

RESEARCH COMMUNICATION

Mechanism of P-glycoprotein Expression in the SGC7901 Human Gastric Adenocarcinoma Cell Line Induced by Cyclooxygenase-2

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Abstract

Objective: To investigate possible signal pathway involvement in multi-drug resistant P-glycoprotein (P-gp) expression induced by cyclooxygenase-2 (COX-2) in a human gastric adenocarcinoma cell line stimulated with paclitaxel (TAX). **Methods:** The effects of TAX on SGC7901 cell growth with different doses was assessed by MTT assay, along with the effects of the COX-2 selective inhibitor NS-398 and the nuclear factor-KB (NF-KB) pathway inhibitor pyrrolidine dithiocarbamate (PDTC). **Influence on COX-2, NF-KB p65 and P-gp expression was determined by Western blotting.** **Results:** TAX, NS-398 and PDTC all reduced SGC7901 growth, with dose-dependence. With increasing dose of TAX, the expression of COX-2, p65 and P-gp showed rising trends, this being reversed by NS-398. PDTC also caused decrease in expression of p65 and P-gp over time. **Conclusion:** COX-2 may induce the expression of P-gp in SGC7901 cell line via the NF-kappa B pathway with paclitaxel stimulation.

Keywords: COX-2 - P-gp - NF-kappa B - paclitaxel - gastric adenocarcinoma cell

Asian Pacific J Cancer Prev, 13, 2379-2383

Introduction

COX-2 is an important rate-limiting enzyme in the process of synthesis of prostaglandins, playing a key role in the occurrence and development of tumor (Aruna and David., 2011). A large number of studies show that COX-2 participates in the regulation and control of multidrug resistance, and there exists correlation between the expression of COX-2 and P-gp expression (Nardone et al., 2004; Miller et al., 2006; Liu et al., 2009) in many types of tumors. It is not clear how COX-2 stimulates the expression of P-gp. It is found that in the process of using prostaglandin H₂ to treat resistant breast cancer cell lines, NF-KB pathway can be activated to result in the expression of P-gp (Hyung et al., 2011), which recovers its resistance for ADM.

This study adopts chemotherapeutic drugs paclitaxel to stimulate gastric cancer cell lines SGC-7901, which induces high expression of COX-2, and combines with COX-2 selective inhibitors of NS-398, NF- κ B inhibitor PDTC to check the expression changes of COX-2, NF-KB p65 and P-gp, which probes into the possible signal transduction pathway of gastric cancer cell lines multidrug resistance P protein expression resulted in by COX-2.

Materials and Methods

Material

Human gastric adenocarcinoma cell line SGC-7901

(gifted by the Medical University Of Anhui immunology laboratory); DMEM (high glucose) medium, pancreatin (product of GIBCO company); paclitaxel (product of BMS); MTT, NS-398 and PDTC (Sigma company); COX-2 monoclonal antibody (Bioworld Technology company), P-gp and NF-KB p65 monoclonal antibody (SANTA CRUZ company), β -actin and PIKA second antibody (Beijing Zhong Shan Jin Qiao Company); maker and Luminescence Agent (Fermentas Thermo scientific company); BCA Protein Quantitation Kit (YuanPingHao Biology Technology Limited Company).

Cell culture

Human Gastric Adenocarcinoma Cell Line (SGC7901) is inoculated into DMEM medium containing 10% calf serum glucose type and 37 °C, 5% CO₂ saturated humidity incubator to be routinely cultured. Wash twice by PBS 5 ml, carry on 0.25% trypsinization, passage occurs every other day.

The effects of NS-398 different doses on cell growth inoculate logarithmic phase cells into 96 orifice plates with 10⁴/hole to be routinely cultured for 24 hours, culture fluid including TAX (0, 0.1, 0.2, 0.3, 0.4 and 0.5 μ mol/L) was added respectively after cells adhere to the wall, final volume of fluid was 200 μ l/hole, and made PBS hole equal zero. Final concentration 20 μ l 5 mg/mlMTT solution was added to after being cultured for 24 hours, and continued to be cultured for 4 hours, 150 μ lDMSO was added to every hole to be shook up slowly for 10

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minutes after carefully getting supernatant, measuring 490nm wavelength absorption value (A value) by enzyme-labeling instrument.

The effects of NS-398 different doses on cell growth

NS-398 nutrient fluid (0, 5, 10, 20, 40 and 100 umol/L) is added to SGC-7901 cells inoculated into 96 orifice plate (final volume of fluid 200 ul/hole), measuring 490 nm wavelength A value by the enzyme-labeling instrument of above method.

The effects of different doses of PDTC on cell growth

Add PDTC nutrient fluid (0, 0.04, 0.16, 0.6, 2.5, 10 and 40 umol/L) to SGC-7901 cells inoculated into 96 orifice plate (final volume of fluid 200 ul/hole), and determine 490 nm wavelength A value by the enzyme-labeling instrument of above method. Every hole is installed 5 multiple pores, taking the average. Calculating cell viability = (experimental group A value/control group A value) × 100%, cell death rate = 1 - cell viability.

The effect of TAX and its respectively combining with NS-398 and PDTC on SGC-7901 cell and COX-2, P-gp and the expression of p65 protein

Based on above experiment, respectively collect SGC-7901 cell induced by TAX (0, 0.1, 0.3 and 0.5 umol/L) for 24 hours and cells jointly treated by NS-398 (0, 5 and 10 umol/L) and TAX (0.3 umol/L) for 24 hours, 48 hours, and cells of PDTC (0, 0.2 umol/L) pre-acted for one hour functioning with TAX (0.3 umol/L) for 24 hours, 48 hours, add denaturing solution on ice and keep it on ice for 30min, centrifugation occurs when it is 4 degree, extract supernatant protein; extract corresponding nucleoprotein according to operating steps of nuclear protein extraction kit (BIYUNTIAN company), which is used to measure p65 level. Conduct protein excretion by BCA method, 120 °C, 5min denaturalization treatment, respectively undergo 12% (COX-2 and p65) and 6% (P-gp) SDS-PAGE after sample. Cut off corresponding molecular weight gel according to pre-staining marker, semi-dry transmembrane, voltage 100V, conduct transmembrane for 1h, close TBST fluid containing 5%skim milk for 2 hours, incubate COX-2, P-gp and p65 monoclonal antibody overnight at 4°C, use TBST to clean for three times, each time for 15 min. Incubate horseradish peroxidase-labeled mouse or rabbit secondary antibody for 1 hour, after cleaning by TBST, exposure meter is coated with luminescence agent and exposure occurs, and save the image. Use Jetta 801 professional digital gel imaging as objective band and make optical density analysis with analysis system 3.3.2, regarding the ratio between objective band and β-actin optical density as relative content of protein expression.

Statistical analysis

Take the average of three independent experiments as statistics, which is expressed by mean number ± standard deviation (X±SD), adopt spass17.0 statistical software, use t to test the single variable between two groups data, use single factor analysis of variance to compare several groups data. There exists statistical significance based on p<0.05 as difference.

Table 1. Effect of TAX on the Growth of SGC-7901 Cells for 24 Hours ($\bar{X} \pm SD, n=3$)

concentration of TAX (umol/L)	A490	Survival of SGC-7901 cells (%)
0	1.32±0.05	
0.1	1.18±0.05* Δ	89.61±0.57
0.2	1.08±0.03* Δ	82.07±2.27
0.3	0.99±0.04* Δ	74.95±1.56
0.4	0.84±0.03* Δ	63.80±0.31
0.5	0.77±0.03* Δ	58.49±1.30

*p<0.05, compared with control; Δ p<0.05, compared with former group

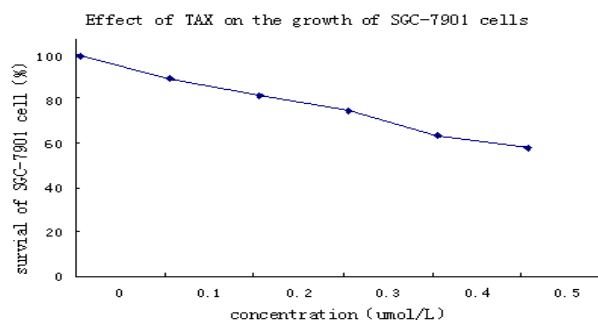


Figure 1. The Effect of TAX at Different Concentrations on the Growth of SGC-7901

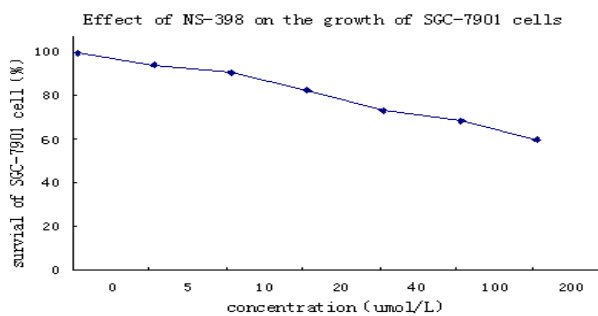


Figure 2. The Effect of NS-398 at Different Concentrations on the Growth of SGC-7901 Cells

Results

The effect of TAX on cell growth

Use different concentration TAX to function on cell lines for 24hours, use MTT method to determine the impact of SGC-7901 cell lines growth. The results show that cell viability decreases as TAX drug concentration increases (Figure 1, Table 1). TAX shows obvious proliferation inhibition effect on growth of gastric cancer cell lines, which presents dose-dependent manner (p<0.05).

The effect of NS-398 on SGC-7901 cell growth

NS-398 has an inhibitory effect on SGC-7901 cell growth, and shows dose-dependent manner (Figure 2, Table 2). When the final concentration of NS-398 is 10 umol/L, survival rate < 90%, it is non cytotoxic concentration. 5umol/L and 10umol/L are selected in this experiment as test dose to observe the effect on COX-2, P65 and P-gp protein expression of cell after it functions with TAX.

The effect of PDTC on SGC-7901 cell growth

PDTC has an inhibitory effect on SGC-7901 cell

Table 2. Effect of NS-398 on the Growth of SGC-7901 Cells for 48 Hours ($\bar{X} \pm SD, n=3$)

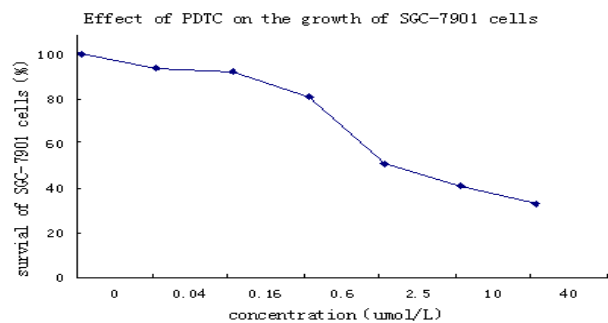
Concentration of NS-398 (umol/L)	A490	Survival of SGC-7901 cells(%)
0	1.35±0.08	
5	1.28±0.09	94.31±1.24
10	1.23±0.09	90.85±1.01
20	1.12±0.10*	82.93±2.56
40	1.00±0.04*	73.70±1.48
100	0.93±0.08*	68.66±2.33
200	0.82±0.06*	60.34±0.78

*p<0.05, compared with control; Δ p<0.05, compared with former group

Table 3. Effect of PDTC on the Growth of SGC-7901 Cells for 48 Hours ($\bar{X} \pm SD, n=3$)

Concentration of PDTC (umol/L)	A490	Survival of SGC-7901 cells (%)
0	1.39±0.17	
0.04	1.31±0.19	93.84±2.09
0.16	1.28±0.18	92.21±1.59
0.6	1.12±0.11	80.77±2.43
2.5	0.71±0.17* Δ	50.68±6.18
10	0.58±0.24*	40.79±11.70
40	0.47±0.15*	33.05±6.24

*p<0.05, compared with control; Δ p<0.05, compared with former group

**Figure 3. The Effect of PDTC at Different Concentrations on the Growth of SGC-7901**

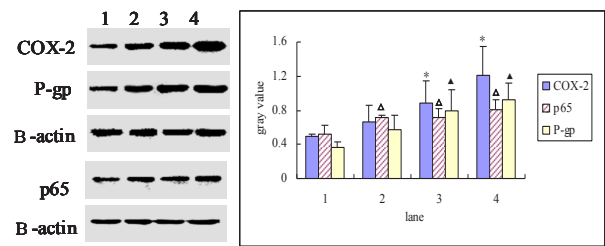
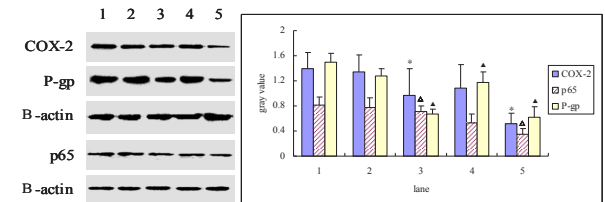
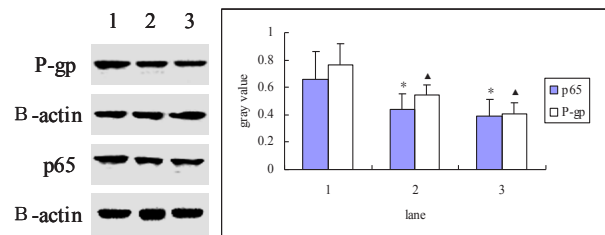
growth, and shows dose-dependent manner (Figure 3, Table 3). What the pre-experiment shows, after 0.2 umol/L PDTC acts on SGC-7901 cell for 48 hours, cell survival rate < 90%, is non cytotoxic concentration. So 0.2 umol/L test dose is selected in this experiment to observe the effect on P65 and P-gp protein expression of cell after it functions with TAX.

The effect of TAX on SGC-7901 cell and protein expression of COX-2, p65 and P-gp

After adopting different concentration TAX (0, 0.1, 0.3 and 0.5 umol/L) to function with SGC-7901 cell for 24 hours, use Western blot method to test its effect on COX-2, p65 and P-gp protein expression. The result shows that SGC-7901 cell COX-2, p65 and P-gp protein all have certain expression, as TAX dose increases, all proteins showing an increasing trend (Figure 4).

The effect of TAX and NS-398 on SGC-7901 cell COX-2, p65 and P-gp protein expression

After adopting different concentration NS-398 (0, 5,

**Figure 4. The Effects of TAX on COX-2, p65 and P-gp Expression in SGC-7901 Cells.** lane 1-4: SGC-7901 cells+0,0.1,0.3,0.5umol/L Tax for 24h; *P<0.05, COX-2 expression in lane 3 and 4 compared with lane 1; Δ P<0.05, p65 expression in lane 2, 3 and 4 compared with lane 1; \blacktriangle P<0.05, P-gp expression in lane 3 and 4 compared with lane 1**Figure 5. The Effects of NS-398 on COX-2, p65 and P-gp Expression in SGC-7901 Cells.** lane 1:SGC-7901 cells+0.3 umol/L TAX for 48 h;lane 2-3: 0.3 umol/L Tax combined with 5, 10 umol/L NS-398 for 24 h; lane 4-5: 0.3 umol/L Tax combined with 5, 10 umol/L NS-398 for 48 h; *P<0.05, COX-2 expression in lane 3 and 5 compared with lane 1; *P<0.05, p65 expression in lane 3 and 5 compared with lane 1; \blacktriangle P<0.05, P-gp expression in lane 3, 4 and 5 compared with lane 1**Figure 6. The Effects of PDTC on p65 and P-gp Expression in SGC-7901 Cells.** lane 1: SGC-7901 cells+0.3 umol/L TAX for 48h; lane 2-3: 0.3 umol/L Tax combined with 0.2 umol/L PDTC for 24h, 48h; *P<0.05, p65 expression in lane 2 and 3 compared with lane 1; \blacktriangle P<0.05, P-gp expression in lane 2 and 3 compared with lane 1

10 umol/L) and TAX (0.3 umol/L) to act on SGC-7901 cells for 24 hours, 48 hours, use Western blot method to test its effect on COX-2, p65 and P-gp protein expression. Results show that, through the effect of NS-398, the increased COX-2, p65 and P-gp protein level induced by TAX can be inhibited obviously, and as the dose of NS-398 increases and acting time prolongs, three kinds of protein expression levels all show decreasing trend (Figure 5).

The effect of TAX and PDTC on SGC-7901 cell p65 and P-gp protein expression

After adopting PDTC (0, 0.2 umol/L) to pre-action for 1 hour, combine with TAX to function for 24 hours, 48 hours, use Western blot method to test its effect on p65 and P-gp protein expression. The results show that after cooperating with PDTC, SGC-7901 cell p65 and P-gp protein expression decreases, inhibitory function increases as function time prolongs (Figure 6).

Discussion

In normal circumstances, COX-2 doesn't express itself in most of tissue and cells, it can be induced by hypoxia, cytokine, certain drugs and other stimulating factors. Many studies find that antitumor drug can induce tumor cell to express COX-2. Eichele etc (Eichele et al., 2008). Use cisplatin, Paclitaxel to act respectively on cervical cancer cells, finding that both can induce cervical cancer cells COX-2 protein to express, and the increasing of expression level shows time-dependent manner. The study results of Zhang Mengxi etc (Zhang et al., 2009) show that paclitaxel can induce COX-2 expression of gastric adenocarcinoma cell lines BGC-823 to increase.

The production of MDR is a main reason of tumor chemotherapy failure, but studies suggest that the over-expression of P-gp encoded by MDR gene family is the main reason of the expression of MDR (Breier et al., 2005). P-gp is a kind of ATP enzyme- active transmembrane pump encoded by MDR gene, and combines with drugs in cell (Hyung et al., 2011). At the same time hydrolysis of ATP happens and gets energy, pump out drug inverse concentration gradient from the cell, drugs concentration in cell is declining constantly, making the toxic injury of drugs for cell weaken until it disappears and finally drug resistance appears. In recent years, many studies (Yu et al., 2009) find that there exists correlation between MDR and COX-2, COX-2 may participate in the production of MDR through P-gp. Vitro Studies (Zhang et al., 2009) suggest that when paclitaxel induces gastric cancer cell lines COX-2 to express highly, P-gp also increases, inhibit COX-2 expression, P-gp expression also decreases.

NF-KB is a kind of protein which can produce specific binding with a variety of gene promoters or enhancer site KB sites and can promote their transcriptional protein (Humberto and Alun, 2011). It can regulate multiple genes, such as cytokine, chemokine, immune factors, transcription factor etc, thus playing several biological roles, such as involvement in the inflammatory response, regulation of cell apoptosis, its own transcription, tumor genesis and drug resistance etc. Many researches (Liu et al., 2006; Yang et al., 2010) found that NF-KB can regulate expressions of many solid tumors and leukemia cell line P-gp, NF- κ B inhibitor can decrease its expression. Others (Bentires-Alj et al., 2003) demonstrate that first exon in MDR1 gene promoter region contains a purified NF- κ B combination sequence (5:CCTFFCGGGG-3), and NF-KB can indeed activate reporter gene transcription connecting with mdrl promoter. This shows that MDR1 is likely to be one of downstream target genes, activating transcription under the regulation of NF-KB, over-expressing P-gp and causing drug resistance of tumor cell.

This study adopts TAX, NS-398 and PDTC to act on gastric cancer cell line SGC-7901, on the basis of obtaining its cell poisonous dose, discussing relevant protein expression level and possible mechanism of COX-2 regulating P-gp expression. The most common functional form of NF- κ B in cells is NF- κ B hetero dimer composed of p65 and p50, which exists in the form of non-activation, and combines with I κ B, when it is stimulated, I κ B is hydrolyzed, let it recover transcriptional

activity and transfer it from cytoplasm to cell nuclear, regulate relevant gene expression, so the detection of p65 expression are all for nuclear levels in this study. Western blot detection finds that COX-2, p65 and P-gp all have a certain level of expression, after exposure to TAX, three kinds of protein all show the increasing trend, the three are obviously higher than basic level ($p < 0.05$) after exposure to the high concentration (0.3, 0.5 $\mu\text{mol/L}$).

This experiment suggests that cell death rate is close to 50% after TAX 0.3 $\mu\text{mol/L}$ acting for 48 hours, this concentration is close to IC50 concentration, so this study chooses this concentration to combine with NS-398 and PDTC. Western blot test shows that, COX-2, p65 and P-gp all can be inhibited, when NS-398 (10 $\mu\text{mol/L}$) functions for 24 hours, three kinds of protein levels begin to decrease obviously, and further decreases ($p < 0.05$) as the function time prolongs. When combining with non-cell toxic dose PDTC (0.2 $\mu\text{mol/L}$), p65 and P-gp expression both decrease, and show time-dependent manner ($p < 0.05$).

The results of this study show that, in gastric cancer cells, there exists correlation between p65 and P-gp, NF- κ B inhibitors can inhibit the expression of P-gp; paclitaxel can induce COX-2, NF-KB p65 and P-gp expressions of gastric cancer cell line SGC-7901 to increase, and above three expressions all can be inhibited by COX-2 selective inhibitor NS-398. This is similar to the finding of studying prostaglandin regulating chemotherapy resistance in breast cancer by Zattelli MC etc, which suggests that TAX induces SGC-7901 cell to produce multidrug resistance, maybe through inducing COX-2 expression to increase, makes prostaglandin synthesis increase, it further activates NF-KB in the cell, and transfers into nucleus, activates MDR1 gene transcription, thus making P-gp expression increase, and this kind of multidrug resistance can be inhibited by COX-2 selective inhibitor.

The correlation between COX-2 and multidrug resistance P-gp in most of the tumors has been clear, but its concrete regulation mechanism is lack of research. This study suggests that inducing P-gp mechanism by COX-2 may relate to the activation of NF-KB pathway, NF-KB can be regarded as a new target of enhancing the effect of chemotherapy to make further research and exploration. Moreover, this study further supports the combination of COX-2 selective inhibitor and chemotherapeutic drugs in gastric cancer, reinforcing the hypothesis of anti-carcinogenesis effects.

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