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Polyene Phosphatidylcholine reverses Oxaliplatin resistance in human gastric cancer BGC823/L-OHP cell lines

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Abstract: **This study investigated the effect of polyenoic phosphatidylcholine (PPC) on the proliferation and apoptosis of BGC823/L-OHP cells in an attempt to reverse the mechanism of drug resistance in these cells. Cell counting (CCK-8) was performed to test the ability of PPC and Oxaliplatin (L-OHP; 1, 5, 10, 20, 30, 40μg/ml) can inhibit the growth of gastric cancer cell lines BGC823 and BGC823/L-OHP. The IC50 was calculated. PPC promoted the growth of BGC823/L-OHP cells in the range of 0.5–24μM and inhibited cell growth in the range of 48–86μM. The L-OHP IC50 in BGC823 cells was 3.52μg/ml, while in BGC823/L-OHP cells was 28.53μg/ml. The results indicated that BGC823/L-OHP cells had a strong drug resistance (P<0.05). The L-OHP IC50 of BGC823/L-OHP cells treated with 5μmol/L PPC was 6.63μg/ml. Flow cytometry examination showed that PPC promoted apoptosis in BGC823/L-OHP cells. We also examined the expression levels of TLR4, Nanog, and ABCF2 by RT-PCR and Western blot, and determined that PPC can downregulate the expression of TLR4, Nanog and ABCF2 in resistant strains. PPC can increase the sensitivity of human gastric cancer resistant cells to oxaliplatin. Reversing the effect of oxaliplatin resistance, and this reversal occurs with a reduction in the levels of TLR4, Nanog, and ABCF2.**

Keywords: Polyene Phosphatidylcholine; Oxaliplatin; BGC823/L-OHP; Drug resistance

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1. Introduction

In terms of the global incidence of malignant tumors, gastric cancer is ranked as the fifth most common, and its mortality rate is third after lung cancer and liver cancer[1]. China has a high incidence rate of gastric cancer, as it is the third mobility and second mortality rate among malignant tumors in China[2,3]. At present, surgery is effective way for most patients with gastric cancer. Chemotherapy plays an important role in the comprehensive treatment of this cancer, especially gastric cancer patients with advanced stages[4,5]. However, there has been a great bottleneck in the treatment of gastric cancer due to chemotherapeutic resistance, most notably multi-drug resistance (MDR)[6-8]. Platinum drugs (cisplatin and oxaliplatin) are the main chemotherapy treatments for advanced gastric cancer, showing the greatest effect on this disease[9]. However, the effect is far from ideal because it is largely limited by endogenous or acquired resistance[10].

Polyenoic phosphatidylcholine (PPC) is widely used as a clinical protectant of the liver. Through its integration with the liver cell membrane and organelle membranes, PPC can increase the integrity, stability, and mobility of the membrane and restore normal cell function while reducing oxidative stress and lipid peroxidation, inhibiting apoptosis, and reducing the inflammatory response, among other functions[11, 12]. The ATP-binding cassette (ABC) transporter protein is overexpressed in tumor cells. It caused drugs to be pumped outside of the cell. This results in tumor resistance and is one of the most important causes of chemotherapeutic failure[13]. Studies have shown that toll-like receptor 4 (TLR4) proteins can activate the Nanog gene in tumor stem cells to upregulate the expression of the ABC protein family[14]. Phosphatidylcholine analogues can inhibit TLR4 in tumor cells[15,16]. Our previous studies have shown that PPC has a synergistic effect with oxaliplatin. It works to inhibit the proliferation of gastric cancer cells[17]. However, there is no report showing the role of PPC in reversing multidrug resistance in human gastric cancer. Therefore, we investigated the effect of PPC in reversing drug resistance in oxaliplatin-resistant BGC823/L-OHP cells.

2. Materials and Methods

2.1. Reagents

RPMI 1640 was purchased from the Hyclone (Fisher Scientific International, U.S.A). Standard fetal bovine serum (FBS) was purchased from BI (Biological Industries, Israel). Oxaliplatin (L-OHP) was a generous gift from the Jiangsu Hengrui Pharmaceutical Co., Ltd. Effective Company (Jiangsu, China). Polyene phosphatidylcholine (PPC) injections were purchased from the Beijing Sanofi Pharmaceutical Co., Ltd. (Beijing, China). The rabbit anti-human TLR4 antibody, rabbit anti-human Nanog antibody, and rabbit anti-human ABCF2 antibody were all purchased from Abcam (London, U.K.). CCK-8 kit was purchased from Guangzhou Yi Yuan Biological Technology Co., Ltd. (Guangzhou, China). The reverse transcription kit and RT-PCR kit were purchased from TaKaRa (Japan). Primers were synthesized by Qingdao Qingke Zi Xi Biotechnology Co., Ltd.

2.2. Cell line and culture conditions

Human gastric cancer parental cell line BGC823 was purchased from Shanghai Zhong Qiao Xin Zhou Biotechnology Co. Ltd. (Shanghai, China). The human gastric cancer BGC823/L-OHP cell line was a generous gift from the Beijing Friendship Hospital laboratory. The cells were maintained in RPMI 1640 medium containing 10% FBS, 100U/ml streptomycin, and 100U/ml penicillin at 37˚C in a humidified atmosphere containing 5% CO_2. To maintain the oxaliplatin-resistant phenotype, the medium for BGC823/L-OHP was additionally supplemented with 1μg/ml L-OHP.

2.3. Growth and proliferation assay

The CCK-8 cell-counting assay was used to measure the ability of PPC to inhibit the growth of BGC823/L-OHP cells. BGC823/L-OHP cells in the logarithmic growth phase were plated in 96-well plates (5×10^3 cells/well) in 100μl of RPMI 1640 with 10% FBS and incubated at 37℃ for 24h. The cells were treated with PPC at final concentrations of 0.0, 0.5, 1.5, 3.0, 6.0, 12.0, 24.0, 48.0, 64.0 and 128.0μM, with four wells per concentration, for 48h, at which time the cells were incubated with 10μl of the CCK-8 solution for 3h at 37℃. An ELISA microplate reader was used to measure the absorbance of each well at 490nm. The average absorbance of the four wells was calculated in each experiment. The experiment was repeated three times.

2.4. In vitro drug sensitivity assay

The cells were divided into five groups: group I (BGC823), group II (BGC823/L-OHP), group III (BGC823/L-OHP with 1μM PPC), group IV (BGC823/L-OHP with 5μM PPC), and group V (BGC823/L-OHP with 10μM PPC). The appropriate cells were seeded into 96-well plates (5×103cells/well) and incubated at 37℃ in a humidified 5% CO_2 atmosphere for 24h. Groups I and II were treated with L-OHP to final concentrations of 1.0, 5.0, 10.0, 20.0, 30.0, and 40.0μg/ml in the culture medium. At 48h after L-OHP administration, the cell viability was assessed using the CCK-8 assay. The IC_{50} of the two groups of cells was calculated. The IC_{50} from group I was selected as the safe concentration for the next experiment. The cells in groups III, IV, and V were treated with L-OHP to final concentrations of 1, 5, 10, 20, 30, and 40μg/ml. The IC_{50} in BGC823/L-OHP cells was calculated at 48h after the administration of PPC. The PPC concentration at which the IC_{50} decreased was selected as the concentration for future experiments, and the fold change of the IC_{50} was then calculated.

2.5. Apoptosis

To measure the rates of apoptosis, the BGC823/L-OHP cells were divided into different groups as above and were cultured for 48h. After harvesting with trypsin digestion, the cells were suspended in PBS and the cell density was adjusted to 1×10^6cells/ml. Next, the cells were stained with Annexin V-FITC and propidium iodide in the dark at room temperature for 20 minutes, and the level of apoptosis was measured by flow cytometry.

2.6. Real-Time Reverse Transcription-Polymerase Chain Reaction

The TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract the total RNA from the BGC823/L-OHP cells treated with PPC. The concentration and purity of the RNA were measured by A260/A280 using a UV spectrophotometer. cDNA was synthesized from 500ng of total RNA using 10μl of the reaction mixture and a cDNA reverse transcription kit (TaKaRa Biotechnology Co. Ltd., Japan). Real-time (RT)-PCR was performed on a SYBR® Premix Ex Taq™ system (TaKaRa Biotechnology Co. Ltd. Japan). The gene primer sequences are cited in the GeneCards database. Finally, the samples were quantified using the 2−ΔΔCt method.

Table 1. Relative mRNA primers

Relative mRNA	Forward Sequence	Reverse Sequence
TLR4	5’-CCCTGAGGCATTTAGGCAGCTA- 3’	5’-AGGTAGAGAGGTGGCTTAGGCT-3’
Nanog	5’-CTCCAACATCCTGAACCTCAGC- 3’	5’-CGTCACACCATTGCTATTCTTCG-3’
ABCF2	5’-GAGGTTTCACTGGGAGCAAGATC-3’	5’-CTGTAGCGTCTTCTCCTTGCTC-3’

2.7. Western blot analysis

The cells were lysed with RIPA buffer (Solarbio Biotechnology Co. Ltd., China) supplemented with PMSF (Riche, CA, USA) and a phosphatase inhibitor. The total protein concentration of each sample was determined by the Protein Assay Kit (Beyotime, China). An equal amount of protein (30μg) was electrophoresed on a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was blocked with 5% skimmed milk powder (Mengniu Dairy Co. Ltd., China) in 0.1% Tween 20 in Tris-buffered saline (TBST) and was incubated at room temperature for 1h. It was then probed with antibodies specific for β-actin (1:5,000; Boster Biological Technology Co. Ltd., China), ABCF2 (1:1,000; Abcam, UK), Naong (1:1,000;

Abcam, UK), or TLR4 (1:1,000; Abcam, UK) overnight at 4 ℃. The secondary antibody was a goat anti-rabbit IgG (1:5,000; Abcam, UK). The Immobilon™ Western chemiluminescent substrate kit (Mai Bio Co. Ltd., China) was used to detect the presence of the bands.

2.8. Statistical analyses

Statistical analyses were performed using the one-way ANOVA in the SPSS 21.0 software, and the data were expressed as mean ± standard deviation. All experiments were repeated three times with similar results, $P<0.05$ was considered statistically significant.

3. Results

3.1. Effect of PPC on inhibiting the growth of BGC823/L-OHP cells

Using the CCK-8 test with the 48h PPC-treatment concentration gradient in BGC823/L-OHP cells, the cell growth inhibition rate can be obtained. 0.5, 1.5, 3.0, 6.0, 12.0, 24.0, 48.0 μM concentrations of PPC can promote cell growth, while high concentrations such as 64 μM can inhibit cell growth (Figure 1).

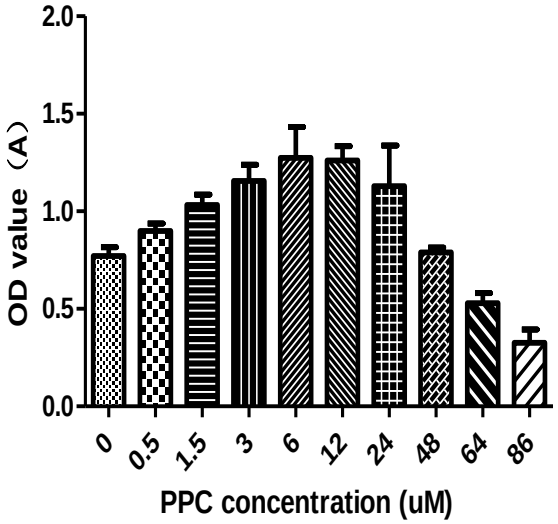

Figure 1. Growth of BGC823/L-OHP cells in the presence of PPC.

3.2. Reversal of drug resistance in human gastric cancer BGC823/L-OHP cell line by PPC

The cytotoxicity of oxaliplatin to groups I (BGC823), II (BGC823/L-OHP), III (BGC823/L-OHP + 1 μM of PPC), IV (BGC823/L-OHP + 5 μM of PPC), and V (BGC823/L-OHP + 10 μM of PPC) was detected using the CCK-8 method. The IC50 of each group was calculated (Figure 2). The IC50 of groups I, II, III, IV and V were 3.52 μg/ml, 28.53 μg/ml, 10.95 μg/ml, 6.63 μg/ml, and 11.21 μg/ml, respectively (Table 2). The fold change of the IC50 in untreated BGC823/L-OHP cells comparasion with the control was calculated to be 7.5. The fold reversals of BGC823/L-OHP cells treated with the various concentrations of PPC were calculated to be 2.60, 1.88, and 2.54 respectively. Group II showed a strong resistance to drug-resistant cells, which was significantly different from group I (BGC823) ($P<0.01$, Table 3). The IC50 of resistant strains treated with different concentrations of PPC was significantly lower than that of the susceptible strain. PPC significantly increased the sensitivity of oxaliplatin to BGC823/L-OHP cells at 5 μM ($P<0.01$; Table 4). Finally, 5 μM of PPC was selected as the concentration to be used in further experiments.

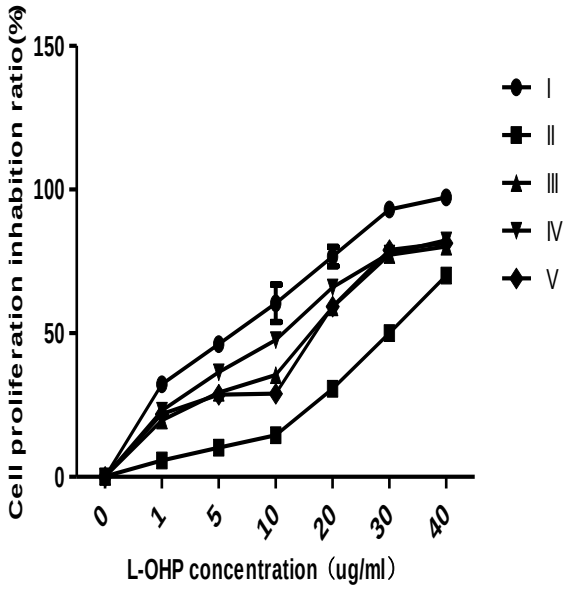

Figure 2. Effects of oxaliplatin on the inhibition rate of five groups of cells after 48 h. Group I: BGC823; group II: BGC823/L-OHP; group III: BGC823/L-OHP + 1 μM of PPC; group IV: BGC823/L-OHP + 5 μM of PPC; and group V: BGC823/L-OHP + 10 μM of PPC.

3.3. PPC combined with oxaliplatin can promote apoptosis in BGC823/L-OHP cells

Tumor cells can exhibit drug resistance by reducing their rate of apoptosis. To study the mechanism of PPC resistance reversal, the apoptosis of BGC823/L-OHP cells treated with PPC was detected using an Annexin V binding assay. The number of apoptotic cells observed in PPC-treated cells was not significantly different from that in the control group compared with the blank control cells. The treatment of BGC823/L-OHP cells with 5 μg/ml of L-OHP and 5 μM of PPC combined with oxaliplatin (5 μg/ml) showed the same rate of apoptosis (Figure 3a, b). In contrast, the apoptotic rate of the combination group was higher than that of the oxaliplatin group alone ($P<0.01$). Promoting apoptosis is a means by which PPC inhibits the growth of oxaliplatin-resistant gastric cancer cells.

Table 2. Effects of oxaliplatin on susceptible and resistant strains, and the IC_{50} calculated with different concentrations of PPC in resistant strains.

Group	IC_{50} (μg/ml) ($\bar{x}$ ± s, n = 3)
I (BGC823)	3.52 ± 0.27
II (BGC823/L-OHP)	28.53 ± 0.69*
III (BGC823/L-OHP + 1 μM of PPC)	10.95 ± 0.78**
IV (BGC823/L-OHP + 5 μM of PPC)	6.63 ± 0.34**
V (BGC823/L-OHP + 10 μM of PPC)	11.21 ± 0.77**

*Compared to group I, P<0.05; **compared with group II, P<0.05.

Table 3. Effects of oxaliplatin on BGC823 and BGC823/L-OHP cell inhibition rates (48h)

L-OHP (μg/ml)	Group I	Group II*
1	33.34 ± 1.15	5.72 ± 0.15
5	48.44 ± 2.37	10.13 ± 1.26
10	63.77 ± 6.54	14.53 ± 1.15
20	77.80 ± 3.36	30.63 ± 0.78
30	93.54 ± 1.29	50.05 ± 1.08
40	97.27 ± 1.13	70.06 ± 1.57

*Compared with group I (BGC823), P<0.01.

Table 4. Comparison of the inhibition rates of BGC823/L-OHP cells treated with different concentrations of PPC and oxaliplatin (48h)

L-OHP (μg/ml)	II	III*	IV*	V*
1	5.72 ± 0.15	19.64 ± 0.59	23.09 ± 1.04	21.86 ± 0.77
5	10.13 ± 1.26	29.32 ± 1.17	36.41 ± 0.84	28.62 ± 0.95
10	14.53 ± 1.15	35.52 ± 1.20	47.65 ± 1.05	28.99 ± 0.82
20	30.63 ± 0.78	59.07 ± 0.89	65.95 ± 1.23	59.38 ± 0.92
30	50.05 ± 1.08	77.34 ± 0.49	77.82 ± 0.61	79.00 ± 0.70
40	70.06 ± 1.57	80.17 ± 1.38	82.40 ± 0.78	81.41 ± 1.10

*Compared with group II (BGC823/L-OHP), P<0.01.

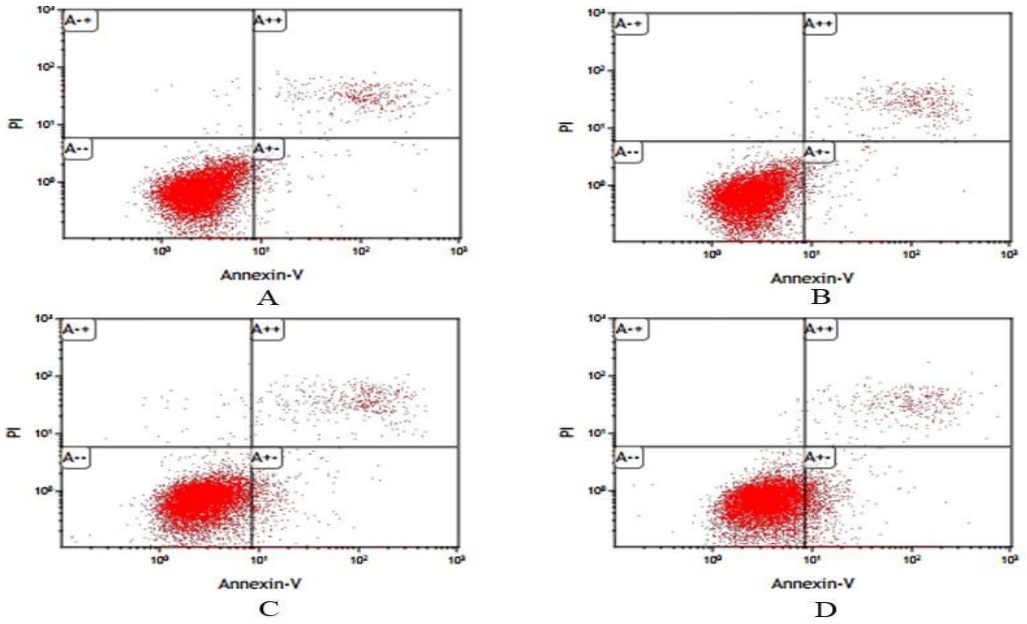

Figure 3(a). Effect of PPC on the apoptosis of human gastric cancer cells. (A) BGC823/L-OHP cells; (B) BGC823/L-OHP cells + 5 μM of PPC; (C) BGC823/L-OHP cells + 5 μg/ml of oxaliplatin; (D) BGC823/L-OHP cells + 5 μM of PPC + 5 μg/ml of oxaliplatin. Flow cytometry was used to detect the effect of apoptosis.

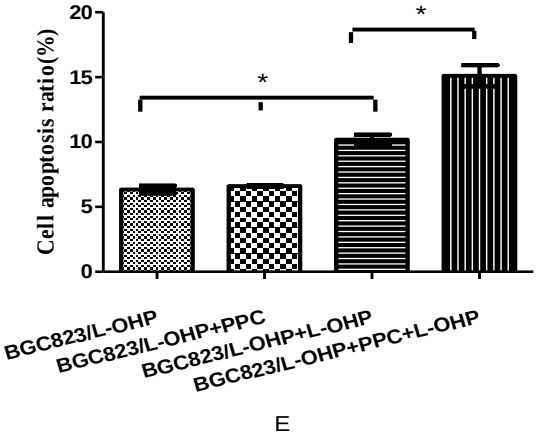

Figure 3(b). (E) Compared with the control group, *P<0.01.

3.4. Expression levels of TLR4, Nanog, and ABCF2 in BGC823 and BGC823/L-OHP cells

To understand the mechanism of PPC increases cell apoptosis, we evaluated the reversal of drug resistance by PPC in conjunction with the expression of TLR4, Nanog, and ABCF2 proteins. The tests were detected by RT-PCR and Western blot. The experimental selection of 5μg/ml of oxaliplatin as a safe concentration was used in these experiments. TLR4, Nanog and ABCF2 were shown to be highly expressed in drug-resistant cells by RT-PCR. The expression of TLR4, Nanog, and ABCF2 decreased after treatment with PPC, and the results were consistent with the mRNA expression levels (Table 5, Figure 5).

Statistically, the expression of TLR4 and Nanog mRNA levels in BGC823/L-OHP cells was higher than those in BGC823 cells, indicating that BGC823/L-OHP cells were associated with TLR4, Nanog, and ABCF2 (P<0.05, Figure 4). The expression of TLR4 and Nanog in BGC823/L-OHP cells was not significantly different from the negative control (BGC823/L-OHP + 5μg/ml of L-OHP). However, the expression levels of TLR4, Nanog, and ABCF2 decreased significantly after PPC treatment in combination with oxaliplatin in BGC823/L-OHP cells (P<0.05, Figure 6). The result indicates that TLR4, Nanog, and ABCF2 were involved in BGC823/L-OHP cell drug-resistance (P<0.05).

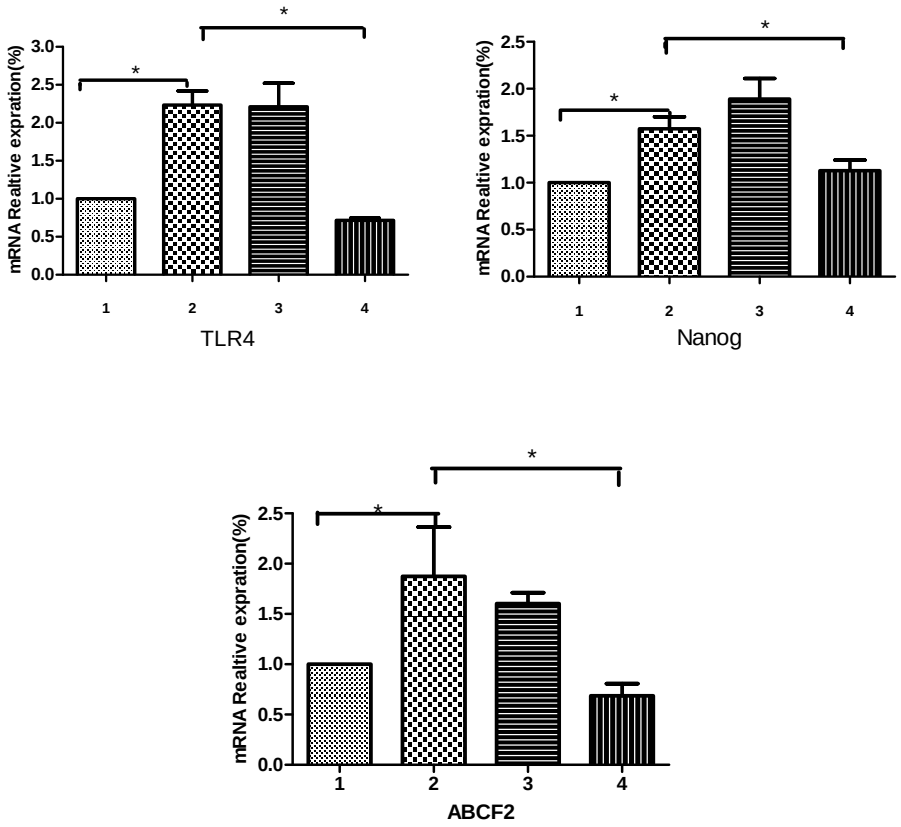

Figure 4. Cells in group 1 (BGC823), group 2 (BGC823/L-OHP), group 3 (BGC823/L-OHP + L-OHP [5μg/ml]), and group 4 (BGC823/L-OHP + L-OHP [5μg/ml] + PPC [5μM]) were cultured for 48h, and the relative expression levels of TLR4, Nanog, ABCF2 mRNA in the four groups were measured,*P< 0.05.

Table 5. Real-Time PCR for detecting mRNA expression of TLR4, Nanog, ABCF2

Gene	1	2	3	4
TLR4	1±0	2.24±0.32	2.21±0.54	0.72±0.05
Nanog	1±0	1.47±0.37	1.89±0.38	0.69±0.23
ABCF2	1±0	1.47±0.32	1.87±0.84	0.68±0.20

The amount of mRNA expression by $2^{-\Delta\Delta CT}$

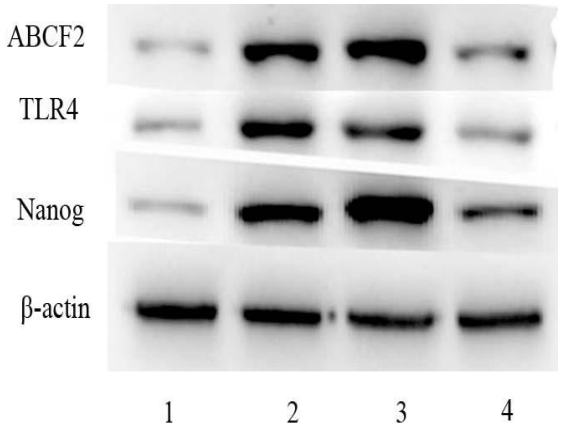

Figure 5. Western blot analysis of TLR4, Nanog, and ABCF2 expression levels in BGC823 and BGC823/L-OHP cells for group 1 (BGC823), group 2 (BGC823/L-OHP), group3 (BGC823/L-OHP + L-OHP [5μg/ml]), and group 4 (BGC823/L-OHP + L-OHP [5μg/ml] + PPC [5μM]).

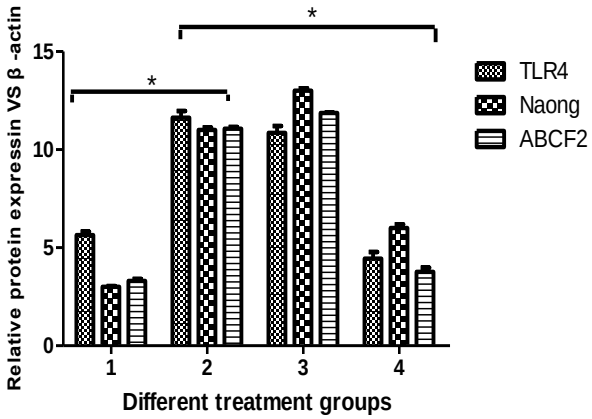

Figure 6. Relative protein expression in groups 1–4. Group I: BGC823; group II: BGC823/L-OHP; group III: BGC823/L-OHP + L-OHP (5μg/ml); and group IV: BGC823/L-OHP + L-OHP (5μg/ml) + PPC (5μM). *P< 0.05.

4. Discussion

Chemotherapy is the most common treatment option for advanced gastric cancer. The multidrug resistance of these cancer cells has become a major obstacle that seriously affects clinical efficacy. Chemotherapy often leads to a certain degree of liver damage in patients. So PPC as a liver protectant is widely used in clinical practice. We have determined that PPC can reverse the resistance of human gastric cancer BGC823/L-OHP cells to oxaliplatin, but the mechanism of this resistance reversal is not yet clear. Our experiments showed that PPC could promote the proliferation of BGC823/L-OHP cells at low concentrations. Cell growth was inhibited at high concentrations. Additionally, PPC combined with oxaliplatin increased tumor cell apoptosis.

The ABC transporter gene superfamily be divided into seven subtypes (ABCA-ABCG). Many members of this family are involved in tumor multidrug resistance[18-22]. The ABC transporter protein is overexpressed in tumor cells, and it pumps the drugs out of the cell to cause tumor drug resistance. It is one of the major causes of chemotherapeutic failure[13]. The ABCF2 protein lacks the transmembrane region and cannot perform this transmembrane transport function[23]. Studies have shown that ABCF2 may be involved in translation regulation, antibiotic resistance, and RNase L inhibition[24], but its role in tumor drug resistance is unclear[25,26].

We demonstrated that PPC reverses the resistance of gastric cancer cells to oxaliplatin, and the expression levels of TLR4, Nanog, and ABCF2 in resistant strains were related to the resistance of human gastric cancer cells to oxaliplatin. It is possible that this mechanism may be related to the redistribution of the main phospholipids in the cell-membrane bilayer, which leads to the rearrangement of the structure of the cell membrane. ABCF2 presents on the surface of the cell membrane. This membrane rearrangement could result in the destruction of the ABCF2 protein structure. The permeability and mobility of the cellular and organelle membranes can determine the ability of the cell to pump out molecules, which is the inhibitive mechanism. It removes the chemotherapy drug from the cell.

TLR4, as a pattern-recognition receptor, is from an important class proteins involved in nonspecific immunity. The main function of this receptor is to mediate endogenous immunity response, and to participate in the expression of inflammatory factors. At the same time, TLR4 is also involved in tumor cell proliferation, anti-apoptosis, immune escape, and drug resistance[27]. The TLR4 receptor in tumor cells acts as the first signal for the production of lipopolysaccharide (LPS) and other stimulating factors, and this acts the activation of the transcription factor E2F1 by phosphorylation to start the downstream signaling of Nanog. It plays an important biological

role in drug resistance[28] by upregulating the expression of ABC-family proteins[14]. Studies have shown that phosphatidylcholine analogues can inhibit TLR4 in tumor cells[15, 16], can competitively inhibit the stimulation of LPS by TLR4[29] and can prevent it from activating stem cells through the pluripotent start-up gene, Nanog[30]. In this study, the expression of TLR4 and Nanog in BGC823/L-OHP cells was higher than that in BGC823 cells. Its expression in BGC823/L-OHP cells decreased at both the protein and gene level after PPC treatment. This indicated that the decreasing expression, activity of TLR4 and Nanog in BGC823/L-OHP cells could helpe to reverse the resistance of gastric cancer cells to oxaliplatin.

It has been demonstrated that PPC can increase the sensitivity of human gastric cancer resistant cells to oxaliplatin, thereby reversing the effect of oxaliplatin resistance, and that this reversal occurs with a reduction in the levels of TLR4, Nanog, and ABCF2. However, the specific mechanism of this reversal of resistance still needs to be studied, and whether PPC acts as a specific protein pathway need be explored. In the future, we will study this mechanism of the PPC reversal of drug resistance in gastric cancer cells and provide new ideas for reversing this resistance to platinum drugs for individualizing treatments to improve the outcomes in gastric cancer.

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