

## Original Article

# Rosiglitazone sensitizes hepatocellular carcinoma cell lines to 5-fluorouracil antitumor activity through activation of the PPAR $\gamma$ signaling pathway

Liang-qi CAO<sup>1,3,#</sup>, Xiao-li WANG<sup>2,#</sup>, Qian WANG<sup>1,\*</sup>, Ping XUE<sup>3</sup>, Xing-yuan JIAO<sup>3</sup>, He-ping PENG<sup>3</sup>, Hai-wu LU<sup>3</sup>, Qiang ZHENG<sup>3</sup>, Xi-lin CHEN<sup>1</sup>, Xiao-hui HUANG<sup>1</sup>, Xin-hui FU<sup>1</sup>, Jing-song CHEN<sup>1</sup>

<sup>1</sup>Department of Hepatobiliary Surgery, the First Affiliated Hospital of Sun Yat-Sen University, Guangzhou 510080, China; <sup>2</sup>Department of Ophthalmology, Guangzhou No.8 People's Hospital, Guangzhou 510060, China; <sup>3</sup>Department of Hepatobiliary Surgery, the Second Affiliated Hospital of Guangzhou Medical College, Guangzhou 510260, China

**Aim:** Resistance to 5-fluorouracil (5-FU) is a major cause of chemotherapy failure in advanced hepatocellular carcinoma (HCC). Rosiglitazone, a peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) agonist, has a crucial role in growth inhibition and induction of apoptosis in several carcinoma cell lines. In this study, we examine rosiglitazone-induced sensitization of HCC cell lines (BEL-7402 and Huh-7 cells) to 5-FU.

**Methods:** The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to evaluate cell viability. Western blotting analysis was performed to detect the protein expression (PPAR $\gamma$ , PTEN, and COX-2) in BEL-7402 cells. Immunohistochemistry staining was used to examine the expression of PTEN in 100 advanced HCC tissues and paracancerous tissues. In addition, small interfering RNA was used to suppress PPAR $\gamma$ , PTEN, and COX-2 expression.

**Results:** Rosiglitazone facilitates the anti-tumor effect of 5-FU in HCC cell lines, which is mediated by the PPAR $\gamma$  signaling pathway. Activation of PPAR $\gamma$  by rosiglitazone increases PTEN expression and decreases COX-2 expression. Since distribution of PTEN in HCC tissues is significantly decreased compared with the paracancerous tissue, over-expression of PTEN by rosiglitazone enhances 5-FU-inhibited cell growth of HCC. Moreover, down-regulation of COX-2 is implicated in the synergistic effect of 5-FU.

**Conclusion:** Rosiglitazone sensitizes hepatocellular carcinoma cell lines to 5-FU antitumor activity through the activation of PPAR $\gamma$ . The results suggest potential novel therapies for the treatment of advanced liver cancer.

**Keywords:** rosiglitazone; hepatocellular carcinoma; PTEN; PPAR $\gamma$ ; 5-fluorouracil

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## Introduction

Rosiglitazone belongs to the thiazolidinedione group of drugs (TZDs) and has been regarded as a synthetic ligand of peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ )<sup>[1]</sup>. Unlike other members, such as troglitazone, pioglitazone, and ciglitazone, rosiglitazone has the highest affinity for PPAR $\gamma$ <sup>[2]</sup>. PPAR $\gamma$  is a ligand-activated nuclear hormone receptor and mediates transcriptional regulation of target genes. PPAR $\gamma$  activation inhibits growth and induces apoptosis of hepatocellular carcinoma cells *in vitro*<sup>[3,4]</sup>. However, it is not yet known whether rosiglitazone could be used as an antitumor agent.

Hepatocellular carcinoma (HCC) is one of the leading causes

of cancer deaths worldwide<sup>[5]</sup>. For advanced HCC, especially with portal tumor thrombi, conventional therapies are not generally effective and have potentially significant complications. Inherent or acquired drug resistance remains a major obstacle to chemotherapy. Therefore, exploring the alternative modality of combination chemotherapy for advanced HCC is desirable.

5-Fluorouracil (5-FU) is an inhibitor of deoxynucleoside triphosphate *de novo* synthesis and is widely used to treat solid tumors, including hepatic, colorectal, gastric and pancreatic cancer in clinical practice. However, the development of drug-resistant phenotypes significantly limits the clinical use of 5-FU. 5-FU alone has been shown to be of limited benefit in enhancing the survival of patients with advanced HCC. Moreover, many patients with HCC have tumors that are inherently resistant to chemotherapy or develop resistance during the course of therapy, leading to failure of HCC chemotherapy<sup>[6]</sup>.

# These authors contributed equally to this work.

\* To whom correspondence should be addressed.

E-mail wangq6@mail.sysu.edu.cn

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Therefore, current strategies focus on studying the molecular mechanisms of chemotherapy resistance, searching for effective methods of overcoming this resistance, and developing novel anti-tumor treatment agents.

The aim of this study is to understand the effects of combining 5-FU and rosiglitazone on HCC cell lines and to elucidate the mechanisms underlying the observed synergistic effect.

## Materials and methods

### Reagents

Rosiglitazone was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). 5-FU was obtained from Sigma Chemical Company (St Louis, MO, USA). These reagents were dissolved in dimethyl sulfoxide (DMSO), and the final concentration of DMSO was maintained at 0.1%. Mouse monoclonal anti-human PPAR $\gamma$  and PTEN, rabbit polyclonal anti-human COX-2 and horseradish peroxidase-conjugated goat anti-mouse/rabbit IgG secondary antibody were provided by Santa Cruz Biotechnology (Santa Cruz, CA, USA).

### Cell culture

Human HCC cell lines BEL-7402 and Huh 7 were kindly donated by Prof Xin-yuan GUAN (Hong Kong University, Hong Kong, China). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Grand Island, NY, USA) containing 10% (*v/v*) fetal bovine serum (Bio-Whittaker, Walkersville, MD, USA), penicillin (100 U/mL) and streptomycin (100 mg/L). The cells were maintained at 37 °C in an incubator with a humidified atmosphere of 5% CO<sub>2</sub>.

### 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide (MTT) assay for cell viability

Exponentially growing cells were diluted to a concentration of  $2.5 \times 10^4$  cells/mL in DMEM, plated in 96-well plates (Corning Inc, Corning, NY, USA) with 200  $\mu$ L/well. After being treated with the appropriate drug and incubated for 48 h (triplicate wells for each sample), the cells were exposed to 20  $\mu$ L/well MTT (5 g/L, Amresco, Solon, OH, USA). The medium was incubated for 4 h and then removed, and DMSO (200  $\mu$ L/well) was added to dissolve the formazan product. Finally, the plate was read in enzyme-linked immunity implement (Bio-Rad 2550, Hercules, CA, USA) at 570 nm.

### Western blotting analysis

Cells were rinsed twice with ice-cold PBS buffer and scraped with lysis buffer containing a protease inhibitor cocktail (Boehringer Mannheim, Lewes, UK) for 30 min at 4 °C. The supernatant was isolated by centrifugation at  $15000 \times g$  for 20 min. The protein concentration was determined with Coomassie brilliant blue G-250. Cell extracts (50  $\mu$ g/lane) were separated via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to polyvinylidene fluoride (PVDF) membranes (Immobilon, Bedford, MA). After being blocked in 20 mmol/L Tris-HCl, pH 7.6 (containing 150 mmol/L NaCl, 0.1% Tween-20, and 5% non-fat dry milk),

membranes were incubated with primary antibodies (used as a sample loading control) overnight at 4 °C and then incubated with a horseradish peroxidase-conjugated secondary antibody. Blots were developed using an enhanced chemiluminescence detection system (ECL, Amersham Pharmacia Biotech) according to the manufacturer's instructions.

### Gene silencing by small interfering RNA

Small interfering RNA and nonspecific control siRNA were purchased from RiboBio (Guangzhou RiboBio Co LTD, China). The cells were plated onto 6-well plates, maintained in antibiotic medium for 24 h, and grown to about 50% confluence. siRNA or control siRNA were transfected with the Lipofectamine<sup>TM</sup> 2000 reagent (Invitrogen, Madison, WI, USA). In short, oligomer-ectamine reagent was diluted at 1:50 in OptiMEMI reduced serum medium (GIBCO, Palo Alto, CA, USA), mixed gently and incubated for 5 min at room temperature. Subsequently, a mixture of siRNA was added and incubated for 20 min. The mixture was diluted by adding medium to each well, and the final concentration of siRNA in each well was set at 100 nmol/L. The cells were then incubated for 48 hours until they were ready for processing.

### Tumor samples for immunohistochemistry

A total of 100 formalin-fixed, paraffin-embedded specimens of human HCC and surrounding non-tumor liver tissues were obtained from the Tumor Tissues Base of the First Affiliated Hospital of Sun Yat-Sen University (Guangzhou, China) from January of 2005 to January of 2007. Consent was obtained before tissue collection, and the study was approved by the Human Ethics Committee of the Sun Yat-Sen University. Of the 100 patients from whom tumor tissue was used, the mean age was 53.5 years, including 82 males and 18 females. Ninety patients were infected with hepatitis B virus and 6 with hepatitis C virus; 4 had other diagnoses. Sections were deparaffinized and rehydrated by routine procedures. Endogenous peroxidase activity was quenched with 1% hydrogen peroxide, and microwave retrieval of antigen was performed. Nonspecific binding was blocked using buffer containing goat serum. The slides were incubated at 4 °C overnight with 1:100 diluted mouse monoclonal anti-human PTEN (Santa Cruz, CA, USA). After being washed three times for 15 min, the slides were incubated with biotin-conjugated secondary antibody (1:200) for 30 min at room temperature. After probing, the ABC complex was added and, finally, 3,3'-diaminobenzidine substrate was used for color development. The slides were then counterstained with hematoxylin and observed under an Advanced Fluorescence Microscope (Nikon 80i, Tokyo, Japan). PTEN expression levels were scored as 0, 1, 2, or 3 according to the immunohistochemical staining intensity, in which 0=no staining; 1=weak staining; 2=moderate staining; and 3=intense staining. The distribution of positive cells was graded as follows: 0 $\leq$ 5%; 1=5%~25%; 2=26%~50%; 3=51%~75%; 4=76%~100%. A final score was obtained by sum of the two scores above. Samples stained with score 0~2 were considered as negative, whereas samples with score  $\geq$ 3 were

considered positive<sup>[7,8]</sup>.

### Statistical analysis

Data were expressed as mean±SD of at least three separate experiments. Differences between groups were assessed with a one-way ANOVA and Student-Newman-Keuls *q* test using SPSS 11.0 for windows (SPSS Inc, Chicago, IL, USA). The difference in PTEN expression was analyzed by a  $\chi^2$ -test. *P* values less than 0.05 were considered statistically significant.

## Results

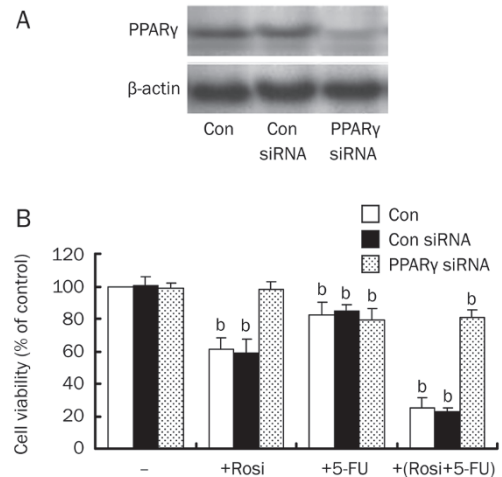
### Rosiglitazone facilitates the anti-tumor effect of 5-FU in HCC cell lines

To investigate the effect of rosiglitazone on fluorouracil-inhibited growth of HCC cell lines, we treated BEL-7402 and Huh7 cells with rosiglitazone in the presence or absence of 5-FU. Cell viability was assessed by the MTT assay. As shown in Figure 1, markedly decreased cell viability was observed in both cell lines when they were treated with 5-FU and rosiglitazone, compared with the results of 5-FU alone or rosiglitazone alone. The cell viabilities for BEL-7402 and Huh7 treated with 5-FU alone (10  $\mu$ mol/L) were (82.56±8.01)% and (87.29±9.38)% at 48 h, respectively. After we co-administered 5-FU with 10, 30, and 50  $\mu$ mol/L rosiglitazone to both cell lines for 48 h, rosiglitazone significantly enhanced the antitumor activities of 5-FU in a dose- and time-dependent manner. It is noteworthy that the viability of cells with 30  $\mu$ mol/L rosiglitazone plus 10  $\mu$ mol/L 5-FU decreased to (25.18±6.00)% and (42.56±7.79)%. These data indicate that a combination of rosiglitazone and 5-FU synergistically inhibits cell growth in HCC cell lines.

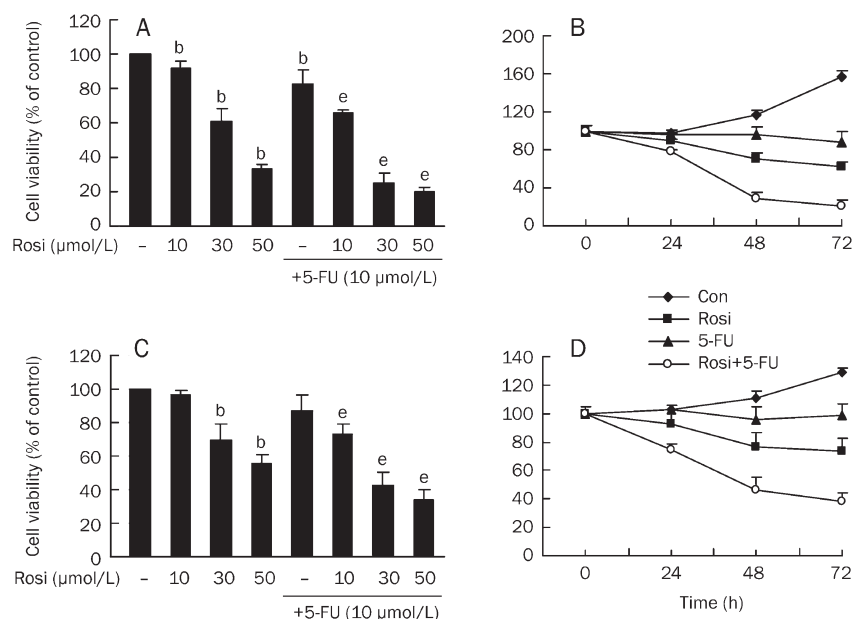
### Rosiglitazone enhances 5-FU-inhibited cell growth in BEL-7402 by the PPAR $\gamma$ pathway

To examine whether the synergistic effect of rosiglitazone plus 5-FU is mediated by the activation of PPAR $\gamma$ , BEL-7402 cells

were transfected with PPAR $\gamma$  siRNA or non-specific control siRNA. Cells treated with PBS were used as a control. Unlike control siRNA, PPAR $\gamma$  siRNA almost completely eliminated endogenous PPAR $\gamma$  protein expression (Figure 2A). Subsequently, the cell viability was assessed by MTT assay. We found that 30  $\mu$ mol/L rosiglitazone did not decrease cell viability after the PPAR $\gamma$  gene was silenced, the cytotoxic effects



**Figure 2.** Rosiglitazone's contribution to the anti-tumor effect of 5-FU by the PPAR $\gamma$  signaling pathway. (A) Cellular protein was isolated from BEL-7402 cells transfected with control or PPAR $\gamma$  siRNA for 48 h and was then subjected to Western blotting analysis for PPAR $\gamma$  protein. PPAR $\gamma$  siRNA inhibits PPAR $\gamma$  protein expression. (B) BEL-7402 cells were transfected with control or PPAR $\gamma$  siRNA for 48 h before exposing the cells to rosiglitazone (30  $\mu$ mol/L) in the presence or absence of 5-FU (10  $\mu$ mol/L). Afterwards, the cell viabilities were determined by MTT assay up to 48 h. Data are expressed as the mean±SD of three independent experiments. <sup>b</sup>*P*<0.05 vs control group; Con, cells treated with 0.1% DMSO; Con siRNA, non-specific siRNA, as a negative control.



**Figure 1.** The cell death effect of rosiglitazone plus 5-FU on BEL-7402 and Huh7 cell lines. BEL-7402 cells were treated with varying concentrations of rosiglitazone for 48 h in the presence or absence of 5-FU (A) and with the combination of rosiglitazone and 5-FU for the indicated periods of time (B). Huh7 cells were treated with varying concentrations of rosiglitazone for 48 h in the presence or absence of 5-FU (C) and with the combination of rosiglitazone and 5-FU for the indicated periods of time (D). Cell viability was determined by MTT assay. Data are expressed as the mean±SD of three independent experiments. <sup>b</sup>*P*<0.05 vs control group; <sup>e</sup>*P*<0.05 vs 5-FU alone group. Con, cells treated with 0.1% DMSO.

were exhibited only for 10  $\mu\text{mol/L}$  5-FU with or without rosiglitazone. This suggests that rosiglitazone increases the growth inhibitive effect of 5-FU in BEL-7402 cells through the activation of the PPAR $\gamma$  signaling pathway. Similar results were obtained in Huh7 cells (data not shown).

#### Activation of PPAR $\gamma$ by rosiglitazone increases PTEN expression and decreases COX-2 expression

Chemoresistance is an active process of tumor cell survival and a consequence of multiple factors, including reduction of intercellular drug accumulation, repair of tumor cell DNA damage, activation of PI3K/Akt, Ras or MAPK signaling pathways, and dysfunction of the tumor suppression gene<sup>[9, 10]</sup>. PTEN is a tumor suppressor gene that inhibits the PI3K/Akt mediated cell survival pathway<sup>[11]</sup>. Over-expression of PTEN sensitizes prostate cancer cells to drug-induced apoptosis<sup>[12]</sup>. Moreover, previous studies have demonstrated that COX-2 expression is increased in chemo-resistant cancer cells. Clinically, COX-2 inhibitors such as celecoxib are used as cancer treatment drugs<sup>[13, 14]</sup>. Thus, we tested the expression of PTEN and COX-2 in BEL-7402 and Huh7 cells by Western blotting analysis. As shown in Figure 3A, compared with the control (0.1% DMSO), rosiglitazone alone or combined with 5-FU increases the expression of PTEN and decreases the expression of COX-2 in BEL-7402 cells, whereas 5-FU alone had little effect on COX-2 and PTEN expression. Furthermore, we found that rosiglitazone alone or combined with 5-FU inactivated the expression of both proteins after the PPAR $\gamma$  gene was silenced by PPAR $\gamma$  siRNA (Figure 3B). These data indicate that the activation of PPAR $\gamma$  by rosiglitazone increases PTEN expression and decreases COX-2 expression. Similar results were obtained in Huh7 cells (Figure 3C and Figure 3D).

#### Over-expression of PTEN by PPAR $\gamma$ sensitizes HCC cell lines to 5-FU antitumor activity

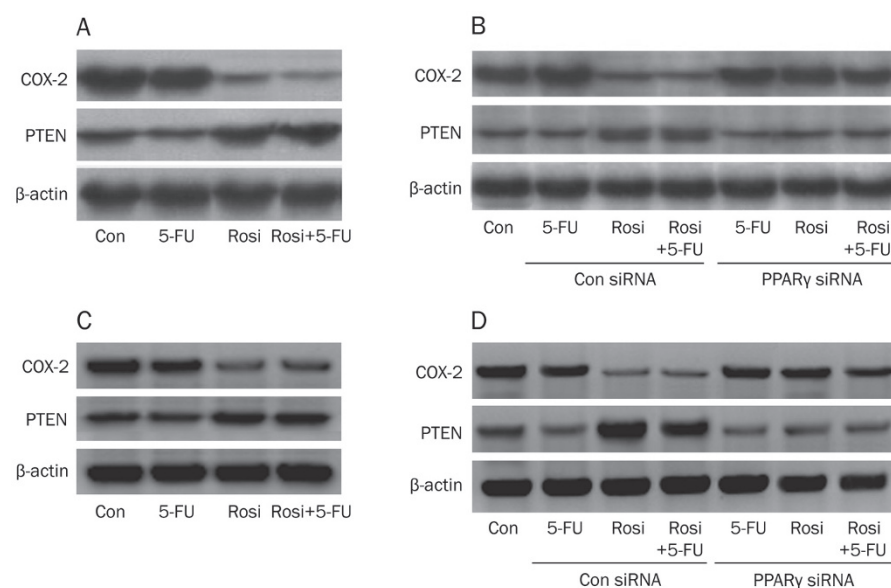
5-FU is widely used to treat advanced HCC, but chemo-

resistance remains a major obstacle to its use in the clinical setting. To investigate the relationship between the expression of PTEN and advanced HCC, we examined the expression of PTEN in 100 advanced HCC tissues and paracancerous tissues. Of 100 cases of HCC, 62 cases were positive for PTEN, and 38 cases were negative. Ninety-two cases of adjacent non-tumor tissues were positive as a control. As shown in Figure 4A-b, the staining distribution of PTEN in HCC tissues was significantly decreased as compared with the paracancerous tissue ( $\chi^2=25.409$ ,  $P<0.05$ ). Furthermore, of the 62 positive samples, 45 showed staining in the cytoplasm of cancer cells (Figure 4A-c), and the other 17 exhibited intensive nuclear staining of cancer cells (Figure 4A-d).

Over-expression of PTEN was detected in the process of rosiglitazone-induced cell growth arrest of HCC cell lines (Figure 1 and Figure 3A). To further confirm the role of PTEN in the rosiglitazone-mediated cell growth inhibition, BEL-7402 cells were transfected with PTEN siRNA or control siRNA, and endogenous PTEN protein expression was completely eliminated (Figure 4B). Then, cells were treated with rosiglitazone, 5-FU, or rosiglitazone plus 5-FU for 48 h. As shown in Figure 4C, the cell viabilities of rosiglitazone alone and rosiglitazone plus 5-FU were (81.46 $\pm$ 6.44)% and (58.91 $\pm$ 7.68)%, respectively, whereas no changes were observed in the control siRNA group. These results suggest that the over-expression of PTEN is involved in the inhibitory effect of rosiglitazone and rosiglitazone plus 5-FU. Similar results were obtained in Huh7 cells (data not shown).

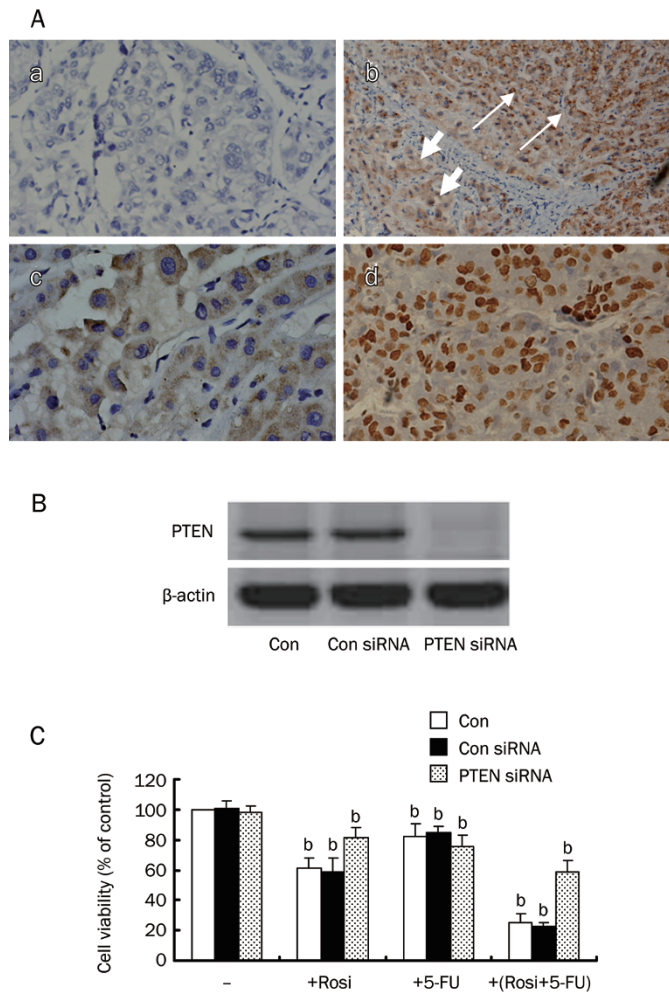
#### Down-regulation of Cox-2 increased 5-FU antitumor activity in HCC cell lines

COX-2 expression is associated with tumor cell proliferation and tumorigenesis<sup>[15, 16]</sup>. Significantly increased COX-2 protein expression was observed in HCC tissues and cell lines<sup>[17, 18]</sup>, which coincides with our results (Figure 3A). To evaluate the significance of down-regulation of COX-2 expression in



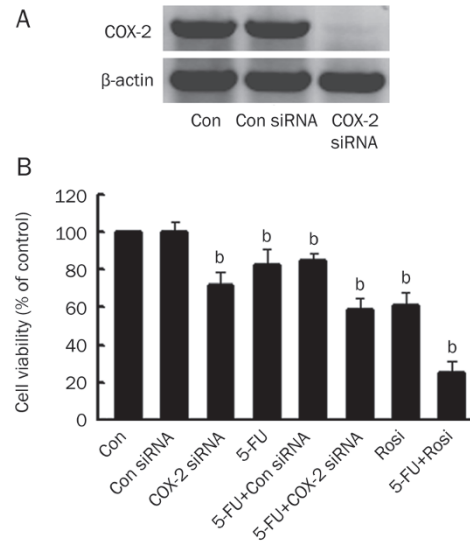
**Figure 3.** The effect of rosiglitazone on PTEN and COX-2 protein expression. Cellular protein was isolated from BEL-7402 cells (A) or Huh 7 cells (C) that were cultured with rosiglitazone (30  $\mu\text{mol/L}$ ) in the presence or absence of 5-FU (10  $\mu\text{mol/L}$ ) for 24 h. Then, western blotting analysis was used to detect the PTEN and COX-2 protein expression. (B) The PPAR $\gamma$  signaling pathway was inactivated by PPAR $\gamma$  siRNA method in BEL-7402 cells (B) or Huh 7 cells (D). Western blotting analysis was used to detect the PTEN and COX-2 protein expression of BEL-7402 cells treated with rosiglitazone (30  $\mu\text{mol/L}$ ) with or without 5-FU (10  $\mu\text{mol/L}$ ) for 24 h. Results are representative of three independent experiments. Con, cells treated with 0.1% DMSO; Con siRNA, non-specific siRNA, as a negative control.





**Figure 4.** Representative sections showing PTEN protein expression by immunohistochemical staining. (A-a) a negative control section of HCC (primary antibody substituted with PBS, original magnification,  $\times 200$ ); (A-b) the positive staining of PTEN was mainly observed in the cytoplasm of cells. The strong staining of PTEN protein was shown in paracancerous tissue (the long arrow), and the weak staining was shown in tumor tissue (the short arrow, original magnification,  $\times 100$ ); (A-c) PTEN protein was expressed in the cytoplasm of carcinoma cells (original magnification,  $\times 200$ ); (A-d) PTEN protein was expressed in the nuclei of carcinoma cells (original magnification,  $\times 200$ ); (B) Cellular protein was isolated from BEL-7402 cells transfected with control or PTEN siRNA for 48 h and was then subjected to Western blotting analysis for PTEN protein. PTEN siRNA inhibits PTEN protein expression. (C) BEL-7402 cells were transfected with control or PTEN siRNA for 48 h before exposing the cells to rosiglitazone (30  $\mu\text{mol/L}$ ) in the presence or absence of 5-FU (10  $\mu\text{mol/L}$ ). Afterwards, the cell viabilities were determined by MTT assay up to 48 h. Data are expressed as the mean $\pm$ SD of three independent experiments. <sup>b</sup> $P < 0.05$  vs control group; Con, cells treated with 0.1% DMSO; Con siRNA, non-specific siRNA, as a negative control.

BEL-7402 cells, we investigated cell viability by MTT assay after BEL-7402 cells were treated with rosiglitazone or COX-2 siRNA (Figure 5A). We found that the knockdown of COX-2 by specific siRNA reduced cell viability (71.49 $\pm$ 6.62)%. Moreover, the cell growth inhibitive effect is dramatically increased



**Figure 5.** (A) Cellular protein was isolated from BEL-7402 cells transfected with control or COX-2 siRNA for 48 h and was then subjected to Western blotting analysis for COX-2 protein. COX-2 siRNA inhibits COX-2 protein expression. (B) After BEL-7402 cells were transfected with control or COX-2 siRNA for 48 h the cells were exposed to rosiglitazone (30  $\mu\text{mol/L}$ ) in the presence or absence of 5-FU (10  $\mu\text{mol/L}$ ). Then, the cell viabilities were determined by MTT assay for 48 h. Data are expressed as the mean $\pm$ SD of three independent experiments. <sup>b</sup> $P < 0.05$  vs control group; Con, cells treated with 0.1% DMSO; Con siRNA, non-specific siRNA, as a negative control.

in the combination of COX-2 siRNA and 5-FU group, in which the cell viability was (59.02 $\pm$ 6.05)% (Figure 5B). Unlike the control siRNA group, this reduction in COX-2 expression by COX-2 siRNA or rosiglitazone correlates with growth inhibition of BEL-7402 cells, indicating that down-regulation of COX-2 is responsible for growth arrest of BEL-7402 cells. Similar results were obtained in Huh7 cells (data not shown).

## Discussion

Multiple factors are involved in increasing resistance to chemotherapeutic agents, reduction of intracellular drug accumulation, and DNA damage repair by the modulation of proliferative or anti-apoptotic proteins, *etc*<sup>[19]</sup>. 5-FU causes cell injury by inhibiting thymidylate synthesis. It has become a mainstay of treatment for advanced HCC. Unfortunately, many advanced-stage cancers are inherently resistant to 5-FU or develop resistance during the course of therapy. This is also the major reason for the treatment failure of advanced HCC chemotherapy<sup>[20]</sup>. There is so far no effective single agent or polychemotherapeutic regimen for the treatment of resistant HCC<sup>[21-23]</sup>. Therefore, a new drug combined with 5-FU for anticancer therapy in advanced HCC is needed.

PPAR $\gamma$  is a nuclear receptor with multiple biologic effects. Recent evidence has demonstrated that PPAR $\gamma$  activation by TZDs inhibits cell growth and induces cell apoptosis in liver cancer cell lines<sup>[24, 25]</sup>. Moreover, rosiglitazone, as a PPAR $\gamma$  synthetic ligand, has been shown to enhance the antitumor

activity of some chemotherapeutic drugs by the modulation of cancer cell agents<sup>[26,27]</sup>. However, the effects of rosiglitazone on hepatoma cells have been poorly understood until now.

Our results showed that rosiglitazone suppressed cell growth in a dose-dependent manner and facilitated the anti-tumor effect of 5-FU in HCC cell lines. Cell viabilities of BEL-7402 and Huh7 cell lines were slightly inhibited by 5-FU alone, and the inhibitive effects for both HCC cell lines were dramatically increased by combining rosiglitazone with 5-FU, indicating that the combination of both drugs synergistically induces cell growth arrest in HCC cell lines. Subsequently, we found that rosiglitazone enhanced 5-FU-inhibited cell growth of HCC via PPAR $\gamma$  activation, because the effect was completely abrogated by the inactivation of PPAR $\gamma$  through PPAR $\gamma$  siRNA. Han *et al* have demonstrated that rosiglitazone inhibits non-small cell lung cancer (NSCLC) cell growth by both PPAR $\gamma$ -dependent and PPAR $\gamma$ -independent signaling pathways<sup>[28]</sup>. Our results show that rosiglitazone increases the antitumor effect of 5-FU via the PPAR $\gamma$ -dependent signaling pathway.

PTEN is a phosphatase with dual-specificity possessing both lipid and protein phosphatases. It decreases the phosphorylation level of Akt and negatively affects the PI3K/Akt pathway. In addition, it plays a critical part in control of normal cell growth<sup>[29]</sup>. The deletion and low expression of PTEN in tumor tissues results in decreased sensitivity of mutant cells to apoptotic stimuli and promotes cellular over-growth and tumorigenesis<sup>[30]</sup>. Our results show that 62% of our HCC tissues were positive for PTEN. Compared with the control (92%), the difference was significant ( $P < 0.05$ ), indicating that HCC tumor tissues have lower expression of PTEN. Interestingly, PTEN protein was localized in the cytoplasm of cells in paracancerous tissues, while 17% of positive cases for PTEN showed nuclear intensive staining of cancer cells. PTEN protein in the cytoplasm of normal cells regulates cell differentiation, proliferation, and apoptosis. The role of PTEN protein in the nuclei of some tumor cells is not clearly understood, which we pursued in further study.

Decreased expression of PTEN might decrease the sensitivity of cancer cells to chemotherapeutic drugs. Over-expression of PTEN sensitizes human ovarian cancer cells to cisplatin-induced apoptosis<sup>[31]</sup>. However, the effect has not been elucidated in HCC cell lines. We found upregulation of PTEN in HCC cell lines with the groups treated with rosiglitazone and rosiglitazone plus 5-FU, which could be one mechanism by which rosiglitazone increases the 5-FU anticancer effect.

COX-2, one isoform of COX, is an enzyme inducible by a number of factors, including cytokines, growth factors, and tumor promoters<sup>[32]</sup>. It is known that over-expression of COX-2 is associated with tumor cell proliferation, escape from apoptosis and tumor angiogenesis<sup>[33]</sup>. Higher levels of COX-2 are identified in hepatocellular carcinoma cells, which increases PGE2 production and growth rate<sup>[17]</sup>. Whereas COX-2 inhibitor enhances cell apoptosis in human carcinoma cell lines<sup>[34]</sup>, non-steroidal anti-inflammatory drugs (NSAIDs), the inhibitors of COX-1 and COX-2, are also shown to reduce the risk of sporadic colorectal, breast, prostate, and lung can-

cers<sup>[35, 36]</sup>. In our study, rosiglitazone reduced COX-2 expression via the PPAR $\gamma$  signaling pathway. The down-regulation of COX-2 has a role in rosiglitazone-induced cell growth inhibition, suggesting that rosiglitazone increases 5-FU's antitumor effect and reduces HCC cell resistance, possibly through the COX-2 pathway.

In summary, the present study demonstrates that a combination of rosiglitazone and 5-FU strongly induces growth inhibition in HCC cell lines. Additionally, our results suggest that rosiglitazone increases PTEN expression and decreases COX-2 expression through the PPAR $\gamma$  signaling pathway, which may explain the underlying mechanisms by which rosiglitazone facilitates 5-FU-inhibited HCC growth. These observations suggest potential novel therapies for the treatment of advanced liver cancer.

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### Author contribution

Qian WANG designed research; Liang-qi CAO and Xiao-li WANG performed research; Ping XUE, Xing-yuan JIAO, He-ping PENG, Hai-wu LU, and Qiang ZHENG contributed new analytical tools and reagents; Xi-lin CHEN, Xiao-hui HUANG, Xin-hui FU, and Jing-song CHEN analyzed data; Liang-qi CAO wrote the paper.

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