere of 5% CO_2 at 37 °C. Cells were in the logarithmic phase of growth for all experiments.

2.2. 50% inhibiting concentration (IC50) and cell proliferation analysis by cell counting kit-8

For cisplatin or salinomycin IC50 analysis in SW620 cells or Cisp-resistant SW620 cells, cells (1×10^4/well) were cultured in 96-well plates and treated with different chemotherapeutics (cisplatin, salinomycin) in different concentrations for 48 h. Then 20 μl of cell counting kit-8 (CCK-8, Dojindo, Kumamoto, Japan) was added into each of the 96-wells. After 4 h incubation at 37 °C, the optical density (OD) values were detected at 450 nm using the scan reader (Labsystems, Santa Fe, NM, USA). Cell growth inhibiting rates were described as cell inhibiting curves and the IC50 parameters (inhibiting concentration of 50% cells) were evaluated by Xlfit 5.2 software (IDBS, UK). For cell proliferation analysis, SW620 cells or Cisp-resistant SW620 cells (5×10^3/well) were also seeded in 96-well plates in serum-containing medium and treated with cisplatin (5 μmol/l, according to the calculated IC50 values of cisplatin in SW620 cells) for 0, 12, 24, 48, 72 and 96 h. Then 20 μl cell counting kit-8 was added into each of the 96-wells. After 4-h incubation at 37 °C, the coloring reactions were also quantified at 450 nm.

2.3. Continuous treatment with cisplatin

SW620 cells were grown in 10-cm culture dishes and treated with proper concentration of cisplatin (5 μmol/l) according to the results of IC50. We replaced the culture medium every 7 day. After continuous exposure to non-lethal levels of cisplatin for 3 months, the Cisp-resistant SW620 cells were obtained (Fig. 1).

2.4. Real-time PCR detection

Total RNA was extracted using Trizol solution (Invitrogen,USA) according to the manufacturer's instructions. The quantities and qualities of isolated RNAs were evaluated using absorbance measurements at 260 and 280 nm. Then reverse transcription (RT) was performed in a 20-μl reaction system using the ReverAid First Stand cDNA Synthesis (Thermo Scientific, Mountain View, CA, USA). Primer sequences for GAPDH were 5′-GGACCTGACCTGCCGTCTAG-3′ (Forward) and 5′-GTAGCCCAGGATGCCCTTGA-3′ (Reverse). Other primer sequences were all listed in Table 1. RT-PCR with SybGreen I (Generay Bio Co., Shanghai, China) was performed using the 7500 real-time PCR system (Applied Biosystems, Hayward, CA, USA) with the follow program: initial denature at 95 °C for 5 min, followed by 40 cycles for 95 °C for 15 s, annealing at 60 °C for 60 s, and 72 °C for 30 s. All samples were processed in triplicate. The mRNA expression of each gene was calculated using the relative quantitative $2^{-\triangle\triangle CT}$ method.

2.5. Cell cycle analysis

Cells were seeded at 5×10^4 per well in 6-well plates in triplicate. 48 h after salinomycin treatment (0.25 μmol/l) or not, SW620 cells and Cisp-resistant SW620 cells were collected, washed twice in 1x PBS, mixed in 200 μl of 1x binding buffer, and incubated at room temperature for 15 min with 250 μg/ml propidium iodide (PI) (Sigma, St. Louis, USA) and 5 μg/ml Rnase (BD Biosciences, USA). The cell cycle was analyzed by FACSscan flow cytometer. Each treatment was performed in triplicate.

2.6. Hoechst33342 staining

Cells were seeded at 5×10^4 per well in 6-well plates in triplicate. 48 h after salinomycin treatment (0.25 μmol/l) or not, SW620 cells and Cisp-resistant SW620 cells (2×10^5 ml^{-1}) were harvested and added 10 μl of Hoechst33342 solution (Keygen Biotech, Nanjing, China) for 15 min in the dark at 37 °C. After centrifuging for 5 min and washing with 1x PBS, 1 ml of Buffer A solution (Keygen Biotech, Nanjing, China) was added at room temperature and mixed. Finally, 20 μl of cell pellet was dropped on a glass slide and the image was observed under an inverted phase-contrast fluorescence microscope (Olympus, Japan).

2.7. Annexin-V-PE staining

Cells were seeded at 5×10^4 per well in 6-well plates in triplicate. 48 h after salinomycin treatment (0.25 μmol/l) or not, SW620 cells and Cisp-resistant SW620 cells were collected. Cells were double stained with Annexin-V-PE (BD Pharmingen, San Jose, CA, USA) and propidium iodide (PI) (Sigma, St. Louis, USA) following the manufacturer's instructions. Cell apoptosis was determined by FACSscan flow cytometer. Each treatment was performed in triplicate.

2.8. LDH release assay

LDH activity was evaluated using a colorimetric LDH assay. Cell were seeded at 1×10^4cells/200 μl in each 96-well plate and pre-treated with salinomycin (0.25 μmol/l) for 48 h. Briefly, 100 μl supernatant was transferred from each well to a 96-well plate and 100 μl freshly reaction mixture was added to each well. After 30 min of incubation at room temperature in the dark, the optical density (OD) values were detected at 490 nm using the scan reader (Labsystems, Santa Fe, NM, USA). The amount of LDH was calculated as a percent compared to the total amount of LDH present in cell treated with 1% Triton-X 100 (Solarbio, Shanghai, China).

2.9. Detections of oxidative stress markers (MDA, SOD and GSH-PX)

Cells were seeded at 5×10^4 per well in 6-well plates in triplicate. 48 h after salinomycin treatment (0.25 μmol/l), cells were harvested and the extracted proteins were quantified. We measured the MDA contents, SOD and GSH-PX activities with commercial reagent kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.10. Western blot analysis

Cells were harvested and lysed with mammalian protein extraction reagent (Pierce, Rockford, IL, USA). Total protein (30 μg) was separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Immobilon-P Transfer Membrane (Millipore, Ballerica, USA). Then the membranes were blocked for 2 h with 5% non-fat dry milk in PBS and incubated with primary antibodies (Caspase-3, Caspase-8 and Caspase-9, Bioworld Technology, USA; Bax and Bcl-2, Cell Signaling Technology, USA). Second antibodies were conjugated to
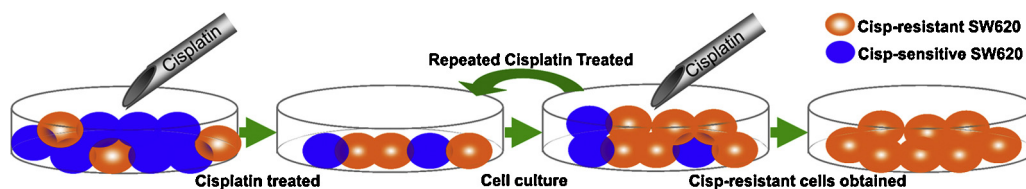
Fig. 1. After continuous exposure to cisplatin (5 μmol/l) for 3 months, Cisp-resistant SW620 cells were obtained.

horse radish peroxidase (HRP) and signals were detected by chemiluminescence. Protein levels were normalized to the total glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using a rabbit monoclonal anti-GAPDH antibody (Sigma, USA).

2.11. Statistical analysis

Statistical analysis was performed with SPSS 17.0 software (SPSS Inc, Chicago, USA). Statistically significant differences were estimated using one-way analysis of variance followed by Student t-test. Data was presented as the mean ± SD and $p < 0.05$ was considered as significant.

3. Results

3.1. Identification of Cisp-resistant SW620 cells

Based on the IC50 value of SW620 cells to cisplatin(6.11 ± 1.52 μmol/l), we selected a proper concentration (5 μmol/l) to induce Cisp-resistant cells. After continuous treatment with cisplatin for 3 months, we successfully obtained the Cisp-resistant SW620 cells (Fig. 1). Then we used a series of experiments to identify that whether our induced cells were relatively resistant to cisplatin. As shown in Fig. 2A and B, we compared the IC50 values between SW620 (6.11 ± 1.52 μmol/l) and Cisp-resistant SW620 cells (51.12 ± 4.98 μmol/l). Our results demonstrated that Cisp-resistant SW620 cells were relatively tolerant to the toxic effects of cisplatin. Moreover, with a certain concentration of cisplatin treatment (5 μmol/l), we compared the optical density (OD) values, and found that the proliferation activity of Cisp-resistant cells was very stable, while the proliferation activity of SW620 cells decreased at 12, 24, 48, 72 and 96 h ($p < 0.05$) (Fig. 2C). Our results manifested that the Cisp-resistant SW620 cells could be relatively resistant to cisplatin treatment and suitable for our further studies.

3.2. Expressions of CSCs related genes in Cisp-resistant SW620 cells

In order to better understand the possible and unique traits of Cisp-resistant SW620 cells that distinguished from the original SW620 cells. We detected the mRNA expressions of some key CSCs related genes by Real-time PCR, including stem cell related transcription factors (Sox2, Oct4, Nanog, Klf4 and Hes1), surface biomarkers (CD24, CD26, CD44, CD133, CD166, Lgr5), functional markers (ALDH1A1 and ALDH1A3), and drug resistance-related proteins (MDR1, MRP1 and LRP) in SW620 cells and Cisp-resistant SW620 cells. Our results in Fig. 2D and E showed that the expressing levels of Sox2, Oct4, Nanog, Klf4, Hes1, MDR1, MRP1, LRP, CD24, CD26, CD44, CD133, CD166, Lgr5, ALDH1A1 and ALDH1A3 were significantly higher in Cisp-resistant SW620 cells than those in SW620 cells respectively ($p < 0.05$), but there was no difference of ALDH2 mRNA expressions between SW620 cells and Cisp-resistant SW620 cells ($p > 0.05$).

3.3. Salinomycin did not change cell cycle in Cisp-resistant SW620 cells

After salinomycin treatment or not, we examined the change of cell cycle between SW620 and Cisp-resistant SW620 cells. As shown in Fig. 3A, Our results showed that repeated treatment of cisplatin could block the cell cycle and induce G0/G1 phase arrest, while cells of S and G2/M phase decreased accordingly ($p < 0.05$). This phenomenon meant that Cisp-resistant SW620 cells maintained a relative quiescent state compared to original SW620 cells. We also examined the change of cell cycle after salinomycin treatment both in SW620 and Cisp-resistant SW620 cells by flow cytometry. Our data indicated that salinomycin did not change the process of cell cycle including

Table 1
Real-time PCR primer sequences.

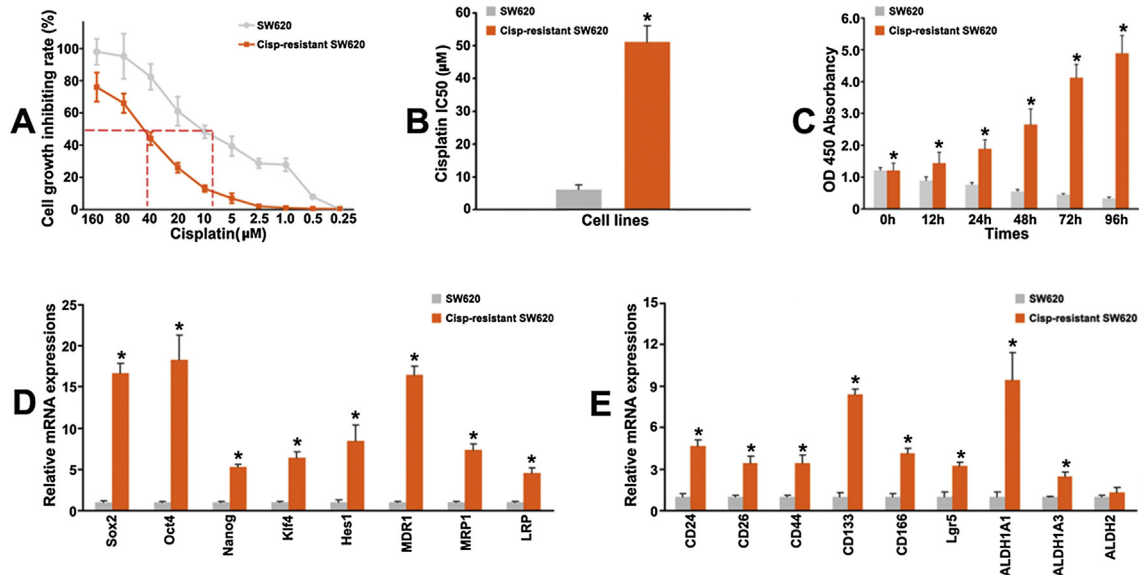| Gene name		Primer sequence (5′–3′)	Gene name		Primer sequence (5′–3′)
Sox2	F	GCCGAGTGGAAACTTTTGTCG	CD133	F	TTCTTGACCGACTGAGACCCA
	R	GCAGCGTGTACTTATCCTTCTT		R	TCATGTTCTCCAACGCCTCTT
Oct4	F	CTTGAATCCCGAATGGAAAGGG	CD166	F	TCCTGCCGTCTGCTCTTCT
	R	GTGTATATCCCAGGGTGATCCTC		R	TTCTGAGGTACGTCAAGTCGG
Nanog	F	TAACCTTGGCTGCCGTCTCTGG	Lgr5	F	CTCCCAGGTCTGGTGTGTTG
	R	AAGCCTCCCAATCCCAAACAATACG		R	GAGGTCTAGGTAGGAGGTGAAG
Klf4	F	CGGACATCAACGACGTGAG	ALDH1A1	F	AGCCTTCACAGGATCAACAGA
	R	GACGGCCTTCAGCACGAACT		R	GTCGGCATCAGCTAACACAA
Hes1	F	TCAACACGACACCGGATAAAC	ALDH1A3	F	GCCCTTTATCTCGGCTCTCT
	R	GCCGCGAGCTATCTTTCTTCA		R	CGGTGAAGGCGATCTTGT
MDR1	F	CTGTGGATCACCGAGAGGG	ALDH2	F	ACCTGGTGGATTTGGACATGGTCC
	R	ACAGGCGATGGTCCAGTAGA		R	TCAGGAGCGGGAAATTCCACGGA
MRP1	F	GAGGAACCATATTACAGGTCCGT	Caspase-3	F	CATGGAAGCGAATCAATGGACT
	R	AGGGGATCATCGAAGAGGTAAAT		R	CTGTACCAGACCGAGATGTCA
LRP	F	CCCGCGACAGTTTCCAATGA	Caspase-8	F	CGGACTCTCCAAGAGAACAGG
	R	GGCGTTGAGACCAGGTTTCA		R	TCAAAGGTCGTGGTCAAAGCC
CD24	F	CTCCTACCCACGCAGATTTATTC	Caspase-9	F	CTCAGACCAGAGATTCGCAAAC
	R	AGAGTGAGACCACGAAGAGAC		R	GCATTTCCCCTCAAACTCTCAA
CD26	F	TACAAAAGTGACATGCCTCAGTT	Bcl-2	F	GGTGGGGTCATGTGTGTGG
	R	TGTGTAGAGTATAGAGGGGCAGA		R	CGGTTCAGGTACTCAGTCATCC
CD44	F	CTGCCGCTTTGCAGGTGTA	Bax	F	CCCGAGAGGTCTTTTTCCGAG
	R	CATTGTGGGCAAGGTGCTATT		R	CCAGCCCATGATGGTTCTGAT

Fig. 2. (A) Cell inhibiting curves were drawn after treatment with different concentrations of cisplatin for 48 h. (B) The IC50 values of cisplatin for 48 h between SW620 cells and Cisp-resistant SW620 cells were compared. (C) Cisplatin (5 μmol/l) could inhibit the growth of SW620 cells by CCK-8 assay, but could not inhibit the growth of Cisp-resistant SW620 cells. (D) Real-time PCR analysis showed that the expressing levels of Sox2, Oct4, Nanog, Klf4, Hes1, MDR1, MRP1, LRP were significantly higher in Cisp-resistant SW620 cells than those in SW620 cells. (E) Real-time PCR analysis showed that the expressing levels of CD24, CD26, CD44, CD133, CD166, Lgr5, ALDH1A1 and ALDH1A3 were significantly higher in Cisp-resistant SW620 cells than those in SW620 cells, but there was no difference in ALDH2 mRNA expressions. (* $p < 0.05$).
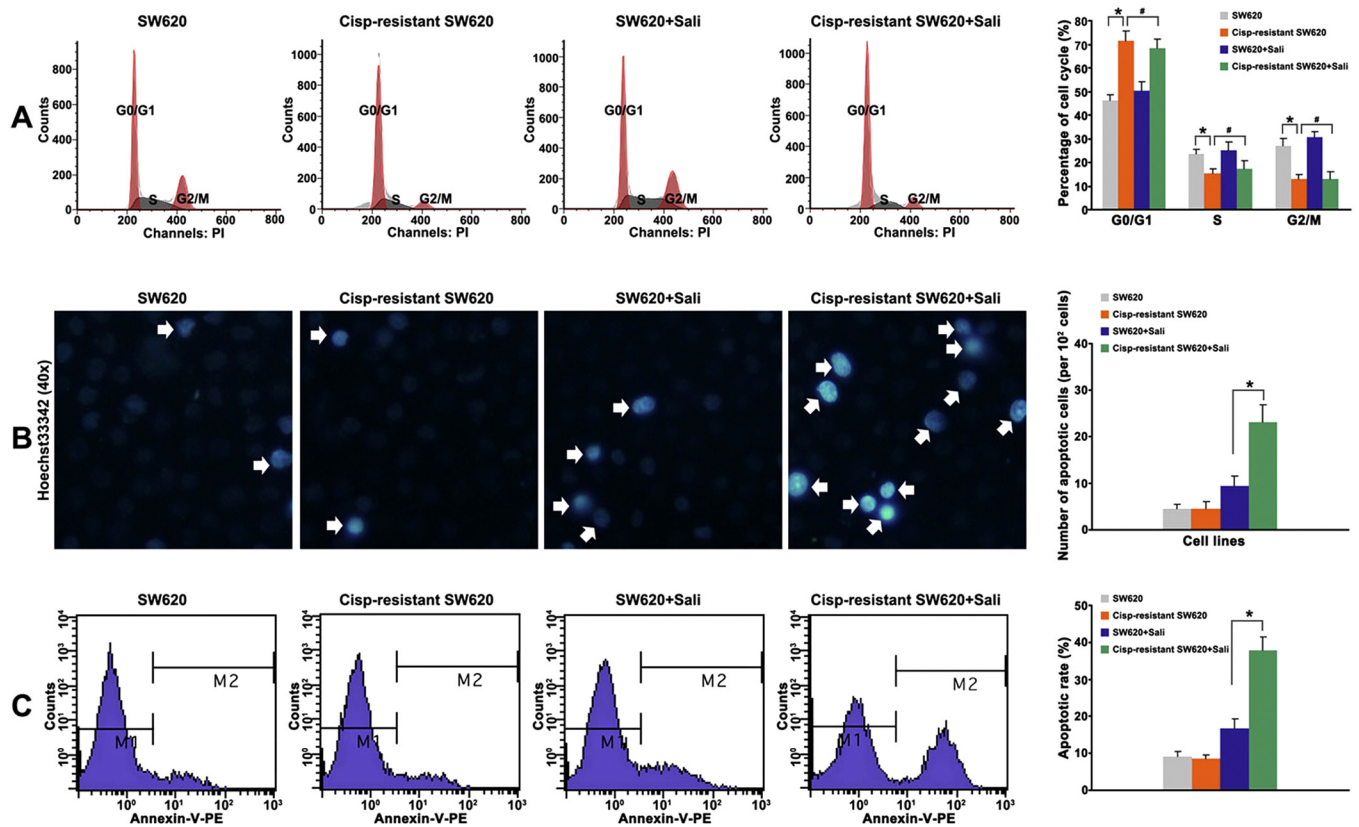

Fig. 3. (A) Compared to original SW620 cells, Cisp-resistant SW620 cells maintained a relative quiescent state. Cells of G0/G1 phase increased, while cells of S and G2/M phase decreased in Cisp-resistant SW620 cells. But salinomycin did not change the cell cycle of SW620 cells and Cisp-resistant SW620 cells. (B) By Hoechst33342 staining, salinomycin increased apoptotic cells in Cisp-resistant SW620 cells than those in SW620 cells. Arrows represented apoptotic cells. (C) By Annexin-V-PE staining, salinomycin significantly increased cell apoptosis in Cisp-resistant SW620 cells than those in SW620 cells. (* $p < 0.05$; # $p > 0.05$).
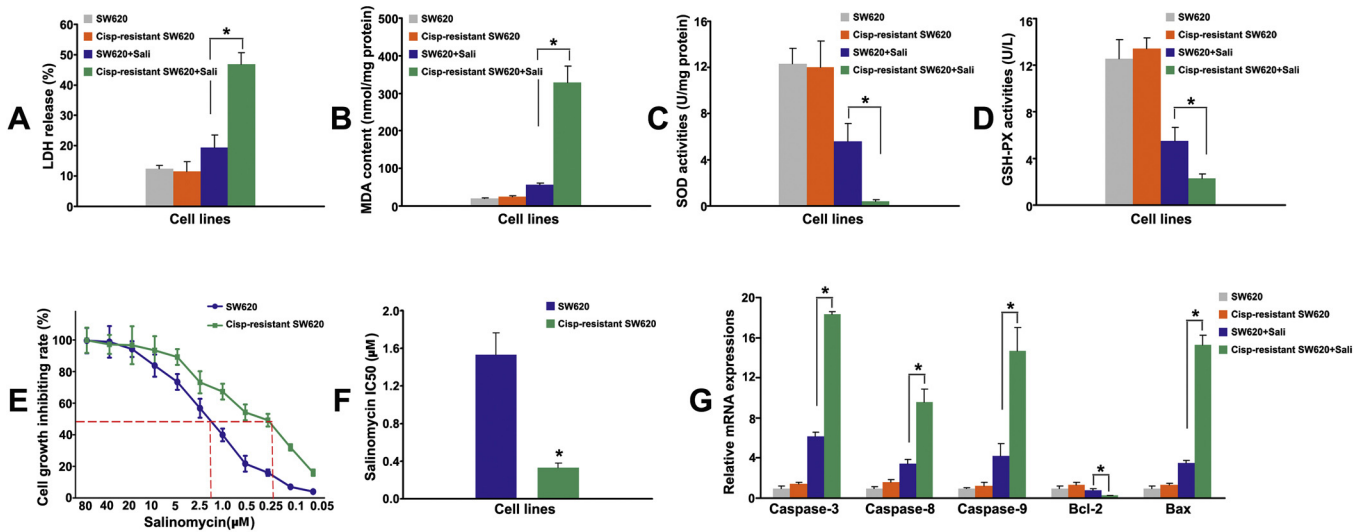
Fig. 4. (A) Compared to original SW620 cells, salinomycin treatment increased the release of LDH in Cisp-resistant SW620 cells. (B) Salinomycin treatment significantly increased MDA contents in Cisp-resistant SW620 cells. (C) and (D) Salinomycin treatment down-regulated SOD and GSH-PX activities in Cisp-resistant SW620 cells than those in SW620 cells. (E) Cell inhibiting curves were drawn after treatment with different concentrations of salinomycin for 48 h in SW620 cells and Cisp-resistant SW620 cells. (F) The IC50 values of salinomycin for 48 h between SW620 cells and Cisp-resistant SW620 cells were compared, and the results showed that salinomycin was extremely sensitive to Cisp-resistant SW620 cells. (G) Real-time PCR analysis showed that after salinomycin treatment, the expressing levels of Caspase-3, Caspase-8, Caspase-9 and Bax were significantly up-regulated in Cisp-resistant SW620 cells than those in SW620 cells, while Bcl-2 mRNA expression was down-regulated. ($* \ p < 0.05$).

G0/G1, S and G2/M phase in SW620 cells ($p > 0.05$). Meanwhile, salinomycin also did not influence the cell cycle in Cisp-resistant SW620 cells ($p > 0.05$).

3.4. Salinomycin increased cell apoptosis in Cisp-resistant SW620 cells by Hoechst33342 staining

After continuous salinomycin treatment for 48 h, the apoptotic cells were observed under the microscope and counted randomly at least 100 cells in one field. Our results showed that the number of apoptotic cells which were stained by Hoechst33342 was significantly increased in Cisp-resistant SW620 cells (20.20 ± 3.72) than that of SW620 cells (9.40 ± 2.07) per 100 cells ($p < 0.05$) (Fig. 3B).

3.5. Salinomycin induced cell apoptosis in Cisp-resistant SW620 cells by Annexin-V-PE staining

After treatment with salinomycin for 48 h, we used flow cytometric analysis to detect the cell apoptosis both in SW620 cells and Cisp-resistant SW620 cells. We found that the cell apoptotic rate in Cisp-resistant SW620 cells ($37.82 \pm 3.63\%$) was significantly higher than that of SW620 cells ($16.78 \pm 2.56\%$) ($p < 0.05$) (Fig. 3C).

3.6. Salinomycin increased LDH release and MDA contents, down-regulated SOD and GSH-PX activities in Cisp-resistant SW620 cells

To investigate the effect of salinomycin on the plasma membrane instability, we conducted the LDH retention assay. Our results showed that the LDH release of Cisp-resistant SW620 cells ($46.78 \pm 3.82\%$) was higher than that of SW620 cells ($19.32 \pm 4.12\%$) after treatment with salinomycin for 48 h ($p < 0.05$) (Fig. 4A). It demonstrated that salinomycin could greatly impact the plasma membrane structure of Cisp-resistant SW620 cells. To better investigate the change of oxidative stress caused by salinomycin, we detected MDA contents, SOD and GSH-PX activities both in SW620 cells and Cisp-resistant SW620 cells. Compared to original SW620 cells, the MDA contents in Cisp-resistant SW620 cells increased

significantly ($p < 0.05$) (Fig. 4B), while SOD and GSH-PX activities were decreased ($p < 0.05$) (Fig. 4C and D).

3.7. Cisp-resistant SW620 cells were extremely sensitive to salinomycin by IC50 comparison

By CCK-8 assay, the cell growth inhibiting curves were drawn and showed in Fig. 4E, and the IC50 values of salinomycin both in SW620 and Cisp-resistant SW620 cells were calculated. As shown in Fig. 4F, the IC50 of salinomycin in SW620 cells was $1.54 \pm 0.23 \ \mu mol/l$, while that in Cisp-resistant SW620 cells was only $0.32 \pm 0.05 \ \mu mol/l$ ($p < 0.05$). This data indicated that Cisp-resistant SW620 cells were relatively sensitive to salinomycin compared to SW620 cells.

3.8. Salinomycin influenced mRNA and protein expressions of Caspase-3, Caspase-8, Caspase-9, BCL-2 and Bax in Cisp-resistant SW620 cells

We further compared the Caspase-3, Caspase-8, Caspase-9, BCL-2 and Bax mRNA expressions in SW620 cells and Cisp-resistant SW620 cells. Compared to SW620 cells, we found that salinomycin could up-regulate Caspase-3, Caspase-8, Caspase-9 and Bax mRNA expressions in Cisp-resistant SW620 cells ($p < 0.05$). On the contrary, the Bcl-2 mRNA expressions in Cisp-resistant SW620 cells were down-regulated ($p < 0.05$). Furthermore, the ratio of Bcl-2/Bax in Cisp-resistant SW620 cells was significantly disordered (Fig. 4G). To gain further insight into the protein expressing levels of Caspase-3, Caspase-8, Caspase-9, BCL-2 and Bax in SW620 cells and Cisp-resistant SW620 cells, western blot was performed. As expected, salinomycin increased the protein expressions of Caspase-3, Caspase-8, Caspase-9 and Bax, but decreased Bcl-2 protein expression in Cisp-resistant SW620 cells, compared to SW620 cells (Fig. 5).

4. Discussion

Cisplatin is one the most commonly used chemotherapeutic agents in treatment of colorectal cancers. The cytotoxic effects of
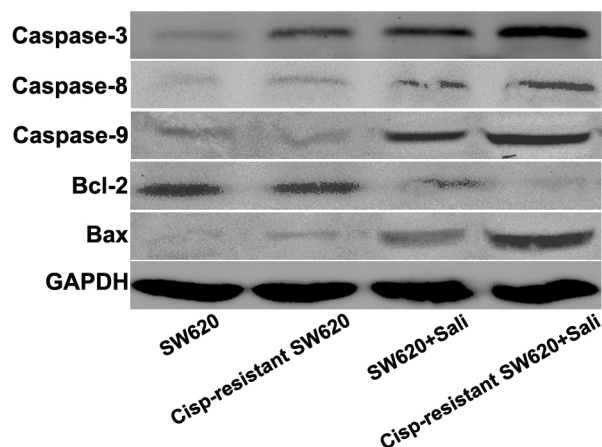
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Fig. 5. Western blot analysis showed that salinomycin could increased the protein expressions of Caspase-3, Caspase-8, Caspase-9 and Bax, but decreased Bcl-2 protein expressions in Cisp-resistant SW620 cells, compared to SW620 cells.

cisplatin are mediated by its interaction with DNA, resulting in the formation of DNA adducts which activate several signal transduction pathways and lead to cell apoptosis (Crul et al., 2002). Though it's widely indications and satisfactory anti-cancer effects in colorectal cancers, we still encounter lots of cisplatin-resistant cases. So there is an urgent need for a better understanding of the molecular mechanisms underlying the cisplatin resistance. Barr et al. illustrated that cisplatin-resistant non-small cell lung cancer cells showed a characteristic of stem cells, and that might be the direct cause of cell resistance (Barr et al., 2013). Wintzell et al. also found that repeated cisplatin treatment could induce a multi-resistant tumor cell population with stem cell features (Wintzell et al., 2012). These stem-like cells are likely to maintain a relative quiescent state, have the natural features that could be resistant to chemotherapy, and overexpress some drug-resistance related proteins, which efficiently transport the chemotherapeutics out of cancer cells (Barr et al., 2013; Moore and Lyle, 2011).

In our present study, we compared the original SW620 cells and Cis-resistant SW620 cells, and found that Cisp-resistant SW620 cells also displayed a stem-like signature in colorectal cancers with increased expressions of some stem cell related transcription factors (Sox2, Oct4, Nanog, Klf4 and Hes1), surface biomarkers (CD24, CD26, CD44, CD133, CD166, Lgr5), functional markers (ALDH1A1, ALDH1A3), and drug resistance-related proteins (MDR1, MRP1 and LRP) (Fig. 2D and E). We also confirmed that the Cisp-resistant SW620 cells presented a relative quiescent state (G0/G1 arrest) compared to the original cells (Fig. 3A). Though there was no difference in ALDH2 mRNA expressions between original SW620 cells and Cisp-resistant SW620 cells ($p > 0.05$), the reason might be that the main coding gene for ALDH is ALDH1 isoforms (ALDH1A1 and ALDH1A3) (Moreb et al., 2007). All data indicated that the potential stem-like traits of Cisp-resistant SW620 cells might be one of the main factors for drug-resistance.

Previously studies have showed that salinomycin could specifically induced cell apoptosis of CSCs. But until now, reports that reveal the relationship between salinomycin and drug-resistant cancer cells are rare. Zhang et al. found that salinomycin could significantly and effectively inhibit the cisplatin-resistant human ovarian cancer cell line growth through the induction of apoptosis, potentially associated with the p38 MAPK activation (Zhang et al., 2013). In our present study, we compared the IC50 values in original SW620 cells and Cisp-resistant SW620 cells, and found that salinomycin was extremely sensitive to the Cisp-resistant SW620 cells (Fig. 4E and F). Meanwhile, by flow cytometry analysis, salinomycin did not influence the cell cycle both in original SW620

cells and Cisp-resistant SW620 cells (Fig. 3A). But after salinomycin treatment, the cell apoptotic rate of Cisp-resistant SW620 cells was significantly increased (Fig. 3B and C). We speculated that salinomycin could greatly induce the apoptpsis of Cisp-resistant cells, as these drug-resistant colorectal cancer cells might presented more stem-like signatures.

To further explore the potential mechanisms that salinomycin could specifically induce apoptosis in drug-resistant cells, we detected some classical and specific oxidative stress indicators that could reflected the levels of lipid peroxidation in cells, such as LDH release, MDA contents, SOD and GSH-PX activities (Cong et al., 2012; Del Rio et al., 2005; Qin et al., 2008). Our results demonstrated that salinomycin could cause abundant of lipid peroxides with increased LDH release and MDA contents in Cisp-resistant SW620 cells. Salinomycin also down-regulated SOD and GSH-PX activities in Cisp-resistant SW620 cells (Fig. 4A, B, C and D). We also detected the downstream apoptosis-related key genes, such as Caspase-3, Caspase-8, Caspase-9, Bcl-2 and Bax. A large number of studies have showed that Bcl2 is a key anti-apoptotic gene, while Caspase-3, Caspase-8, Caspase-9 and Bax are considered as pro-apoptotic genes, the decreased ratio of Bcl2/Bax is involved in different kinds of tumor cell apoptosis (Chai et al., 1999; Mohan et al., 2012; Wyllie, 2010). Our results showed that salinomycin could effectively up-regulate both mRNA and protein expressing levels of caspase-3, Caspase-8, caspase-9 and Bax, but down-regulated the Bcl-2 mRNA and protein expressions in Cisp-resistant SW620 cells (Fig. 4G and Fig. 5). Meanwhile, the ratio of Bcl-2/Bax in Cisp-resistant SW620 cells was seriously disordered. Our study manifested that high levels of oxidative stress caused by accumulated reactive oxygen species and dysregulations of apoptosis-related genes or proteins might ultimately led to apoptosis in Cisp-resistant SW620 cells.

In summary, we firstly obtained Cisp-resistant SW620 cells from original colorectal cancer cells by repeated treatment with 5 μmol/l of cisplatin. These Cisp-resistant SW620 cells, which maintained a relative quiescent state and displayed stem-like signatures, were relatively sensitive to salinomycin. Salinomycin could specifically increase the apoptotic rate of Cisp-resistant SW620 cells, by accumulated reactive oxygen species and down regulation of some apoptosis-related genes or proteins. These findings will provide new clue for novel and selective chemotherapy on drug-resistant colorectal cancer cells.

Conflict of interest

The authors declare that there are no conflicts of interest.

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