Reversing effect of NOTCH1 inhibitor LY3039478 on drug-resistance cells SGC7901/DDP of human gastric cancer and its mechanism

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Abstract. – **OBJECTIVE:** To investigate the reversing effects of NOTCH1 inhibitor LY3039478 on cancer of the stomach's drug-resistance cells SGC7901/DDP and its relevant mechanism.
MATERIALS AND METHODS: Drug-resistance cells SGC7901/DDP of human gastric cancer before and after reversal via NOTCH1 inhibitor LY3039478 were used as objects of study. Changes in the expression of Hes protein in cells were detected via Western blotting; the inhibitory effect of drugs on cell multiplication was detected via cell counting kit-8 (CCK-8), and the Rhodamin123 (Rh123) efflux and P-glycoprotein (P-GP) expression level in cells were detected by flow cytometry.
RESULTS: NOTCH1 inhibitor LY3039478 could inhibit the expression of Hes protein in SGC7901/DDP cells. Under the effect of 1 µmol/L and 2 µmol/L NOTCH1 inhibitor LY3039478, drug sensitivity of SGC7901/DDP cells to cisplatin was increased by 2.2 times and 2.86 times, respectively. The content of Rh123 in cells was increased by 1.41 times and 2.62 times, respectively, but the P-GP expression level was decreased by 67.5% and 45%, respectively.
CONCLUSIONS: NOTCH1 inhibitor LY3039478 can inhibit or even reverse the multidrug resistance-associated protein in SGC7901/DDP cells. The mechanism of drug resistance may be related to the decrease of Rh123 efflux and P-GP expression level in cancer cells.

Key Words:
    NOTCH1 inhibitor LY3039478, SGC7901/DDP, Multidrug resistance-associated protein, Rh123, P-GP.

Introduction

Currently, gastric cancer is among the most malignant tumors, along with high morbidity and mortality rates, as well as a kind of frequently-occurring malignant tumor in China, third only to lung cancer and liver cancer[1]. Gastric cancer is characterized by rapid onset, fast progression and poor treatment effect, which is also the cause of death of the second major malignant tumors in the world[2]. Tumor cells frequently display initial sensitivity to anti-tumor therapeutic drugs, but acquire resistance during the treatment.

At present, one of the most widely used anti-cancer drugs clinically in the world is cisplatin. The production of multidrug resistance (MDR) is one of the major causes of failure of cisplatin treatment, and there is no exception in gastric cancer[3]. Cisplatin resistance of gastric cancer is multifactorial; accumulating evidence has suggested that the aberrant expression of proteins, which associated with decreased cellular accumulation, increased DNA repair capacity, increased drug inactivation, playing an important role in the acquisition of cisplatin resistance. With the emergence of targeted therapeutic drugs, variety of targeted drugs have been used in the treatment of malignant tumors in succession, and Her-2 and other drugs have started to be clinically applied in gastric cancer cells as the targeted treatment[4]. Her-2 is overexpressed in 25% of gastric cancer patients, who would supposedly benefit from

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trastuzumab therapy. However, it is also found in the application of drugs that significant drug resistance occurs in gastric cancer therapeutic drugs[5]. Therefore, how to find effective measures to reverse drug resistance of gastric cancer cells to chemotherapy drugs and targeted drugs is extremely important for improving the therapeutic effect on malignant gastric tumor[6]. Recently, researchers have found that the mechanism of drug resistance in cancer of stomach is related to the activation of signal transducers and activation of transcription 1 (STAT3)/Jagged-1/interleukin-6 (IL-6)/Notch positive feedback loop[7]. Evidence showed that the acquisition of resistance is associated with the formation of EMT/CSC phenotype. This study aimed at finding possible drugs that can reverse drug resistance of gastric cancer.

Materials and Methods

Main Reagents

Human gastric cancer drug-resistant cells SGC7901/DDP were purchased from Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). Selective NOTCH1 inhibitor LY3039478 was purchased from Selleck (Houston, TX, USA), Cell Counting Kit-8 (CCK-8) from Dojindo (Kumamoto, Japan), Hes protein antibody from Cell Signaling Technology (Danvers, MA, USA), and flow cytometer from Beckman Coulter (Miami, FL, USA).

Cell Culture

SGC7901/DDP cells were cultured in 5% CO_2 at 37°C, Roswell Park Memorial Institute (RPMI)-1640 culture solution containing 10% fetal bovine serum, and digested with 0.25% trypsin-ethylene diamine tetraacetic acid (EDTA), followed by passage every 2-3 d once. Logarithmic growth phase cells were used for all experiments.

Detection of Inhibitory effect of LY3039478 on SGC7901/DDP Cell Proliferation via CCK-8

Cells were inoculated into a 96-well plate at a density of 1×10^4/mL (50 μL per well), incubated in 5% CO_2 at 37°C for 24 h, and added with 0.125, 0.25, 0.5, 1, 2, 4 and 8 μmol/L LY3039478. The negative control wells and blank control wells were set up, and three replicate wells were set for

each group of samples. After cells were incubated with 5% CO_2 at 37°C for 48 h, each well was added with 10 μL CCK-8, and cells continued to be cultured for 2-3 h. Optical density (OD) value was measured at 450 nm, the cell inhibition curve was drawn, and inhibitory rate of LY3039478 on SGC7901/DDP cells proliferation was calculated according to the formula: inhibitory rate = (1-OD drug / OD control) × 100%. The experiment was repeated for at least 3 times.

Detection of Reversing Effect of LY3039478 on SGC7901/DDP cell MDR via CCK-8

SGC7901 cells were inoculated into the 96-well plate at a density of 1×10^4/mL (50 μL per well), incubated in 5% CO_2 at 37°C for 1 day, and added with 0.125, 0.25, 0.5, 1, 2, 4 and 8 μmol/L cisplatin. SGC7901/DDP cells were inoculated into the 96-well plate at a density of 1×10^4/mL (50 μL per well), incubated in 5% CO_2 at 37°C for 1 day, and added with 0, 1 and 2 μmol/L NOTCH1 inhibitor LY3039478 and 0.125, 0.25, 0.5, 1, 2, 4 and 8 μmol/L cisplatin. The negative control wells (cells and medium) and blank control wells (medium) were set up, and three replicate wells were set for each group of samples. After cells were incubated in 5% CO_2 at 37°C for 48 h, each well was added with 10 μL CCK-8, and cells continued to be cultured for 2-3 h. OD value was measured at 450 nm, the cell inhibition curve was drawn, and half maximal inhibitory concentration (IC50) of SGC7901 and SGC7901/DDP cells under the action of 1 μmol/L and 2 μmol/L NOTCH1 inhibitor LY3039478 were calculated, respectively. The experiment was repeated for at least 3 times. Changes in drug resistance of SGC7901/DDP cells under NOTCH1 inhibitor LY3039478 were calculated according to the formula: resistance index (RI) = IC50SGC7901/DDP/IC50SGC7901.

Detection of the effects of LY3039478 on SGC7901/DDP cells via Western blotting

Cells were inoculated into a 6 cm culture dish, and incubated using 1 μmol/L and 2 μmol/L NOTCH1 inhibitor LY3039478 at 37°C for 12 h after they covered 80% of the dish. After cells were washed twice with phosphate-buffered saline (PBS), they were added with lysis buffer and centrifuged at 10000 rpm at 0°C for 5 min. The concentration of protein extracted was measured via bicinchoninic acid (BCA) protein assay. 50 μg

protein were subjected to 12% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis, transferred onto a nitrocellulose membrane, sealed with gelatin for 2 h, and added with rabbit anti-human Hes protein-specific polyclonal antibody (diluted at 1:500, CST, Danvers, MA, USA) and mouse anti-human β-actin monoclonal antibody (diluted at 1:1000, Zhongshan Goldenbridge, Beijing, China) at 4°C overnight. Next, the protein was incubated with specific horseradish peroxidase (HRP)-conjugated secondary antibody (1:10000) for 1.5 h, and washed with Tris-Buffered Saline with Tween-20 (TBST-20) for 3 times. The protein bands were developed using enhanced chemiluminescence (ECL) method.

Detection of the Effects of LY3039478 on Rhodamin123 (Rh123) Efflux Level in SGC7901/DDP Cells via Flow Cytometry

Rh123 is a fluorescent substrate of P-glycoprotein (P-GP); the detection changes in fluorescence intensity of Rh123 in tumor cells can show the expression level of P-GP on surface of cell. The higher fluorescence intensity of Rh123 is, the lower the expression level of P-GP on the cell surface will be, and the weaker the ability to pump drugs out of cells will also be. In this work, cells were inoculated in 6-well plate at a density of 5×10^5/mL, incubated with 1 µmol/L and 2 µmol/L NOTCH1 inhibitor LY3039478 at 37°C for 12 h, and added with 500 µL Rh123 (4 µmol/L) for incubation in a dark place at 37°C for 1 h. Next, cells were collected, rinsed with pre-cooled PBS twice, and detected *via* flow cytometry at an emission wavelength of 525 nm to calculate the mean fluorescence intensity (MFI) and detect the changes in Rh123 content in cells immediately.

Detection of the Effects of LY3039478 on P-GP Expression Level in SGC7901/ DDP Cells via Flow Cytometry

The production of MDR of tumor cells is mostly related to high P-GP on the cell surface. As an adenosine triphosphate (ATP)-dependent pump, P-GP can pump drugs with different structures and mechanisms out of cells, resulting in MDR of cells. In this study, SGC7901 cells were inoculated into the 6-well plate at a density of 5×105/mL, incubated with 1 µmol/L, 2 µmol/L NOTCH1 inhibitor LY3039478 at 37°C for 6 h, collected and added with 10 µL PE-UIC2 for incubation in a dark place at 4°C for 15 min. Then cells were rinsed with pre-cooled PBS twice and immediately detected *via* flow cytometry at an emission

wavelength of 575 nm to calculate the MFI in cells and detect the changes in P-GP expression on the cell surface.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 16.0 software (SPSS Inc., Chicago, IL, USA) were used for statistical analysis. All quantitative data were expressed as mean ± standard deviation. Comparison between groups was done using One-way ANOVA test followed by LSD (Least Significant Difference) Post Hoc-Test. $p < 0.05$ suggested that the difference was statistically significant.

Results

Effects of LY3039478 on SGC7901/DDP Cell Proliferation

The effects of LY3039478 on SGC7901/DDP cell proliferation were dose-dependent (Figure 1). Cell inhibition curve showed that LY3039478 showed no significant toxicity to SGC7901/DDP cells (inhibition rate < 5%) at 1 µmol/L concentration, but had slight toxicity to SGC7901/DDP cells (inhibition rate = 10%-15%) at 2 µmol/L concentration. Therefore, 1 µmol/L, and 2 µmol/L LY3039478 were selected for the study on MDR.

Reversing Effect of LY3039478 on SGC7901/DDP Cell MDR

As shown in Table I, IC50 of cisplatin for SGC7901 cells was 3.56 mg/L, while that for SGC7901/DDP cells was 32.38 mg/L. Under the action of 1 µmol/L and 2 µmol/L LY3039478, IC50 of SGC7901/DDP cells was 14.72 mg/L and 11.26 mg/L, respectively, RI of SGC7901/DDP cells was decreased from 9.09 to 8.3 and 3.16, respectively, and drug sensitivity to cisplatin was increased by 2.20 and 2.86 times, respectively.

Inhibitory Effect of LY3039478 on Hes Protein in SGC7901/DDP Cells

Western blotting showed that the NOTCH1 inhibitor LY3039478 could significantly inhibit the Hes protein expression in SGC7901/DDP cells in a dose-dependent manner (Figure 2).

Effects of LY3039478 on Rh123 Efflux and P-GP Expression Level in SGC7901/DDP Cells

The results of flow cytometry showed that LY3039478 could reduce the Rh123 efflux and increased Rh123 concentration in SGC7901/DDP
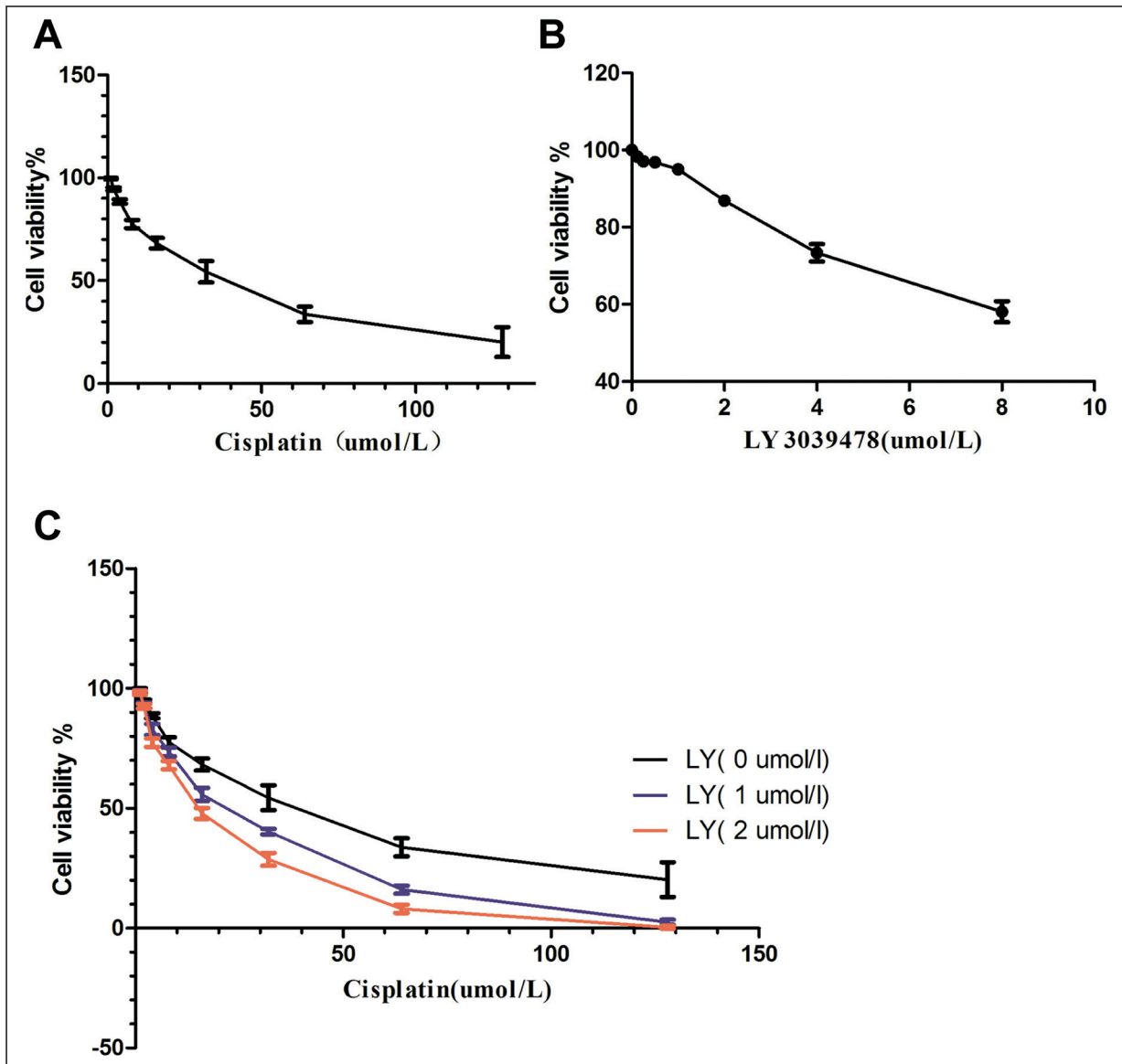
Figure 1. Effects of Cisplatin, LY3039478 on SGC7901/DDP cell proliferation. **A,** Effects of Cisplatin on SGC7901/DDP cell proliferation. **B,** Effects of LY3039478 on SGC7901/DDP cell proliferation. **C,** Effects of Cisplatin and LY3039478 on SGC7901/DDP cell proliferation.

Table I. Reversing effect of LY3039478 on SGC7901/DDP cell MDR.

	IC50 (mg/L)	RI
SGC7901	3.56	–
SGC7901/DDP	32.38**	9.09
SGC7901/DDP (1 μmol/L)	14.72*	4.13
SGC7901/DDP (2 μmol/L)	11.26*	3.16

*Compared with SGC7901, $p < 0.05$; **Compared with SGC7901, $p < 0.01$.

cells (Figure 3) (Table II). Under the action of 1 μmol/L and 2 μmol/L LY3039478, the Rh123 concentration in SGC7901/DDP cells was 1.41 times ($p < 0.05$) and 2.62 times ($p < 0.05$) that in control group (Figure 3A). LY3039478 could also decrease the P-GP expression on the surface of SGC7901/DDP cells. Under the action of 1 μmol/L, 2 μmol/L LY3039478, P-GP expression on the surface of SGC7901/DDP cells was 67.5% ($p < 0.05$) and 45% ($p < 0.05$) of that in control group (Table II).
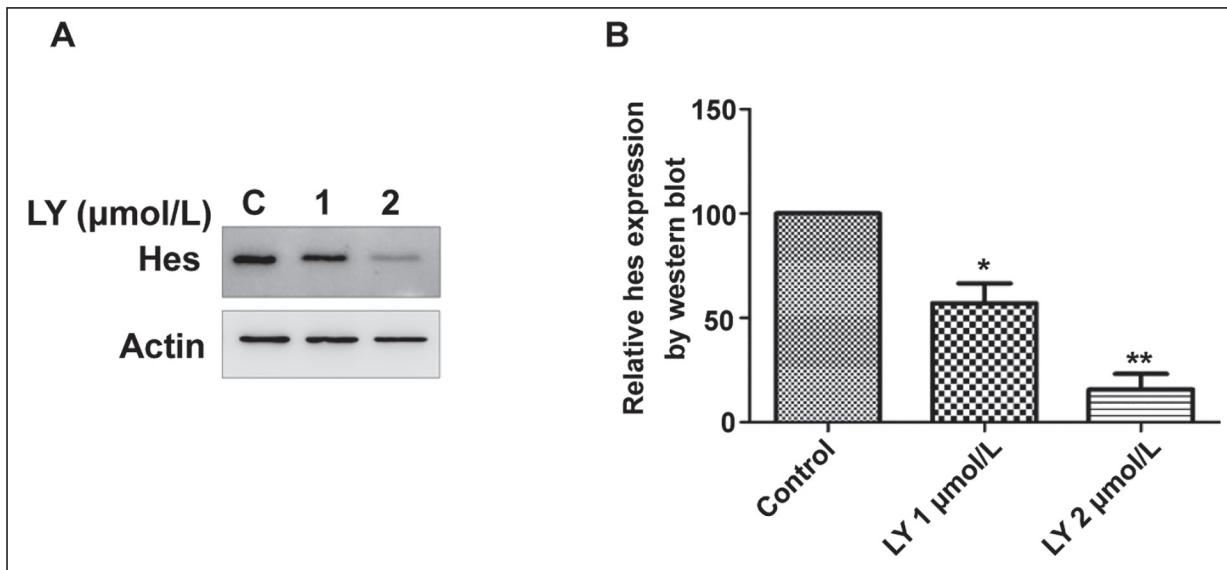
Figure 2. Effect of LY3039478 on Hes protein in SGC7901/DDP cells. ***A,*** Protein expression of Hes-1 by Western blot. ***B,*** Expression level of Hes-1 by Western blot experiment. (**p* < 0.05 control with LY3039478 0 μmol/L group; ***p* < 0.01 control with LY3039478 0 μmol/L group).
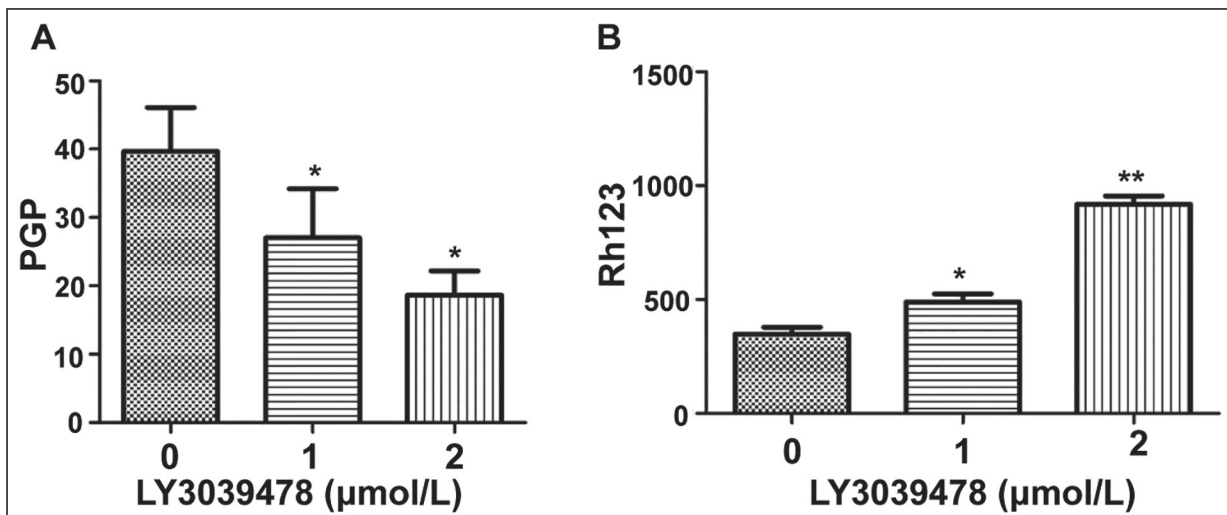
Figure 3. Effects of LY3039478 on Rh123 efflux and P-GP expression level in SGC7901/DDP cells ***A,*** Effects of LY3039478 on Rh123 efflux. ***B,*** Effects of LY3039478 on Rh123 efflux and P-GP expression level (**p* < 0.05 control with SGC7901 cell; ***p* < 0.01 control with SGC7901 cell).

Table II. Effects of LY3039478 on Rh123 efflux and P-GP expression level in SGC7901/DDP cells.

LY 3039478	MF1	
	RH123	**PGP**
0 umol/L	348 ± 27	40 ± 4
1 umol/L	489 ± 34*	27 ± 5*
2 umol/L	912 ± 41**	18 ± 3**

*Compared with SGC7901, *p* < 0.05; **Compared with SGC7901, *p* < 0.01.

Discussion

Gastric cancer is among the several malignant cancers with a higher incidence rate in the world, and its 5-year survival rate is only 20-50%[8]. MDR of gastric cancer cells and delayed diagnosis are the main reasons for the failure of gastric cancer treatment at present[9]. However, most MDR reversal agents used clinically cannot obtain ideal therapeutic effects, so searching for new reversal agents

of MDR protein has become a key link in the anti-tumor research[10].

Up to now, the mechanism of MDR is still unclear and complex, which mainly includes the increased drug efflux, abnormal metabolism, increased DNA damage repair[11], decreased apoptosis, modification or change of target protein of drug activity, theory of tumor stem cell[12], epithelial-mesenchymal transition (EMT), hypoxia and hypoxia-inducible factor-1α[13], and micro ribonucleic acid (RNA)[14]. The increased drug efflux caused by the change in the activity of transporters is one of the important reasons for MDR of tumor cells at present. In drug transporters, the overexpression of adenosine triphosphate binding cassette (ABC) membrane transporter is closely related to MDR of tumor cells[15]. ABC family gene-encoded transmembrane transporters decrease intracellular drug concentration, thus leading to drug resistance. P-GP, a member of the ABC membrane transporter superfamily, can induce MDR[16]. Restraining P-GP overexpression in tumor cells becomes the major mean of reversing MDR. It is reported that MDR of cancer cells is also correlated with EMT[17]. During EMT process, tumor cells obtain the characteristics of interstitial cells, the cell polarity disappears, and the connection with basement membrane is lost, thus obtaining higher migration and metastasis capacities[18]. At the same time, there are changes in cell adhesion molecules, the secretion of matrix metalloproteinase-2, matrix metalloproteinase-9 and N-cadherin are increased, but the E-cadherin expression is decreased, etc[19]. The tolerance of tumor cells to chemotherapeutic drugs (5-FU, cisplatin and doxorubicin) is positively correlated with EMT-related homeobox and high Twist expression[20]. Typical EMT occurs in trastuzumab-resistant gastric cancer cells, and invasion, metastasis capacities *in vitro* and *in vivo* are significantly enhanced[21]. Further mechanism research shows that Notch signaling pathway is activated, the phosphorylation level of serine-threonine kinase is decreased, and the release of IL-6 is increased in trastuzumab-resistant gastric cancer cells, thus activating STAT3[22]. Besides, targeted inhibition of Notch pathway and STAT3 expression can reverse the drug resistance of gastric cancer cells[23].

It was found in this work that Hes protein in drug-resistant SGC7901/DDP cells of stomach tumor was highly expressed, and LY3039478 at a concentration of 1 μmol/L, 2 μmol/L signifi-

cantly inhibited Hes protein expression. Cytotoxicity test showed that LY3039478 can effectively improve the drug sensitivity of SGC7901/DDP cells to cisplatin and set back the drug resistance of SGC7901/DDP cells. P-GP is a kind of glycoprotein encoded by MDR1, which is expressed in most normal tissues in human body, such as intestinal tract, liver, kidney and epithelial cells in blood-brain barrier and placental barrier, regulating the drug absorption, distribution, metabolism and excretion[24]. The P-GP expression level is increased in gastric cancer cells[25]. The specific substrate of P-GP, Rh123, is a kind of membrane potential-sensitive lipophilic cationic fluorescent dye, as well as a mitochondrial transmembrane potential indicator, which can reflect the functional status of P-GP. In this study, the content of Rh123 in tumor cells was detected by flow cytometry. Experimental results showed that LY3039478 could significantly increase the content of Rh123 in SGC7901/DDP cells by a dose-dependent manner, indicating that LY3039478 may set back the MDR of SGC7901/DDP cells by increasing drug concentration in cells.

Conclusions

We showed that LY3039478 inhibited the activity of Notch signaling pathway, decrease the expression of Hes protein and the expression of P-GP on the surface of SGC7901/DDP cells and increased the drug concentration in cells, thereby increasing the drug sensitivity of SGC7901/DDP cells to cisplatin, and reversing drug-resistant cells of gastric cancer. Results of this study provide an experimental basis for reversing P-GP-mediated drug-resistant cells of gastric cancer. The mechanism of LY3039478 in reversing drug-resistant cells of human gastric cancer and its role in downstream signaling pathway remain to be further studied.

Conflict of Interest
The Authors declare that they have no conflict of interests.

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