

# Effect and mechanism of peroxisome proliferator-activated receptor- $\gamma$ on the drug resistance of the U-87 MG/CDDP human malignant glioma cell line

SHAORONG HAN\*, XIAOYAN LV\*, YANMING WANG, HAI GONG,  
CONG ZHANG, ANNA TONG, BAOYI ZHANG and HUI YAO

Department of Radiation Oncology, Jinan Military General Hospital, Jinan, Shandong 250031, P.R. China

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**Abstract.** Peroxisome proliferator-activated receptor- $\gamma$  (PPAR- $\gamma$ ) is important in tumor differentiation, proliferation and apoptosis. However, the effect and mechanism of PPAR- $\gamma$  on the promotion of cisplatin sensitivity in glioma cells remain to be elucidated. The present study established cisplatin-resistant U-87 MG/CDDP cell lines and U-87 MG/CDDP cell lines overexpressing PPAR- $\gamma$ . With upregulated expression of PPAR- $\gamma$ , the sensitivity of cancer cells to cisplatin was increased. Flow cytometry revealed that the intracellular content of rhodamine-123 was increased, expression of P-glycoprotein was downregulated, cell cycle was arrested in G0/G1 phase, apoptosis and oxidative stress was increased, levels of intracellular thymidylate synthase, glutathione and transforming growth factor- $\beta$ 1 were decreased, expression levels of multidrug resistance related gene (MDR), multidrug resistance-associated protein and glutathione S-transferase- $\pi$  were downregulated, expression levels of cell proliferation and apoptosis associated genes, including survivin and B-cell lymphoma-2, were downregulated, p53, p21 and caspase-3/8 were significantly upregulated, phosphorylation of extracellular signal-regulated kinase and small mothers against decapentaplegic 2 were downregulated, and the transcriptional activities of Twist and nuclear factor (erythroid-derived 2)-like 2 were significantly reduced. The results suggested that upregulation of PPAR- $\gamma$  in the U-87 MG/DDP cells increased cisplatin sensitivity, and the underlying mechanisms included the regulation of MDR and apoptosis associated genes, which increased the intracellular

accumulation of the drug, inhibited cell proliferation and promoted cell apoptosis.

## Introduction

Brain tumors grow in a limited space of the cranial cavity and easily cause damage to the central nervous system. These types of tumor frequently incur high risks and are life threatening. Based on the statistics, primary brain tumors account for ~1% of all types of tumor, 2.4% of tumor-associated mortality and 20-25% of pediatric tumors (1). Glioma is the most common primary brain tumor, accounting for ~50% of primary brain tumors and up to 80% of malignant brain tumors (2).

The progress made in understanding glioma has been in three predominant aspects, including molecular etiology and pathology, diagnosis and treatment techniques, and treatment concepts. In the 'Chinese Guideline for the Diagnosis and Treatment of Glioma in the Central Nervous System' issued by the Oncology Group of the Neurosurgery Branch of Chinese Medical Association in 2012 (3), the treatment of glioma was suggested to comprise predominantly a combination of surgery, radiotherapy and chemotherapy. Surgery can remove the gross tumors, while radiotherapy and chemotherapy can destroy or inhibit the residual tumor cells, extending survival rates. Although surgery, as the primary choice for the treatment of a brain tumor, exhibits the advantages of directness and thoroughness, the complex brain structure makes the tumors inaccessible in certain patients and, consequently, obstructs or prevents implementation of the surgical procedures. Radiotherapy destroys tumor cells with radiation, while limiting damage to the normal brain cells. It is the most common measure for the treatment of a secondary brain tumor and is an important complementary method for surgical treatment. Treatment with chemotherapy to destroy tumor cells with compounds is generally used in combination with surgery and radiotherapy to increase the chances of successful treatment (4-6). Despite significant progress in therapeutic methods, curing glioma remains rare, with median survival rates not exceeding 2 years (7).

Cisplatin, as a common chemotherapeutic drug, can inhibit DNA replication and transcription in cancer cells

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*Correspondence to:* Dr Yanming Wang, Department of Radiation Oncology, Jinan Military General Hospital, 25 Normal School Road, Jinan, Shandong 250031, P.R. China  
E-mail: ymwang64@163.com

\*Contributed equally

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through DNA cross-linking, leading to tumor cell growth arrest and apoptosis, however, tumor cells often develop clinical resistance to chemotherapeutic drugs, resulting in treatment failure (8). Therefore, identifying the mechanisms underlying tumor resistance and developing novel methods to reverse drug resistance have important significance for improving the clinical benefits for patients. Previous studies have identified drug-resistance mechanisms of tumor cells, including reducing drug absorption, increasing drug efflux via transporter proteins, detoxifying antitumor drugs via the glutathione system, and causing abnormalities in apoptotic pathways to reduce tumor cell apoptosis (9,10).

Peroxisome proliferator-activated receptor- $\gamma$  (PPAR- $\gamma$ ) is a type II nuclear receptor, which is closely associated with obesity, diabetes, atherosclerosis and other metabolic diseases clinically, as initial studies have revealed that PPAR- $\gamma$  regulates fatty acid storage and glucose metabolism (11). Previous studies have demonstrated the association of PPAR- $\gamma$  with cancer, and a series of preclinical studies have revealed that ligand-activated PPAR- $\gamma$  is able to arrest tumor cell growth, increase cell apoptosis and inhibit tumor metastasis (12,13). The prevention and treatment of glioma remains a significant clinical challenge, and the correlation between PPAR- $\gamma$  and cisplatin-resistant glioma remains to be elucidated. The present study aimed to investigate the expression of PPAR- $\gamma$  in a cisplatin-resistant glioma cell line, whether PPAR- $\gamma$  altered the sensitivity or resistance of the drug-resistant cells to cisplatin, and preliminarily examine the underlying molecular mechanisms.

## Materials and methods

**Cells and cell culture.** The glioma U87 MG cell line was purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were subcultured routinely in Dulbecco's modified Eagle's medium (Corning Life Sciences, Manassas, VA, USA), containing 10% fetal bovine serum, 100 U/ml penicillin and 100 U/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA) at 37°C in a saturated humidity, 5% CO<sub>2</sub> incubator. The U-87 MG cells were cultured in cisplatin-containing media, at an initial concentration of 1/10 the half maximal inhibitory concentration (IC<sub>50</sub>), and the cisplatin concentration was increased gradually with continuous culture. Following continuous exposure to increased concentrations of cisplatin for ~6 months, the growing tumor cells were obtained for the cisplatin-resistant U-87 MG/CDDP cell line.

**Construction of the PPAR- $\gamma$ -overexpressing U-87 MG/CDDP cell line.** A PPAR- $\gamma$  expression plasmid (PPARG-pCMV/hygro plasmid; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and blank plasmid were purchased from FITGene Tech, Inc. and transfected into the U-87 MG/CDDP cells using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. Screening was performed in hygromycin-containing culture media (Sigma-Aldrich), in which cells that had undergone successful transfection with the plasmids were able to grow in the selective culture media.

**Detection of the inhibition of cell proliferation using an MTS assay.** The cells ( $3 \times 10^3$ ) were seeded into 96-well plates and incubated for 24 h at 37°C with 5% CO<sub>2</sub>. Cisplatin (0, 0.1, 0.5, 1, 5, 10, 50 or 100  $\mu$ M; Sigma-Aldrich) was added, and the cells were incubated for a further 72 h. Fresh culture media, containing 20  $\mu$ l MTS (Promega Corporation, Madison, WI, USA), was added to each well and incubated for 2 h. The absorbance was subsequently measured with a microplate reader (Synergy 2; BioTek Instruments, Inc., Winooski, VT, USA) at 492 nm to detect the sensitivity of the tumor cells to cisplatin.

**Detection of the intracellular content of rhodamine (Rh)-123, oxidative stress, expression of P-gp, cell cycle and apoptosis by flow cytometry.** The cells ( $3 \times 10^5$ ) were seeded into 6-well plates and incubated for 24 h, prior to being digested with trypsin (Sigma-Aldrich) at 37°C, centrifuged at 300 x g, washed with cold-phosphate-buffered saline (PBS; Corning Life Sciences) three times, and incubated in the dark at 37°C with 10  $\mu$ g/ml Rh-123 for 30 min. The cells were subsequently washed with cold-PBS three times and analyzed by flow cytometry (FACSARIA; BD Biosciences, San Jose, CA, USA) at 488 nm to detect the intracellular content of Rh-123 in the tumor cells.

To determine the oxidative stress, the cells ( $3 \times 10^5$ ) were seeded into 6-well plates and incubated for 24 h, prior to adding 10  $\mu$ M cisplatin and incubating for a further 24 h. The cells were then digested with trypsin at 37°C, centrifuged at 300 x g, washed with cold-PBS three times and incubated in the dark at 37°C with 20  $\mu$ l DCFH-DA for 30 min. The cells were subsequently washed with cold-PBS three times and analyzed by flow cytometry at 488 nm to detect the oxidative stress of the tumor cells.

To determine the expression of P-gp, the cells ( $3 \times 10^5$ ) were seeded into 6-well plates and incubated for 24 h, followed by being digested with trypsin at 37°C, centrifuged at 300 x g for 5 min, washed with cold-PBS three times, and incubated in the dark at 37°C with 20  $\mu$ l P-glycoprotein (gp)-phycoerythrin (PE) antibody for 15 min. The cells were then washed with cold-PBS three times and analyzed by flow cytometry at 488 nm to detect the expression of P-gp in the tumor cells.

To examine the effects on the cell cycle, the cells ( $3 \times 10^5$ ) were seeded into 6-well plates and incubated at 37°C with 5% CO<sub>2</sub> for 24 h, followed by being digested with trypsin at 37°C, centrifuged at 300 x g for 5 min, fixed using cold alcohol (75%) overnight. The cells were then washed with cold-PBS three times and incubated with 500  $\mu$ l propidium iodide (PI) staining solution with RNaseA (Beyotime Institute of Biotechnology, Shanghai, China) for 30 min. Following staining, the cells were washed with cold-PBS three times and analyzed by flow cytometry at 488 nm to detect the cell cycle of the tumor cells.

To determine the levels of apoptosis, the cells ( $3 \times 10^5$ ) were seeded into 6-well plates and incubated for 24 h at 37°C with 5% CO<sub>2</sub> prior to the addition of 10  $\mu$ M cisplatin for a further 24 h at 37°C with 5% CO<sub>2</sub>. The cells were then digested with trypsin at 37°C, centrifuged at 300 x g for 5 min, washed with cold-PBS three times and incubated in the dark at 37°C with 20  $\mu$ l annexin V-fluorescein isothiocyanate antibody

and PI staining solution for 15 min. The cells were washed with cold-PBS three times and analyzed by flow cytometry at 488 nm to detect the apoptosis of the tumor cells.

**Detection of the intracellular levels of thymidylate synthase and glutathione level using a biochemical assay.** The cells ( $3 \times 10^5$ ) were seeded into 6-well plates and incubated for 24 h. Following incubation, the cells were lysed, centrifuged at  $14,000 \times g$  for 5 min at  $4^\circ\text{C}$  and the supernatant was collected. The intracellular levels of thymidylate synthase and glutathione were measured using a thymidylate synthase and Total Glutathione Assay kit (Beyotime Institute of Biotechnology), according to the manufacturer's instructions.

**Detection of the production of TGF- $\beta$ 1 using an ELISA assay.** The cells ( $3 \times 10^5$ ) were seeded into 6-well plates and incubated for 24 h, prior to the addition of fresh culture medium for 24 h and collection of the supernatant. The levels of TGF- $\beta$ 1 were measured using an ELISA detection kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

**Detection of the protein expression levels of PPAR- $\gamma$ , multidrug resistance gene (MDR)1, multidrug resistance-associated protein (MRP)1, glutathione S-transferase (GST)- $\pi$ , survivin, B-cell lymphoma (Bcl)-2, p53, p21, caspase-3/8, phosphorylated (p)-extracellular signal-regulated kinase (ERK) and p-small mothers against decapentaplegic (Smad)2 by western blotting.** The cells ( $3 \times 10^5$ ) were seeded into 6-well plates and incubated for 24 h at  $37^\circ\text{C}$  with 5%  $\text{CO}_2$ . Following incubation, the cells were lysed, centrifuged at  $14,000 \times g$  for 5 min at  $4^\circ\text{C}$  and the concentration of total protein in the supernatant was measured using a Bradford Protein Assay Kit. The Bradford assay involved solutions of standard or unknown protein samples being placed in two blank tubes, one for the standard curve, to which  $30 \mu\text{l}$   $\text{H}_2\text{O}$  was added, and the other for the unknown protein samples, to which  $30 \mu\text{l}$  protein preparation buffer was added. In addition, 1.5 ml Bradford reagent was added to each tube and they were mixed well and incubated at room temperature for a minimum of 5 min, but for no more than 1 h. The absorbance could then be measured at 595 nm. The total protein ( $80 \mu\text{g}$ ) was separated on 12% SDS-PAGE gels (Beyotime Institute of Biotechnology) and transferred onto a polyvinylidene fluoride membrane (Beyotime Institute of Biotechnology). The membrane was blocked using 5% non-fat milk for 2 h at room temperature and was subsequently incubated at  $4^\circ\text{C}$  overnight with the following monoclonal IgG antibodies from Santa-Cruz Biotechnology, Inc.: Rabbit anti-human PPAR- $\gamma$ , (1:300; sc-7196), mouse anti-human MDR1 (1:300; sc-555), rabbit anti-human MRP1 (H-70) (1:300; sc-13960), rabbit anti-human GST- $\pi$  (110-218) (1:300; sc-33614), mouse anti-human survivin (C-6) (1:300; sc-374616), rabbit anti-human Bcl-2 (1:300; sc-492), rabbit anti-human p53 (FL-393) (1:300; sc-6243), rabbit anti-human p21 (C-19) (1:300; sc-397), rabbit anti-human caspase-3 (H-277) (1:200; sc-7148), rabbit anti-human caspase-8 p18 (H-134) (1:200; sc-7890), rabbit anti-human p-ERK (H-300) (1:200; sc-13073), rabbit anti-human p-Smad2 (Ser 467) (1:200; sc-101801) and rabbit anti-human  $\beta$ -actin

(H-196) (1:5,000; sc-7210). The membrane was washed with Tris-buffered saline (TBS), containing 0.1% Tween 20 (Beyotime Institute of Biotechnology) three times and was subsequently incubated with horseradish peroxidase-labeled secondary antibody (1:2,000; Sigma-Aldrich) at room temperature for 1 h. The membrane was then washed with TBS containing 0.1% Tween 20 three times and visualized using a diaminobenzidine kit (Millipore, Billerica, MA, USA).  $\beta$ -actin was used as an internal loading control.

**Detection of the mRNA expression levels of PPAR- $\gamma$ , MDR1, MRP1, GST- $\pi$ , survivin, Bcl-2, p53 and p21 by reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** The cells ( $3 \times 10^5$ ) were seeded into 6-well plates, incubated for 24 h and the total RNA was extracted using TRIzol reagent (Life Technologies, Carlsbad, CA, USA). qPCR was performed with  $1 \mu\text{g}$  RNA, Taqman Master mix (Life Technologies) and the following primers from [Genscript (Nanjing) Co., Ltd., Nanjing, China]: PPAR- $\gamma$ , sense 5'-CACATCTACAATGCC TACCT-3' and antisense 5'-CTTCTCTGCCTGCCACAA TGTCT-3'; MDR1, sense 5'-AAAAAGATCAACTCGTAC CACTC-3' and antisense 5'-GCACAAAATACACCAACA A-3'; MRP1, sense 5'-ACTTCCACATCTGCTTCGTCA GTG-3' and anti-sense 5'-ATTCAGCCACAGGAGGTAGAG AGC-3'; GST- $\pi$ , sense 5'-ACCTGCCTGTGACATCAT-3' and antisense 5'-TCTCCCTTTGTGCGTTCT-3'; survivin, sense 5'-GCATGGGTGCCCCGACGTTG-3' and antisense: 5'-GCTCCGGCCAGAGGCCTCAA-3'; Bcl-2, sense 5'-ACG GGGTGAAGTGGGGGAGGA-3' and antisense 5'-TGTTTG GGGCAGGCATGTTGACTT-3'; p53, sense 5'-GCCCCA CAACACCAGCTCC-3' and antisense 5'-CCTGGGCAT CCTTGAGTTCC-3'; p21, sense 5'-CACTCCAAACGCCGG CTGATCTTC-3' and antisense 5'-TGTAGAGCGGGCCTT TGAGGCCCTC-3' and  $\beta$ -actin, sense 5'-TGAGCGCGGCTA CAGCTT-3' and antisense 5'-TCCTTAATGTCACGCACG ATTT-3'. PCR was performed with the ABI 7500 Sequence Detection System (Applied Biosystems Life Technologies, Foster City, CA, USA) and the reaction conditions were as follows:  $94^\circ\text{C}$  denaturation for 3 min, followed by 40 cycles of  $95^\circ\text{C}$  for 5 sec,  $65^\circ\text{C}$  for 35 sec and  $72^\circ\text{C}$  for 60 sec.  $\beta$ -actin was used as an internal control, and mRNA expression levels were quantified by comparing target gene and  $\beta$ -actin expression levels.

**Detection of the transcriptional activity of Twist and nuclear factor (erythroid-derived 2)-like 2 (NRF2) using a dual luciferase reporter gene assay.** The cells ( $3 \times 10^5$ ) were seeded into 6-well plates and incubated for 24 h. The luciferase reporter plasmids of Twist and NRF2 (Beyotime Institute of Biotechnology) were transfected into the cells using Lipofectamine 2000 (Invitrogen Life Technologies), according to the manufacturer's instructions, and the cells were cultured for 24 h. The fluorescence intensity was subsequently determined using a Dual-Glo<sup>®</sup> Luciferase assay system (Promega Corporation) to detect the transcriptional activity of Twist and NRF2.

**Statistical methods.** The data are expressed as the mean  $\pm$  standard deviation and were analyzed using SPSS 11.5 software (SPSS, Inc., Chicago, IL, USA) by one-way analysis



of variance. Each experiment was repeated three times.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**PPAR- $\gamma$  increases the cisplatin sensitivity of U-87 MG/DDP cells.** The results of the western blotting and RT-qPCR demonstrated that the expression of PPAR- $\gamma$  in the U-87 MG/DDP cells transfected with the PPARG-pCMV/hygro plasmid (PPAR- $\gamma$  group 1 and PPAR- $\gamma$  group 2) was increased compared with the that in the U-87 MG/DDP cells transfected with the blank plasmid (negative group) and the U-87 MG/DDP cells without transfection (control group). In addition, there was increased expression of PPAR- $\gamma$  in PPAR- $\gamma$  group 2 compared with PPAR- $\gamma$  group 1 (Fig. 1). These results indicated that the PPAR- $\gamma$  overexpressing U-87 MG/DDP cell lines had been successfully established.

The MTS assay indicated that there was increased cisplatin sensitivity in the PPAR- $\gamma$ -overexpressing U-87 MG/DDP cells compared with the control and negative groups. The  $IC_{50}$  of the control group, negative group, PPAR- $\gamma$  group 1 and PPAR- $\gamma$  group 2 was 78.3, 80.5, 53.6 and 36.4  $\mu$ M, respectively, and the reversal fold (RF) was 1.46 and 2.15, respectively, indicating that PPAR- $\gamma$  increased the sensitivity of the U-87 MG/DDP cells to cisplatin (Fig. 2).

**PPAR- $\gamma$  increases intracellular Rh-123 content and oxidative stress, decreases the expression levels of P-gp, thymidylate synthase, glutathione and TGF- $\beta$ 1, arrests the cell cycle and improves the cell apoptosis of U-87 MG/DDP cells.** The results of the flow cytometry demonstrated increased intracellular content of Rh-123 and levels of oxidative stress in the PPAR- $\gamma$ -overexpressing U-87 MG/DDP cell lines compared with the control group, however, the expression levels of P-gp, thymidylate synthase, glutathione and TGF- $\beta$ 1 were reduced in the PPAR- $\gamma$ -overexpressing U-87 MG/DDP cell lines compared with the control group. Additionally, the G0/G1 phase rate and apoptotic rate were increased in the PPAR- $\gamma$ -overexpressing U-87 MG/DDP cell lines compared with the control group (Fig. 3).

**PPAR- $\gamma$  regulates the expression levels of MDR-associated genes in U-87 MG/DDP cells.** The results of the western blotting demonstrated decreased protein expression levels of MDR, MRP, GST- $\pi$ , survivin and Bcl-2 in the PPAR- $\gamma$ -overexpressing U-87 MG/DDP cell lines compared with the control and negative groups. However, the protein expression levels of p53, p21 and caspase-3/8 were increased in the PPAR- $\gamma$ -overexpressing U-87 MG/DDP cell lines compared with the control and negative groups. The RT-qPCR results revealed the same trend (Fig. 4).

**PPAR- $\gamma$  regulates MDR-associated signaling molecules in U-87 MG/DDP cells.** The results of the western blotting revealed that the phosphorylation levels of ERK and Smad2 were decreased in the PPAR- $\gamma$ -overexpressing U-87 MG/DDP cell lines compared with the control and negative groups. In addition, the results of the reporter gene assay demonstrated that there were decreased transcriptional activities of Twist and NRF2 in the PPAR- $\gamma$ -overexpressing

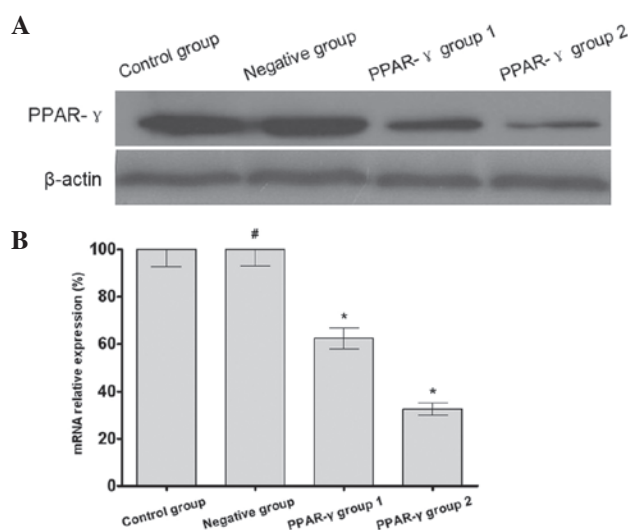


Figure 1. Expression of PPAR- $\gamma$  in different clones of stably-transfected U-87 MG/CDDP cell lines. (A) Western blotting demonstrated the protein expression levels of PPAR- $\gamma$  in the normal U-87 MG/CDDP cell line (control group), blank plasmid-transfected U-87 MG/CDDP cell line (negative group) and PPAR- $\gamma$ -overexpressing cell lines (PPAR- $\gamma$  group 1 and PPAR- $\gamma$  group 2).  $\beta$ -actin was used as an internal loading control. (B) Reverse transcription-quantitative polymerase chain reaction revealed the mRNA expression levels of PPAR- $\gamma$  in the control group, negative group, PPAR- $\gamma$  group 1 and PPAR- $\gamma$  group 2.  $\beta$ -actin was used as an internal loading control. The data are expressed as the mean  $\pm$  standard deviation ( $^{\#}P > 0.05$  vs. control;  $^*P < 0.05$  vs. control;  $n = 5$ ). PPAR, peroxisome proliferator-activated receptor.

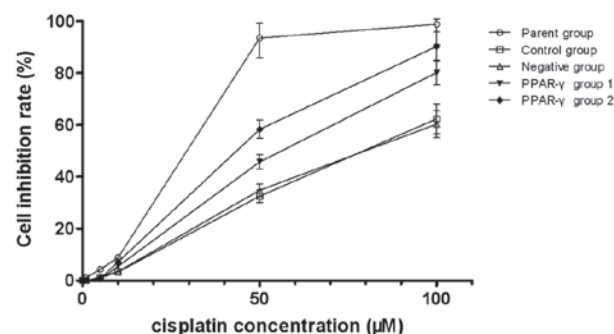


Figure 2. Reversal effect of PPAR- $\gamma$  on the U-87 MG/CDDP cell line. An MTS assay demonstrated the inhibition of cell proliferation in the U-87 MG cell line (parent group), normal U-87 MG/CDDP cell line (control group), blank plasmid-transfected U-87 MG/CDDP cell line (negative group) and PPAR- $\gamma$  overexpressing cell lines (PPAR- $\gamma$  group 1 and PPAR- $\gamma$  group 2) with increasing concentrations of cisplatin. The data are expressed as the mean  $\pm$  standard deviation ( $n = 10$ ). PPAR, peroxisome proliferator-activated receptor.

U-87 MG/DDP cell lines compared with the control group (Fig. 5).

## Discussion

Glioma is the most common type of primary malignant brain tumor. Although surgery is the primary choice in brain tumor treatment, a number of patients are not suitable for surgery for a variety of reasons or surgery cannot achieve the optimal therapeutic effect. In these cases, radiotherapy and chemotherapy are a suitable alternative or adjuvant therapy. Platinum-containing agents are a class of widely used

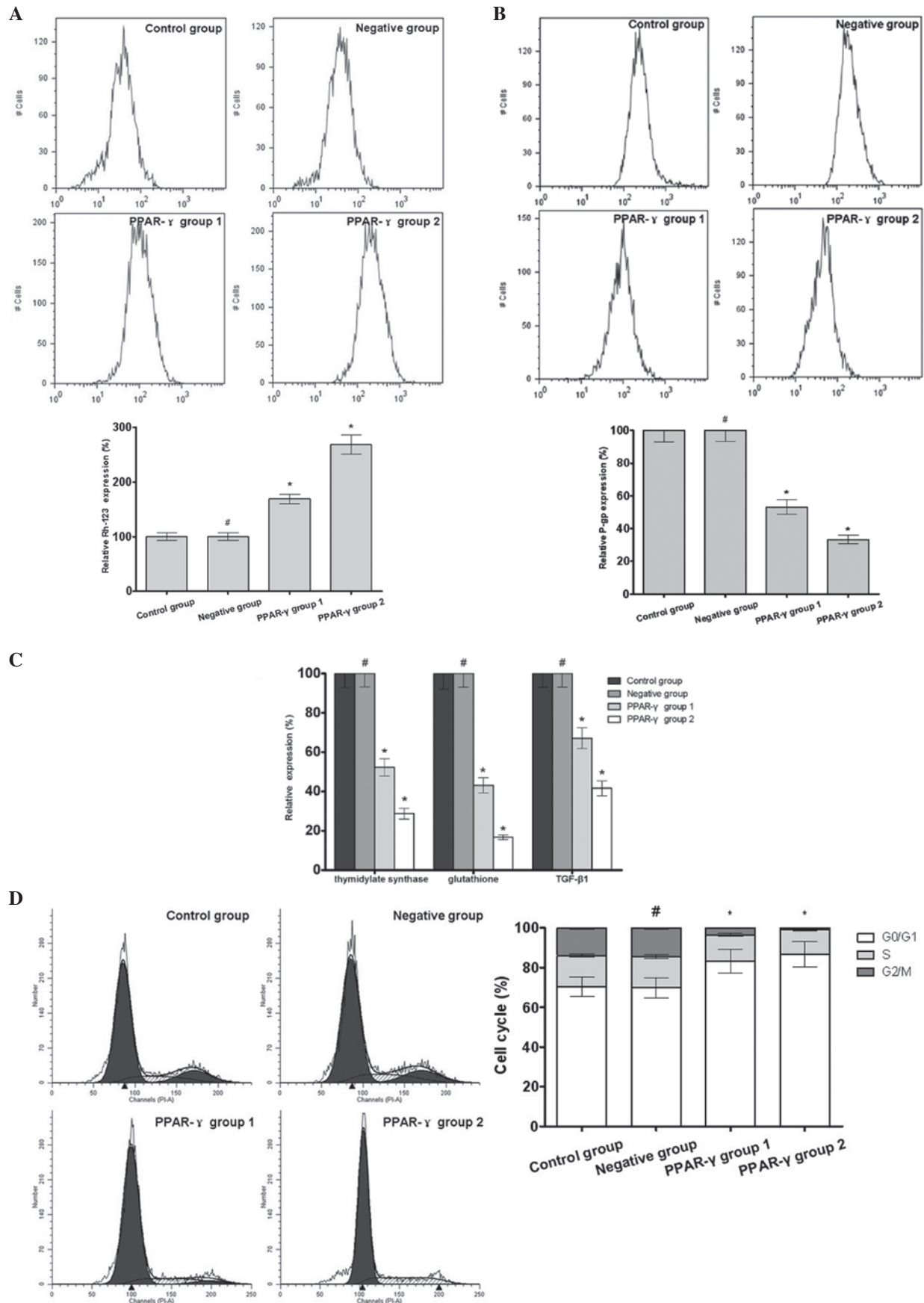


Figure 3. Effect of PPAR- $\gamma$  on the intracellular Rh-123 content, expression levels of P-gp, ROS, thymidylate synthase, glutathione and TGF- $\beta$ 1, cell cycle and apoptosis of the U-87 MG/DDP cell lines. Data are expressed as the mean  $\pm$  standard deviation. (A) Flow cytometry demonstrated an increased intracellular content of Rh-123 in the PPAR- $\gamma$  group 1 and PPAR- $\gamma$  group 2 compared with the control group ( $^{\#}P>0.05$ ;  $^{*}P<0.05$ ;  $n=3$ ). (B) Flow cytometry demonstrated decreased expression of P-gp in PPAR- $\gamma$  group 1 and PPAR- $\gamma$  group 2 compared with the control group ( $^{\#}P>0.05$ ;  $^{*}P<0.05$ ;  $n=3$ ). (C) Expression levels of thymidylate synthase, glutathione and TGF- $\beta$ 1 decreased in PPAR- $\gamma$  group 1 and PPAR- $\gamma$  group 2 compared with the control group ( $^{\#}P>0.05$ ;  $^{*}P<0.05$ ;  $n=5$ ). (D) Flow cytometry demonstrated an increased G0/G1 phase rate in PPAR- $\gamma$  group 1 and PPAR- $\gamma$  group 2 compared with the the control group ( $^{\#}P>0.05$ ;  $^{*}P<0.05$ ;  $n=3$ ).

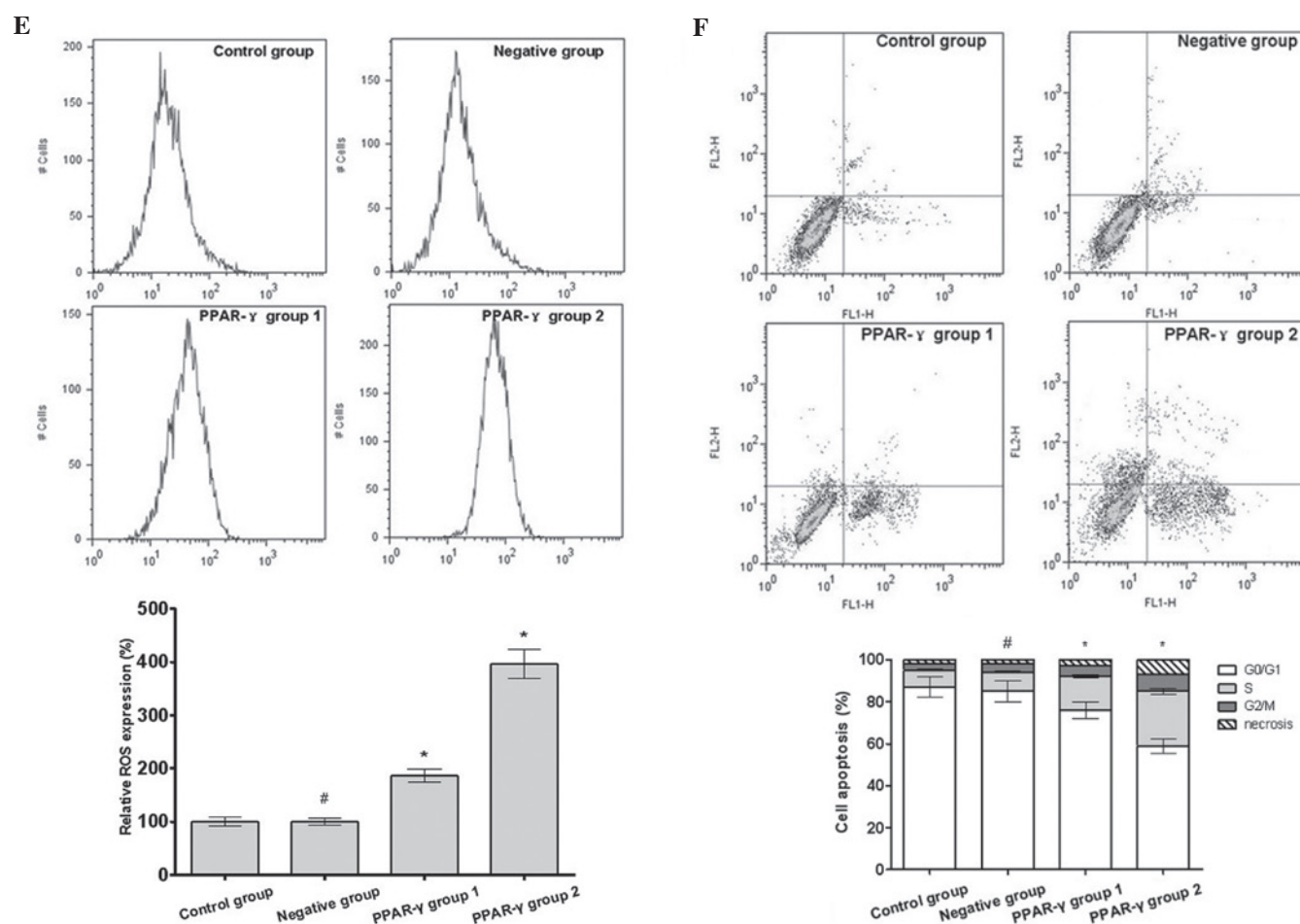


Figure 3. Continued. (E) Flow cytometry demonstrated that there was an increased expression of ROS in the PPAR- $\gamma$  group 1 and PPAR- $\gamma$  group 2 compared with the control group ( $^{\#}P>0.05$ ,  $^*P<0.05$ ;  $n=3$ ). (F) Flow cytometry demonstrated an increased rates of apoptosis rate in PPAR- $\gamma$  group 1 and PPAR- $\gamma$  group 2 compared with the control group. Data are expressed as the mean  $\pm$  standard deviation ( $^{\#}P>0.05$ ,  $^*P<0.05$ ;  $n=3$ ). PPAR, peroxisome proliferator-activated receptor; Rh, rhodamine; ROS, reactive oxygen species; TGF, transforming growth factor.

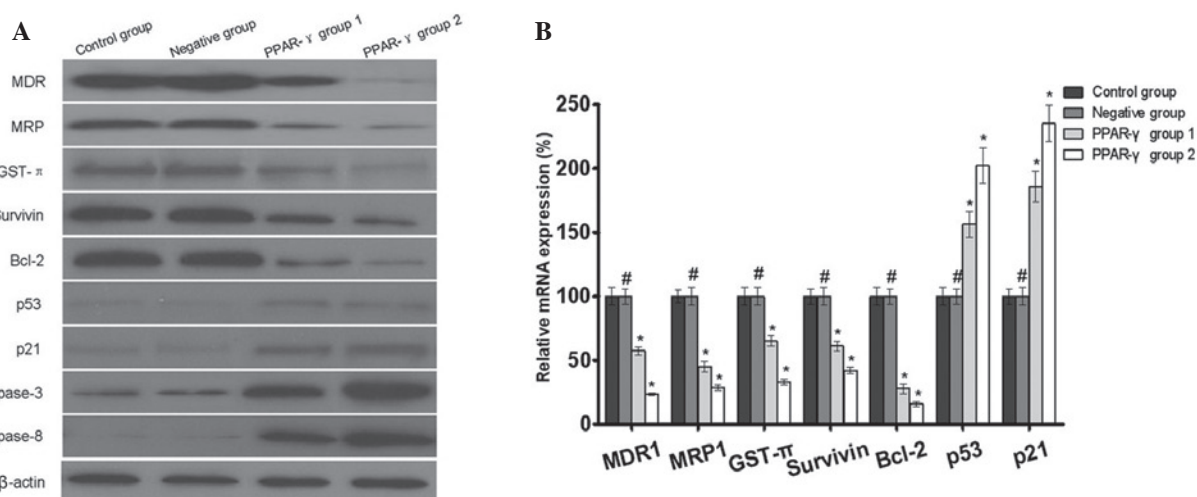


Figure 4. Effect of PPAR- $\gamma$  on the expression levels of multidrug resistance-associated genes of the U-87 MG/DDP cell lines. (A) Western blotting demonstrated decreased protein expression levels of MDR, MRP, GST- $\pi$ , survivin and Bcl-2 in PPAR- $\gamma$  group 1 and PPAR- $\gamma$  group 2 compared with the control and negative groups, however, the protein expression levels of p53, p21 and caspase-3/8 increased in the PPAR- $\gamma$  group 1 and PPAR- $\gamma$  group 2 compared with the control and negative groups.  $\beta$ -actin was used as an internal loading control. (B) Reverse transcription-quantitative polymerase chain reaction assay results demonstrated decreased mRNA expression levels of MDR, MRP, GST- $\pi$ , survivin and Bcl-2 in PPAR- $\gamma$  group 1 and PPAR- $\gamma$  group 2 compared with the control group, however, mRNA expression levels of p53 and p21 were increased in PPAR- $\gamma$  group 1 and PPAR- $\gamma$  group 2 compared with the control group.  $\beta$ -actin was used as an internal loading control. The data are expressed as the mean  $\pm$  standard deviation ( $^{\#}P>0.05$ ,  $^*P<0.05$ ;  $n=5$ ). PPAR, peroxisome proliferator-activated receptor; Bcl, B-cell lymphoma; MDR, multidrug resistance gene; MRP, multidrug resistance-associated protein; GST, glutathione S-transferase.

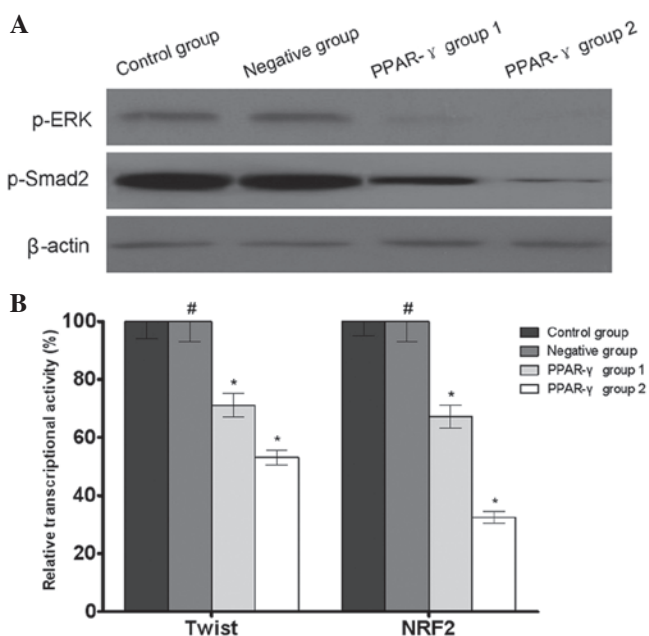


Figure 5. Effect of PPAR- $\gamma$  on multidrug resistance-associated signaling molecules of the U-87 MG/DDP cell lines. (A) Western blotting demonstrated decreased expression levels of phosphorylated ERK and Smad2 in PPAR- $\gamma$  group 1 and PPAR- $\gamma$  group 2 compared with the control and negative groups.  $\beta$ -actin was used as an internal loading. (B) Reporter gene assay results demonstrated a decreased in the transcriptional activity of Twist and NRF2 in the PPAR- $\gamma$  group 1 and PPAR- $\gamma$  group 2 compared with the control group. The data are expressed as the mean  $\pm$  standard deviation ( $^{\#}P < 0.05$ ;  $^*P < 0.05$ ;  $n = 5$ ). PPAR, peroxisome proliferator-activated receptor; ERK, extracellular signal-regulated kinase; Smad, mothers against decapentaplegic; NRF2, nuclear factor (erythroid-derived 2)-like 2.

chemotherapeutic drugs and demonstrate favorable efficacy in the treatment of a variety of malignant types of tumor. However, the tumor cells can develop drug resistance during the therapeutic process, often leading to treatment failure (14). This means that searching for molecular drug targets against drug resistance clinically is urgently required to improve the clinical benefits for the patient.

PPAR- $\gamma$  is a nuclear transcription factor, which can bind to corresponding ligands to induce or inhibit target gene expression. PPAR- $\gamma$  is overexpressed in adipose tissue and expressed in mammary, pulmonary, ovarian, prostate and other types of tissue. Previous studies elucidated the close association between PPAR- $\gamma$  and cancer, for example, the ligand-induced activation of PPAR- $\gamma$  arrests the growth of ovarian cancer cells and non-small cell lung cancer cells, and also induces tumor cell differentiation and apoptosis. Patients with breast cancer overexpressing PPAR- $\gamma$  exhibit longer disease-free survival rates (15-17). However, the role of PPAR- $\gamma$  in cisplatin-resistant glioma remains to be elucidated.

One of the mechanisms of tumor cell resistance to chemotherapeutic drugs is the overexpression of MDR proteins, including MDR1 and MRP1, leading to increased drug efflux to reduce the intracellular drug accumulation and effective drug concentration at the target sites (18,19). The present study established the U-87 MG/CDDP cisplatin-resistant cell line and used a recombinant plasmid transfection technique to stably overexpress PPAR- $\gamma$  in these drug-resistant cells. The subsequent MTS assay revealed that the sensitivity of the U-87

MG/CDDP PPAR- $\gamma$ -overexpressing cell lines to cisplatin was increased, as evidenced by the reduced IC<sub>50</sub>. This sensitivity was positively correlated with the expression of PPAR- $\gamma$ . The present study also used flow cytometry to detect changes in the content of Rh-123 in tumor cells, the results of which demonstrated that a higher level of PPAR- $\gamma$  was correlated with an increase in the intracellular content of Rh-123. Since Rh-123 is a substrate of MDR1 and MRP1, the increase in intracellular Rh-123 content indirectly suggested a reduced efflux of chemotherapeutic drug molecules from tumor cells (20,21). Furthermore, western blotting and RT-qPCR demonstrated that the transcription and expression levels of MDR1 and MRP1 decreased, consistent with the decreased expression of P-gp.

In addition to MDR1 and MRP1, other mechanisms may mediate tumor cell resistance to cisplatin. GST- $\pi$ , thymidylate synthase and glutathione can act as detoxication molecules to detoxify chemotherapeutic drug toxicity (22,23). The present study also found that the expression levels of GST- $\pi$ , thymidylate synthase and glutathione significantly decreased following the overexpression of PPAR- $\gamma$  and there was increased oxidative stress by cisplatin, suggesting that PPAR- $\gamma$  negatively regulated the expression levels of GST- $\pi$ , thymidylate synthase and glutathione, and consequently inhibited the tumor cell drug resistance mediated by the latter. Therefore, PPAR- $\gamma$  reversed U-87 MG/CDDP drug resistance through multiple pathways.

The flow cytometry results demonstrated that PPAR- $\gamma$  arrested the tumor cell cycle at the G0/G1 phase and increased the levels of apoptosis. Investigation of the cell cycle- and apoptosis-associated protein molecules revealed that PPAR- $\gamma$  negatively regulated the expression levels of survivin and Bcl-2, which are tumor cell apoptosis inhibitory proteins (24,25), and positively regulated the expression levels of p21, p53 and caspase-3/8, which are cell apoptosis inducing proteins (26-28). Accordingly, PPAR- $\gamma$  regulated tumor cell apoptosis through coordinated regulation of multiple protein molecules. The fact that p21/p53 can arrest cells at the G1/S checkpoint and previous findings indicate G0/G1 transforming gene-2 as the target gene of PPAR are consistent with the observation in the present study that the overexpression of PPAR- $\gamma$  arrested tumor cells at the G0/G1 phase (29). In addition, western blotting demonstrated that PPAR- $\gamma$  was able to downregulate the levels of TGF- $\beta$ 1 and the phosphorylation of ERK and Smad2, which are important in tumor cell growth (30). It was also observed that PPAR- $\gamma$  was able to downregulate the transcriptional activity of Twist and NRF2 in the U-87 MG/CDDP tumor cells. These transcription factors are all important in the carcinogenesis and progression of tumor cells (31-33).

In conclusion, the present study demonstrated that the overexpression of PPAR- $\gamma$  was able to reverse drug resistance in the U-87 MG/CDDP cisplatin-resistant glioma cell line. The mechanisms of action were found to include: Downregulation of MDR1 and MRP1, increased intracellular drug accumulation, increased tumor cell sensitivity to drugs, regulation of multiple proteins associated with the cell cycle and cell apoptosis, inhibition of tumor cell growth and increased apoptosis through the TGF- $\beta$ 1/ERK/Smad2 pathway.



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