Synergic effect between 5-fluorouracil and celecoxib on hypoxic gastric cancer cells

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Abstract. 5-fluorouracil (5-FU) is commonly used in the treatment of gastric cancer; however, resistance to this drug occurs under hypoxic conditions. Celecoxib may be used to reverse this resistance. The aim of the present study was to elucidate the inhibitory effects and mechanisms of 5-FU and celecoxib on the gastric cancer cell line SGC7901 under hypoxic conditions. SGC7901 cells were divided into four groups: Hypoxic control group, 5-FU group, celecoxib group and 5-FU/celecoxib combination group. Following treatment, the inhibition rates of cells were determined using an MTT assay. Protein and messenger RNA (mRNA) expression of hypoxia-inducible factor 2α (HIF-2α), adenosine triphosphate-binding cassette sub-family G member 2 (ABCG2) and octamer binding protein 4 (Oct-4) were determined using immunohistochemistry, reverse transcription quantitative polymerase chain reaction (RT-qPCR) and western blot analysis. The results demonstrated that the 5-FU/celecoxib combination group had a significantly higher inhibition rate than the individually treated 5-FU and celecoxib groups ($P<0.05$); inhibition rates were 66.09, 52.61 and 46.1%, respectively. mRNA and protein expression levels of HIF-2α, ABCG2 and Oct-4 were significantly lower in the celecoxib and 5-FU/celecoxib combination groups ($P<0.01$) compared with those of the hypoxia control and 5-FU groups. The 5-FU group demonstrated the highest levels of the respective mRNA and proteins. In conclusion, the results of the present study indicated that celecoxib had anti-tumor effects, as it was shown to inhibit tumor cell growth via the inhibition of HIF-2α, ABCG2 and Oct-4. The 5-FU/celecoxib combination had a synergic effect on tumor growth inhibition. This therefore suggested that inhibition of HIF-2α, ABCG2 and Oct-4 may be a potential method of reducing chemotherapy resistance and enhancing the effectiveness of chemotherapy treatment.

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Introduction

Gastric cancer is a prevalent type of cancer with high mortality rates throughout the world, which is often diagnosed at an advanced stage (1,2). The five-year survival rate was reported to be 70-75% for stage I disease, which drops to 35% for stage II (2). Numerous efforts have been taken to improve therapies and survival; at present, chemotherapy is one of the primary treatments for gastric cancer (3). However, chemotherapy treatment is not always effective; hypoxia, a characteristic of solid tumors, including gastric cancer, has been reported to induce chemotherapy resistance (4).

5-fluorouracil (5-FU) is an antimetabolite chemotherapeutic drug which targets thymidylate synthase, blocking the transformation of deoxy-uridine monophosphate into deoxy-thymidine acid. This results in cell death via decreased DNA synthesis and S-phase arrest (5). Clinical trials showed that regimens containing 5-FU improved the survival rate of gastric cancer patients; however, local treatment failure and distant metastases still occur (3,6). Previous studies have demonstrated that hypoxic conditions induced cancer cell resistance to 5-FU treatment *in vitro* (7,8).

Celecoxib is a non-steroidal anti-inflammatory drug (NSAID) and a selective cyclooxygenase (COX)-2 inhibitor with anti-inflammatory and analgesic effects (9). Previous studies indicated that celecoxib may have a promising novel use in the treatment of cancer; however, its mechanism of action remains to be elucidated (10-12).

The aim of the present study was to assess the effects of celecoxib on hypoxic gastric cancer SGC7901 cells and determine whether celecoxib reduced the hypoxia-induced

resistance of these cells to 5-FU. Furthermore, the present study aimed to elucidate the underlying mechanisms of action in order to improve the treatment of gastric cancer and increase the survival rate of patients.

Materials and methods

Materials. Human gastric cancer cells SGC7901 (Shandong Academy of Sciences, Jinan, China)and cobalt chloride ($CoCl_2$) were provided by Professor Feng from the Affiliated Hospital of Weifang Medical University (Weifang, China). The cells tested negative for mycoplasmic infection. 5-FU was obtained from Zhenguo Pharmaceutical Co., Ltd. (Jiangsu, China). RPMI 1640 medium was purchased from Gibco-BRL (Carlsbad, CA, USA). MTT kits were purchased from Sigma (St Louis, MO, USA). Fetal bovine serum (FBS) was obtained from Hyclone (Thermo Fisher Scientific, Waltham, MA, USA). Rabbit anti-hypoxia-inducible factor (HIF)-2α, anti-octamer binding protein (Oct)-4 and anti-adenosine triphosphate-binding cassette sub-family G member 2 (ABCG2) antibodies and immunohistochemical kits were for purchased from Abcam (Cambridge, MA, USA). TRIzol® reagent was purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Oligo-deoxy-thymine(dT), Moloney murine leukemia virus (M-MLV) reverse transcriptase, 5X reverse transcription buffer and 10X polymerase chain reaction (PCR) buffer were obtained from Fermentas (Waltham, MA, USA). A protein extraction kit was purchased from Biyuntian Biotech, Co. (Shanghai, China). Finally, the western blot enhanced chemiluminescence (ECL) reagent kit was obtained from Thermo Fisher Scientific (Waltham, MA, USA).

Cell culture. SGC7901 cells were inoculated in RPMI 1640 medium containing FBS (100 ml/l), penicillin and streptomycin (10^5 U/l). Cells were subcultured regularly at 37˚C in a 5% CO_2 incubator. The chemical hypoxia-inducing agent $CoCl_2$ (150 μmol/l) was used to simulate the hypoxic microenvironment of solid tumors.

Proliferation inhibition rate. The proliferation inhibition rates of different concentrations of 5-FU and celecoxib in gastric cancer cells under hypoxia were determined by MTT assay. Cells in the logarithmic growth phase were inoculated in 96-well culture plates at a cell density of $2x10^4$/l (200 μl). Cells were divided into four groups: The hypoxia control group, 5-FU group, celecoxib group and 5-FU/celecoxib combination group. $CoCl_2$ was used to simulate a hypoxic microenvironment following the cells becoming adherent. The hypoxic control group was not treated with any drug. Cells in the 5-FU group were exposed to numerous concentrations of 5-FU (25, 50, 100 and 200 mg/l). The celecoxib group was exposed to different concentrations of celecoxib (50, 100, 200 and 300 μmol/l). Cells were cultured for 24, 48 or 72 h at 37˚C in a 5% CO_2 incubator. Optical density (OD) for each well was measured using a microplate reader (Bio-rad 680; Bio-rad Laboratories, Inc., Hercules, CA, USA) at 490 nm. Cell growth inhibition rates were calculated as: [(control OD-experimental OD)/control OD]x100%. The half inhibitory concentrations (IC_{50}) of 5-FU and celecoxib under hypoxic conditions were calculated. The 5-FU/celecoxib combination group was subjected to 5-FU and celecoxib using their respective IC_{50}. Cell growth inhibition rates were calculated following culturing the cells for 24, 48 and 72 h at 37˚C in a 5% CO_2 incubator.

Immunohistochemical detection of HIF-2α, ABCG2 and Oct-4. SGC7901 cells in the logarithmic growth phase were prepared into a $4x10^4$ cells/ml suspension (0.5 ml) and added to 24-well plates with cover glasses. Cells were separated into identical groups and subjected to identical conditions to those of the proliferation inhibition rate experiment. The cover glasses were removed following 48 h in culture. Cells were fixed using cold acetone for 10-15 min and then washed with PBS. Immunohistochemistry kits for HIF-2α, ABCG2 and Oct-4 were used according to the manufacturer's instructions. MDA-MB-231 breast cancer cells (Shanghai Baili Biological Technology Co., Shanghai, China) were used as the positive control and PBS in place of the primary antibodies was used as the negative control. Cytoplasms stained with yellowish brown pellets indicated a positive result.

HIF-2α, ABCG2 and Oct-4 reverse transcription quantitative PCR (RT-qPCR). Cells were grouped and subjected to identical conditions as in the proliferation inhibition rate and immunohistochemistry experiments. TRIzol® was used to extract total RNA from the cells, RNA was then dissolved in 30 μl 0.1% diethylpyrocarbonate water (Biyuntian Biotech,Co., Shanghai, China). Reverse transcription was performed in 20 μl to obtain cDNA: RNAase-free deionized water (9 μl), RNA template (2 μl), Oligo-(dT)-18 (1 μl), 5X reaction buffer (4 μl), RNase inhibitor (20 U/μl; 1 μl), dNTP mix (10 mmol/l; 2 μl), and M-MLV RT (1 μl). Reaction conditions were: 70˚C for 5 min, then immediately put on ice for 5 min; 25˚C for 5 min; 37˚C for 60 min; and 70˚C for 10 min. Samples were kept on ice if used immediately, or kept at -150˚C if used later.

Primer sequences for semi-quantitative PCR were: HIF-2α forward, 5'-CTT GGA GGG TTT CAT TGC TGT GGT-3' and reverse, 5'-GTG AAG TCA AAG ATG CTG TGT CCT-3' (123 bp); ABCG2 forward, 5'-CCC TTA TGA TGG TGG CTT ATT C-3' and reverse, 5'-GTG AGA TTG ACC AAC AGA CCA T-3' (132 bp); Oct-4 forward, 5'-CCC GAA AGA GAA AGC GAA CC-3' and reverse, 5'-CAG AAC CAC ACT CGG ACC AC-3' (151 bp); and GAPDH forward, 5'-GCA CCA CCA ACT GCT TAG CAC-3' and reverse, 5'-GCA GCG CCA GTA GAG GCA GG-3' (1143 bp). PCR reaction (50 μl) was performed using cDNA template (1 μl), forward and reverse primers (1 μl each), Taq DNA polymerase (1 μl), dioxynucleotide triphosphates (2 mmol/l, 5 μl), $MgCl_2$ (25 mmol/l, 2 μl), 10X PCR buffer (5 μl) and double distilled H_2O (34 μl). Conditions were as follows: 94˚C for 5 min, 94˚C for 30 sec, 50˚C for 30 sec and 72˚C for 60 sec, for 40 cycles and then 72˚C for 10 min. Fragments were separated using 1.5% agarose gel electrophoresis. The MiniLumi digital photo gel imaging system (DNR Bio-Imaging Systems Ltd., Jerusalem, Israel) and Image J 1.26t (National Institutes of Health, Bethesda, MD, USA) were used to capture images of the gels. HIF-2α, ABCG2 and Oct-4 messenger RNA (mRNA) expression was determined based on the OD value using GAPDH as reference.

Table I. Effect of treatment time and 5-FU concentration on proliferation of SGC7901 cells under hypoxic conditions.

	24 h		48 h		72 h	
Group	OD	Inhibition rate (%)	OD	Inhibition rate (%)	OD	Inhibition rate (%)
Hypoxia	0.531±0.020		0.672±0.021		0.860±0.026	
5-FU						
25 mmol/l	0.416±0.017	21.66	0.517±0.019	23.07	0.652±0.025	24.19
50 mmol/l	0.388±0.016	26.93	0.468±0.022	30.36	0.546±0.018	36.51
100 mmol/l	0.349±0.015	34.27	0.403±0.015	40.03	0.459±0.016	46.63
200 mmol/l	0.298±0.016	43.88	0.334±0.010	50.29	0.406±0.017	52.80

Mean ± standard deviation (n=4). 5-FU half inhibitory concentration, 200 mmol/l 48 h following treatment. 5-FU, 5-fluorouracil; OD, optical density at 490 nm.

Table II. Effect of treatment time and celecoxib concentration on proliferation of SGC7901 cells under hypoxic conditions.

	24h		48h		72h	
Group	OD	Inhibition rate (%)	OD	Inhibition rate (%)	OD	Inhibition rate (%)
Hypoxia	0.520±0.020		0.678±0.023		0.840±0.022	
Celecoxib						
50 μmol/l	0.476±0.018	8.46	0.534±0.019	21.24	0.737±0.019	12.26
100 μmol/l	0.295±0.020	43.27	0.348±0.016	48.67	0.466±0.018	44.52
200 μmol/l	0.244±0.017	53.08	0.284±0.017	58.11	0.385±0.014	54.17
300 μmol/l	0.188±0.012	60.50	0.207±0.013	69.50	0.319±0.016	62.02

Mean ± standard deviation (n=4). Celecoxib half inhibitory concentration, 100 μmol/l 48 h following treatment. OD, optical density at 490 nm.

Table III. Inhibition ratio using half inhibitory concentrations of each drug alone or in combination.

	24h		48h		72h	
Group	OD	Inhibition rate (%)	OD	Inhibition rate (%)	OD	Inhibition rate (%)
Hypoxia	0.506±0.023		0.690±0.022		0.888±0.024	
5-FU	0.272±0.018	46.25	0.358±0.019	52.61	0.467±0.022	47.41
Celecoxib	0.306±0.027	39.53	0.367±0.019	46.81	0.504±0.021	43.24
Combination	0.210±0.011	58.50	0.234±0.015	66.09	0.390±0.014	56.08

Mean ± standard deviation (n=4). 5-FU, 5-fluorouracil; OD, optical density at 490 nm.

Western blot analysis. Cells were grouped and treated as described above. Cells were washed twice with chilled PBS following 24 h in culture. Radio-immunoprecipitation assay cell lysis solution (Biyuntian Biotech, Co.) was added, then kept in an ice bath for 30 min. Cells were centrifuged (100 x g) for 10 min at 4˚C. The supernatant was collected and stored at -70˚C. Proteins (25 μg) were separated using SDS-PAGE, transferred to nitrocellulose membranes, and incubated for 2 h with 5% skimmed milk powder at 37˚C. Primary rabbit anti-HIF-2α, Oct-4 and ABCG2 polyclonal antibodies (dilution 1:50) and GAPDH were added and incubated overnight at 4˚C. Secondary horseradish peroxidase-labeled antibodies were added and incubated for 2 h at 37˚C. The antibodies were purchased from Abcam (Cambridge, MA, USA). Blots were quantified using an ECL reagent. The ratio of the absorbance value of HIF-2α, ABCG2 and Oct-4 was determined relative to GAPDH using the digital photo gel imaging system (Image J).

Statistical analysis. SPSS 17.0 (IBM, Armonk, NY, USA) was used for data processing and analysis. Continuous data are presented as the means ± standard deviation and analyzed using a one-way analysis of variance, followed by a Dunnett's post-hoc T3 test. $P<0.05$ was considered to indicate a statistically significant difference between values.

Results

5-FU and celecoxib, alone or in combination, inhibit the proliferation of hypoxic SGC7901 cells. The proliferation of hypoxic SGC7901 gastric cancer cells was significantly inhibited in a dose-dependent manner by 5-FU (Table I) and celecoxib (Table II), ($P<0.05$ for comparisons of all concentrations for 5-FU as well as celecoxib). Cells were in the logarithmic growth phase within 48 h following inoculation. The IC_{50} of 5-FU was 200 mg/l, while the IC_{50} of celecoxib was 100 μmol/l. The respective IC_{50} of 5-FU and celecoxib were used in combination for the treatment of the 5-FU/celecoxib combination group. The combination treatment inhibited cell proliferation to a greater extent at each time-point than each treatment alone (Table III).

5-FU-treated cells express the highest levels of HIF-2α, ABCG2 and Oct-4. Following 48 h in culture, immunohistochemical analysis revealed that the expression of HIF-2α, ABCG2 and Oct-4 proteins were the highest in the 5-FU group, followed by the hypoxia control group. The celecoxib and 5-FU/celecoxib combination groups demonstrated the lowest expression of the proteins (Figs. 1-3).

HIF-2α, ABCG2 and Oct-4 expression levels are significantly reduced by celecoxib and 5-FU/celecoxib combination treatments. RT-qPCR was used to observe changes in HIF-2α, ABCG2 and Oct-4 mRNA expression in each group following 48 h in culture. HIF-2α, ABCG2 and Oct-4 expression levels were the highest in the 5-FU group, followed by the hypoxia control group, and significantly lower in the 5-FU/celecoxib combination and celecoxib groups ($P<0.01$) (Figs. 4 and 5).

HIF-2α, ABCG2 and Oct-4 levels by western blot. Western blot analysis was used to observe the changes in HIF-2α, ABCG2 and Oct-4 expression following 48 h in culture. HIF-2α, ABCG2 and Oct-4 expression were the highest in the 5-FU group, followed by the hypoxia control group, and significantly lower in the 5-FU/celecoxib combination and celecoxib groups ($P<0.01$) (Figs. 6 and 7).

Discussion

5-FU is one of the most commonly used chemotherapeutic drugs, which is also employed to test the tumor susceptibility of gastric cancer cells *in vitro* (7,8). The mechanism of 5-FU proceeds through inducing apoptosis via blocking DNA synthesis, which is done by restricting the progression of cells in the S phase of the cell cycle (13). However, cells have been shown to develop resistance to 5-FU, particularly solid tumor cells under hypoxic conditions (4,7,8). Therefore, determining novel strategies to overcome this resistance is of prime importance.

The present study demonstrated that celecoxib or 5-FU alone were able to inhibit gastric cancer cell growth. Of note, the combination of the two drugs had a synergistic effect, further inhibiting the growth of tumor cells. The results also revealed that the expression levels of HIF-2α, ABCG2 and Oct-4 were involved in the growth suppression of these tumor cells.

Previous studies have reported that small amounts of cancer stem cells were present in tumor tissues; these cells had an unlimited self-renewal ability and unlimited differentiation potential. Cancer stem cells are increasingly thought to be the cause of metastases and tumor recurrence as well as drug and radiation resistance (14,15). However, little is currently known about these cells and their phenotypic marker profile has not yet been defined; therefore, purification of cancer stem cells is difficult.

HIF was reported to be closely associated with a malignant phenotype, which was involved in tumor angiogenesis, invasion and metastasis as well as drug and radiation resistance (16). HIF-2α, in comparison to HIF-1α, was shown to be more closely associated with cancer stem cells and maintains the stem cell phenotype via the regulation of several associated pathways (17). ABCG2, a member of the superfamily of transport proteins, was reported to be involved in the excretion of numerous chemotherapeutic drugs from cells; therefore, high expression of ABCG2 may be a significant contributing factor in tumor multi-drug resistance (18). Studies have shown that ABCG2 expression was high in numerous cancer stem cells, and that ABCG2 was a direct target gene of HIF-2α. This therefore indicated that high expression of HIF-2α and ABCG2 may lead to the multi-drug resistance observed in tumor stem cells and hypoxic cells (19). Oct-4, a member of the Pit-Oct-Unc (POU) family of transcription factors, acts as a marker of cancer stem cell pluripotency (20,21). Covello *et al* (17) demonstrated that Oct-4 was also a direct target gene of HIF-2α. This therefore indicated that hypoxia may induce the retention of a stem cell phenotype in tumor cells through activation of the HIF-2α/Oct-4 pathway.

Dallas *et al* (22) reported a high expression of the stem cell phenotype (CD133+/CD44+) in colon cancer cell lines (HT29/5-FUR) resistant to 5-FU, therefore suggesting that the cancer stem cells resistant to 5-FU may be the source of chemotherapy resistance. A previous study showed that expression levels of HIF-2α and ABCG2 were increased when 5-FU was added to the SGC7901 gastric cancer cells under hypoxic conditions, therefore indicating that this resistance may be associated with the induction of the HIF-2α/ABCG2 pathway and promote the maintenance of the stem cell phenotype (23).

Celecoxib is a selective COX-2 inhibitor which has anti-inflammatory and analgesic effects (24). It is primarily used for the treatment of acute or chronic osteoarthritis as well as rheumatoid arthritis; in addition, celecoxib has fewer gastrointestinal side effects than other NSAIDs (9). Celecoxib was also reported to have certain anti-tumor effects (10-12). Steinbach *et al* (25) showed that celecoxib significantly reduced the occurrence of polyps in patients with familial adenomatous polyposis. In addition, chronic NSAID therapy may be able to reduce the risk of colon cancer by 50%, as well as the incidence of esophageal and gastric cancer (26). Studies on animals showed that celecoxib prevented and inhibited gastric cancer carcinogenesis (27,28). The results of the present study demonstrated that celecoxib inhibited the proliferation of SGC7901 gastric cancer cells. The inhibition rate of the combined 5-FU/celecoxib group was significantly increased compared with that of the 5-FU group; these results were consistent with those of a previous

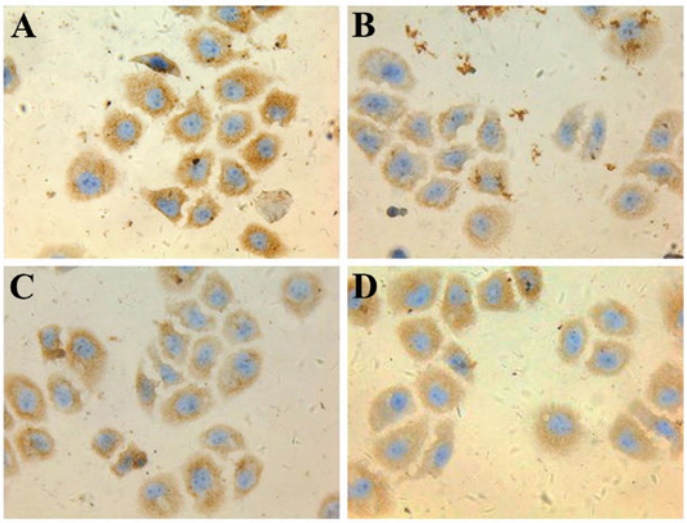

Figure 1. Hypoxia-inducible factor 2α expression in each group by immunocytochemistry (magnification, x400). (A) 5-fluorouracil group; (B) celecoxib group; (C) combination group; and (D) hypoxia control group.

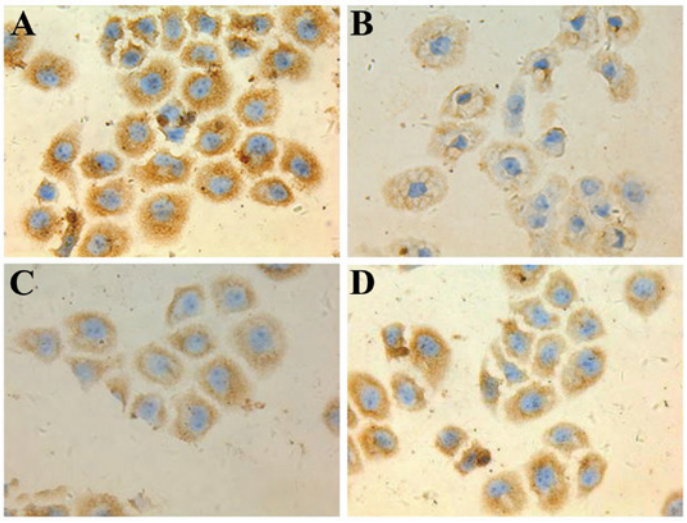

Figure 2. Adenosine triphosphate-binding cassette sub-family G member 2 expression in each group by immunocytochemistry (magnification, x400). (A) 5-fluorouracil group; (B) celecoxib group; (C) combination group; and (D) hypoxia control group.

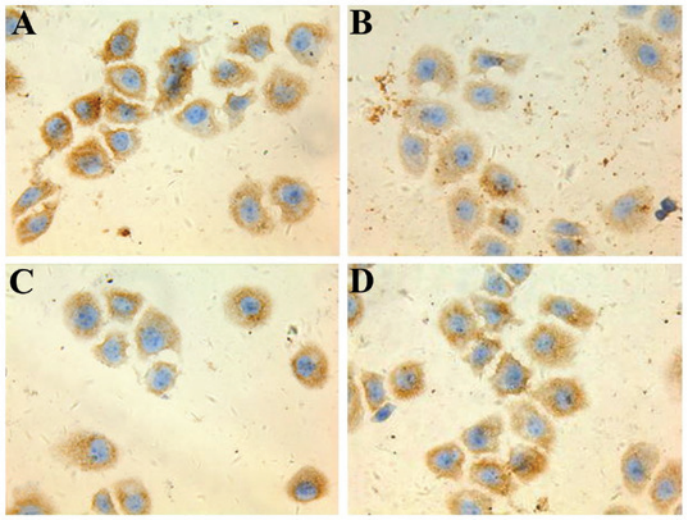

Figure 3. Octomer binding protein 4 expression in each group by immunocytochemistry (magnification, x400). (A) 5-fluorouracil group; (B) celecoxib group; (C) combination group; and (D) hypoxia control group.

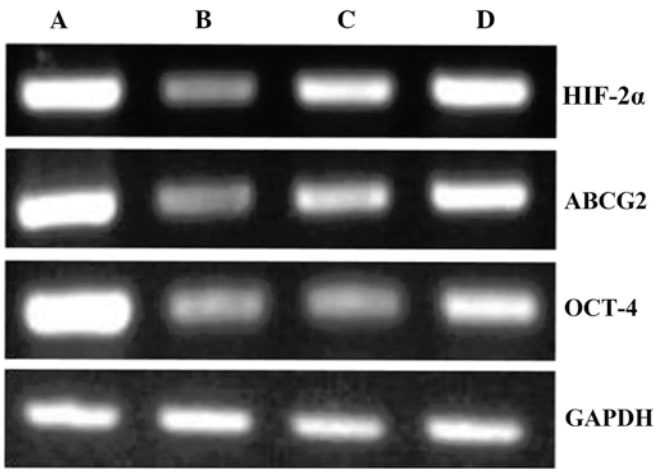

Figure 4. HIF-2α, ABCG2 and Oct-4 messenger RNA expression in each group using reverse transcription quantitative polymerase chain reaction. (A) 5-fluorouracil group; (B) celecoxib group; (C) combination group; and (D) hypoxia control group. HIF-2α, hypoxia-inducible factor 2α; ABSG2, adenosine triphosphate-binding cassette sub-family G member 2; Oct-4, octamer binding protein 4.

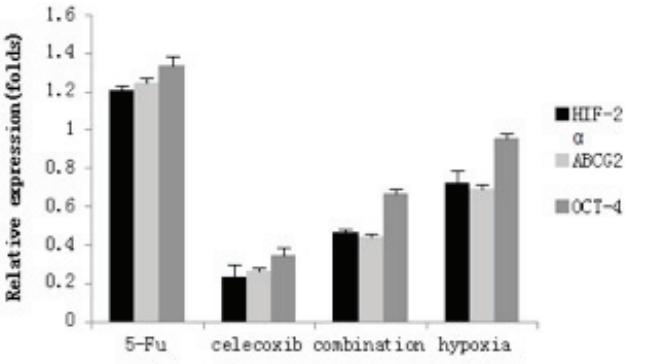

Figure 5. Quantification of HIF-2α, ABCG2 and Oct-4 mRNA expression in each group using reverse transcription quantitative polymerase chain reaction. Expression of HIF-2α, ABCG2 and Oct-4 was different between all groups. HIF-2α, hypoxia-inducible factor 2α; ABSG2, adenosine triphosphate-binding cassette sub-family G member 2; Oct-4, octamer binding protein 4.

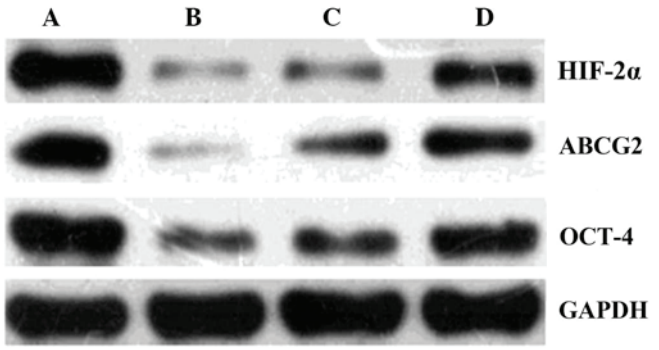

Figure 6. HIF-2α, ABCG2 and Oct-4 protein expression in each group using western blot analysis. (A) 5-fluorouracil group; (B) celecoxib group; (C) combination group; and (D) hypoxia control group. HIF-2α, hypoxia-inducible factor 2α; ABSG2, adenosine triphosphate-binding cassette sub-family G member 2; Oct-4, octamer binding protein 4.

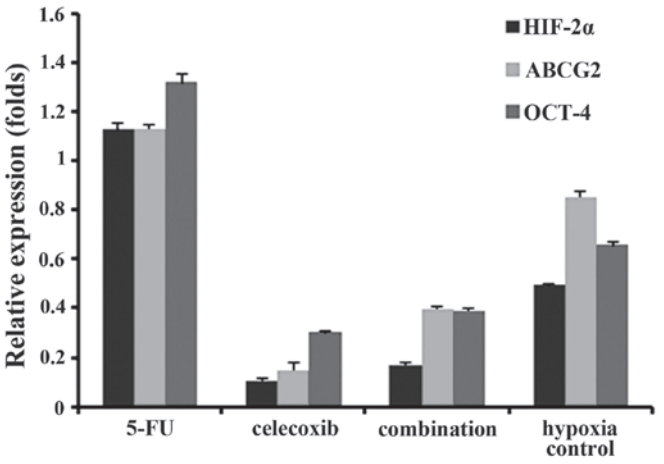

Figure 7. Quantification of HIF-2α, ABCG2 and Oct-4 protein expression in each group using western blot analysis. Expression of HIF-2α, ABCG2 and Oct-4 was different between all groups. HIF-2α, hypoxia-inducible factor 2α; ABSG2, adenosine triphosphate-binding cassette sub-family G member 2; Oct-4, octamer binding protein 4.

study using rofecoxib combined with anti-tumor drugs on gastric cancer (29).

However, the mechanism of the anti-tumor effect of NSAIDs remains to be elucidated. Previous experiments have suggested that NSAIDs induce apoptosis of tumor cells through inhibiting COX-2 activity, therefore reducing the synthesis of prostaglandin E2 (30). However, Ding *et al* (31) observed that the mechanism of the anti-tumor effect of celecoxib occurs prior to the deterioration of oral mucosa cells. Numerous studies have suggested that celecoxib may promote tumor cell apoptosis via COX-2-independent pathways, and its effects on apoptosis may be achieved through the regulation of genes, including p21, Fas, protein kinase B, glycogen synthase kinase 3β, forkhead homolog in rhabdomyosarcoma, caspase-9, B cell lymphoma 2/B cell lymphoma 2-associated X protein, p53 and survivin (32-35). However, further studies are required in order to elucidate the exact mechanisms underlying the effects of celecoxib in tumor cells.

In conclusion, the results of the present study demonstrated that celecoxib reduced mRNA and protein expression of HIF-2α, Oct-4 and ABCG2 in gastric cancer SGC7901 cells under hypoxic conditions. This therefore indicated that elevated expression of HIF-2α, ABCG2 and Oct-4 mRNA may lead to 5-FU resistance. Furthermore, the results showed that celecoxib increased the efficacy of 5-FU in gastric cancer by reducing 5-FU resistance, therefore indicating its potential synergic use in chemotherapy treatment. However, clinical trials are required to confirm this hypothesis.

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