

• Basic Research •

# Role of histone deacetylase in inhibiting invasion of human gastric carcinoma cell line SGC-7901 by PPAR $\gamma$ -mediated pathway

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**[Abstract]** **Background and Objective:** Histone deacetylase (HDAC) can attenuate the function of peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) to drive adipocyte differentiation. PPAR $\gamma$  activation is confirmed to inhibit the development and metastasis of a variety of malignant cells. This study was to investigate the role of HDAC in inhibiting the invasion of human gastric carcinoma SGC-7901 cells through PPAR $\gamma$ -mediated pathway, and explore potential mechanism. **Methods:** SGC-7901 cells were treated with different concentrations of Trichostatin A (TSA) and Rosiglitazone (ROZ) respectively to select the best combination through assessing cell proliferation by MTT assay. Then cells were randomly divided into control group, TSA group, ROZ group, and combination group. Cell proliferation was detected by MTT assay after 48 h; cell invasion was detected by Boyden chamber invasion test. The mRNA levels of PPAR $\gamma$  and matrix metalloproteinase-2 (MMP-2) were assessed by reverse transcription-polymerase chain reaction (RT-PCR), and the protein level of MMP-2 was evaluated by Western blot. **Results:** Both TSA and ROZ inhibited the proliferation of SGC-7901 cells in a dose-dependent manner. A combination of 20 nmol/L TSA and 5  $\mu$ mol/L ROZ synergistically inhibited the invasion of SGC-7901 cells ( $q=1.41$ ). ROZ down-regulated the mRNA and protein expression of MMP-2. TSA and ROZ in combination reduced MMP-2 expression more obviously than ROZ alone. TSA up-regulated the expression of PPAR $\gamma$  mRNA. **Conclusions:** HDAC suppresses the activation of PPAR $\gamma$  through a series of molecular mechanisms. The activity of ROZ in inhibiting invasion of human gastric carcinoma cells can be enhanced after the activity of HDAC is inhibited by TSA.

**Key words:** gastric neoplasm, SGC-7901 cell, histone deacetylase, peroxisome proliferator-activated receptor  $\gamma$ , invasion

Peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) is a member of nuclear hormone receptor superfamily. As a transcription factor, it plays an important part in the process of fat formation and inflammation. Recent studies suggested that binding with its ligand, PP

AR $\gamma$  could not only regulate lipid metabolism, energy balance, and inflammation process, but also inhibit tumor growth, proliferation and invasiveness.<sup>1-3</sup> Rosiglitazone (ROZ) is one of the

PPAR $\gamma$ 's ligands.

Histone acetyltransferase (HAT) and histone deacetylase (HDAC) regulate the acetylation state of histone's lysine residues; thereby they regulate gene transcription and expression. Trichostatin A (TSA) is a typical inhibitor of HDAC. Others have shown that histone deacetylase inhibitor (HDACi) can correct acetylation status of histone and enhance the effectiveness of some traditional chemotherapy drugs, which target DNA or DNA bound enzyme.<sup>4</sup> Combining with ligand, PPAR $\gamma$  becomes a nuclear transcription factor and activates the target DNA transcription and expression. This study aimed to investigate whether HDAC could inhibit the invasion of human gastric carcinoma cells by PPAR- $\gamma$  mediated pathway.

## Materials and Methods

**Cells.** Human gastric cancer cells SGC-7901 were purchased from the foundation of Chongqing Medical Institute and cultured in RPMI-1640 culture medium, containing 10% fetal bovine serum and double-antibiotics (penicillin 100 U/mL and streptomycin 100 U/mL). The serum was put in the incubator at 37 °C, 5% CO<sub>2</sub>, exchanged the next day, and digested to passage every 3 ~ 4 d by 0.25% Trypsin.

**Reagents and equipment.** The fresh fetal bovine serum was produced of Hangzhou Sijiqing Engineering Materials Co.Ltd. ROZ free alkali was produced of base for the Beijing Gaomeng Chemical Co. Ltd. MTT and DMSO were produced of Sigma. RPMI-1640 culture medium and 0.25% Trypsin were purchased from Chongqing Medical University foundation Institute. Trizol reagent and RT-PCR kit were produced of the TaKaRa Company. Rabbit-derived anti-human MMP2 was purchased from eBioscience Company. Rabbit-derived antibody was purchased from Wuhan Boshide Bio-engineering Co. Ltd. Monoclonal Anti- $\beta$ -Actin antibody (mouse) and horseradish enzyme marked goat anti-mouse antibody were purchased from Zhongshan Jinqiao Biotechnology Co. Ltd. TSA, BeyoECL

Plus LED Kit, SDS-PAGE gel preparation kit, and Bradford Protein Assay Kit were purchased from Biyuntian Biotechnology Research Institute.

**The TSA mediated cytotoxicity test to SGC-7901.** Independent effect. The SGC-7901 cells at logarithmic growth phase were incubated in 96 well plates with the density adjusted to  $7 \times 10^4$ /mL ( $7 \times 10^3$ /mL/well). We added TSA (final concentration of 0, 10, 20, 40, 80, 100, 200, 400, 800 and 1000 nmol/L) and ROZ (final concentration of 0, 5, 10, 20, 40, 80, 100  $\mu$  mol/L) separately with medium changed 24 h later. We set zero adjust group at the same time with 5 duplicated wells, and added MMT 20  $\mu$  L (5 mg/L) to each well. After 48-hour action, we disposed the culture medium and put DMSO 150  $\mu$  L into each well, shaken until the purple crystal disappeared completely, then measured the absorbance value (A) of each well by the automated microplate reader with 570 nm wavelength. Cell proliferation inhibition rate = [(negative control group values A<sub>570</sub> - A<sub>570</sub> value of the experimental group) / (A<sub>570</sub> value of negative control group - A<sub>570</sub> value of zero adjust group)]  $\times$  100%.

Combined effec. The SGC-7901 cells at logarithmic growth phase were incubated in 96 well plates with the density adjusted to  $7 \times 10^4$ /mL ( $7 \times 10^3$ /mL/well). The cells were divided into 6 groups: control group, T1 group (20 nmol/L), T2 group (40 nmol/L), R group (5  $\mu$  mol/L), T1+R group, and T2+R group, with medium changed 24 h later. Test the inhibition rate of cell proliferation as steps in 1.3.1.

**In vitro invasion assay.** The effect of TSA and ROZ on invasion of SGC-7901 cell. We set TSA into 20 and 40 nm/L, ROZ into 2.5 and 5  $\mu$  mol/L, which were of no cytotoxicity, and set the control group at the same time. The ECM gel (product of Sigma Company), attenuated by serum-free RPMI-1640 medium, was evenly coated by polycarbonate membrane of chamber Transwell (product of Millipore Company) (pore size 8  $\mu$  m). The chamber was put into 24 well plate, air dried at 4 °C, and hydrated by a small amount of serum-free medium before use. 600  $\mu$  L of RPMI-1640 serum containing 20% fetal bovine serum was put into the inferior chamber,

while single cell suspension of 200  $\mu$  L ( $5 \times 105/\text{mL}$ ) cultured by serum-free RPMI-1640 medium and treated with various concentration of RGZ or TSA 48 h was put into the upper chamber. The chambers were put into the incubator at 37°C, 5% CO $_2$ , and put out 20 h later. The cells on the surface membrane were removed by cotton swabs, fixed by 95% ethanol, and stained in 30 min by 0.1% crystal violet. We counted the number of trans-membrane cells under an optical microscope, chose five high power fields by random, and checked each field of vision to evaluate the invasion and metastasis of tumor cells in vitro.

Effect of TSA combined with ROZ on invasion of SGC-7901 cell. The cells were divided into 4 groups: control group, group T (20 nmol/L), group R (5  $\mu$  mol/L), and group TSA (20 nmol/L) + ROZ (5  $\mu$  mol/L). The cells were treated by the steps in 1.4.1 for 48 h, then their invasion ability was calculated by the following equation: cell invasion inhibitory rate = [(the number of transmembrane cells of control group - the number of transmembrane cells of experimental group) / the number of transmembrane cells of control group]  $\times$  100%. The effect of TSA combined with ROZ on invasion of SGC-7901 cell was calculated by Kim Evaluation:  $q = EA + B / [EA + (1 - EA) \times EB]$ . EA + B was the inhibition rate of the combination of two drugs in cell invasion, EA and EB stood for the inhibition rate of the two drugs acting alone. It indicated that the two drugs had antagonistic effect when  $q < 0.85$ ;  $0.85 \leq q \leq 1.15$  indicated that the role of the two drugs were combined;  $q > 1.15$  indicated that the two drugs had synergistic effect.

**Detection of PPAR $\gamma$ , MMP2 mRNA expression by RT-PCR.** Cells culture, groups dividing and dealing with the cells were done following the steps in 1.4.2 for 48 h. The total RNA was extracted following the Trizol reagent manual by one step, the concentration of which in each group was adjusted to 400 ng/ $\mu$  L. We synthesized cDNA by the AMV reverse transcriptase enzyme as RT-PCR kit requested.

We used the software Primer Premier 5 to design primer sequences of PPAR $\gamma$  (GenBank

serial number registry NM\_138712), MMP2 (gene sequence No. NM\_004530) and  $\beta$ -actin (gene sequence No. NM\_001101). The sequences were synthesized by Dalian Bao bio-engineering company. The primer sequence of PPAR $\gamma$ : sense: 5'-GGAGCAGAGCAAAGAGGTG-3'; antisense: 5'-GAGGACTAGGGTGTTCAG-3'; amplified fragment was 474 bp. The primer sequence of MMP2: sense: 5'-CACAGCCAACTACGATGATGAC-3'; antisense: 5'-GAACACAGCCTTCTCCTCC TG -3'; amplified fragment was 472 bp. The primer sequence of  $\beta$ -actin: sense: 5'-CCACGAAACTACCTTCAACTCC-3'; antisense: 5'-actgctcactctgcttctgct-3'; amplified fragment was 272 bp. Reaction conditions were: PPAR $\gamma$ : 94°C predegeneration 5 min, 94°C denaturation 30 s, 58.5°C annealing 30 s, 72°C extension of 1 min, 35 cycles, 72°C extension a further 10 min; MMP2: 94°C predegeneration 5 min, 94°C change 30 s, 58°C annealing 30 s, 72°C extension of 1 min, 35 cycles, 72°C extension a further 10 min;  $\beta$ -actin: 94°C predegeneration 5 min, 94°C denaturation 30 s, 57°C annealing 30 s, 72°C extension of 1 min, 35 cycles, 72°C extension a further 10 min. Electrophoresis of PCR products was taken by 2% agarose gel. The electrophoresis results were scanned by automated gel scanning to get the relative optical density (ROD) ratio of target gene in each sample and  $\beta$ -actin mRNA.

**The detection of the expression of MMP2 protein by Western blot.** Cells culture, groups dividing and dealing with the cells were done following the steps in 1.4.2 for 48 h. We Collected the treated cells in each group, got the cells lysed by RIPA lysis buffer, extracted total protein, detected the protein content by Bradford Determination, and did vertical SDS-PAGE electrophoresis with separation gel and concentrated gel, which concentrations were of 10% and of 5%. We put 40  $\mu$  g of sample into each well, transferred the electrophoretic separated proteins to PVDF membrane, blocked the membrane by 5% skimmed milk powder for 1 h. The samples were incubated with 1:1000 rabbit anti-human MMP2 antibody at 4°C overnight, washed 3 times with TBST, then were

incubated with 1:1000 goat anti-rabbit IgG/HRP added for 1 h. The membrane was rinsed 3 times with TBST, and imaged by BeyoECL Plus chemiluminescence. The reference used was  $\beta$ -actin (mouse anti-human, dilution to 1:1000).

**Statistical analysis.** SPSS13.0 statistical software was used for the analysis of experimental data. It was expressed with mean  $\pm$  standard deviation (SD) and variance analysis to deal with the test results. Dunnett-t test was used to compare a number of experimental groups with the control group. The comparison between each two groups was done by LSD-t method. The relation of two variable groups was detected by linear regression analysis.  $P < 0.05$  indicated statistically significant difference.

## Results

**The inhibition effect of TSA and ROZ on proliferation of SGC-7901 cell.** The cell proliferation inhibition rates of 0, 10, 20, 40, 80, 100, 200, 400, 800 and 1000 nmol/L of TSA were 0%, 0.55%, 0.71%, 4.88%, 11.46%, 14.09%, 31.38%, 45.5%, 74.18%, and 82.71%, respectively. When the concentration of less than 40 nmol/L of TSA on cell proliferation compared with control group, there was no significant difference ( $P > 0.05$ ); linear regression analysis showed inhibition rate had linear relationship ( $P < 0.01$ ) and strong positive correlation ( $r = 0.98$ ) with concentration.

The cell proliferation inhibition rates of 0, 5, 10, 20, 40, 80, and 100  $\mu$  mol/L of ROZ were 0%, 2.04%, 13.88%, 28.80%, 45.39%, 62.09%, and 66.35%. When the concentration of ROZ was 5  $\mu$  mol/L, there was no significant difference between the cell proliferation inhibition effect of ROZ and that of the control group ( $P > 0.05$ ); when the concentration of ROZ was 10  $\mu$  mol/L, with the increase in the concentration of ROZ markedly, it enhanced the inhibitory effect on cell proliferation. Linear regression analysis showed there was linear correlation between the inhibition rate and the concentration ( $P < 0.01$ ). Also there was strong positive correlation between them ( $r = 0.96$ ).

The cell proliferation inhibition rates of the control group, T1 group, T2 group, R group, T1 + R group, and T2 + R group were 0%, 0.76%, 1.02%, 1.75%, 2.49%, and 5.92%. There was statistically significant difference when T2 + R group was compared with the control group ( $P < 0.01$ ).

**The inhibition effect of TSA and ROZ on invasion of SGC-7901 cell.** Transwell invasion showed that the number of trans-membrane cells of the control group was  $366.67 \pm 12.22$ , when the number of trans-membrane cells in groups treated with 20 and 40 nmol/L of TSA were  $315.00 \pm 11.00$  and  $283.33 \pm 12.50$ , therefore the cell invasion was markedly inhibited compared with the control group. The difference was statistically significant ( $P < 0.01$ ). With the increase in TSA concentration, the number of invasive cells decreased, and the inter-group differences were significant ( $P < 0.05$ ).

According to the proliferation inhibition, we selected drugs concentration with no significant inhibitory effect on cells, which was 20 nmol/L TSA + 5  $\mu$  mol/L ROZ. The numbers of trans-membrane cells of the control group, T group (20 nmol/L), R group (5  $\mu$  mol/L), and the joint group were  $294.33 \pm 19.60$ ,  $235.67 \pm 16.26$ ,  $191.00 \pm 22.91$ , and  $94.33 \pm 8.02$ . Compared with the control group, the treatment group significantly inhibited the invasion, and the difference was statistically significant ( $P < 0.01$ ). The differences were statistically significant between the joint treatment group and individual treatment group ( $P < 0.01$ ),  $q = 1.41 > 1.15$  (calculated by Kim method). It suggested that the two drugs in combination on the inhibition of cell invasion were synergic. Figure 1

**The influence of TSA combined with ROZ on PPAR $\gamma$ , MMP2 mRNA expression.** Semi-quantitative RT-PCR amplification products showed optical density ratios of PPAR $\gamma$  /  $\beta$ -actin in the control group, TSA group, ROZ group and TSA + ROZ group were  $1.72 \pm 0.09$ ,  $2.12 \pm 0.07$ ,  $1.66 \pm 0.10$  and  $2.15 \pm 0.08$ . Compared with the control group, the expression of PPAR $\gamma$ , MMP2 mRNA in TSA group and the joint group were increased significantly, and the difference was



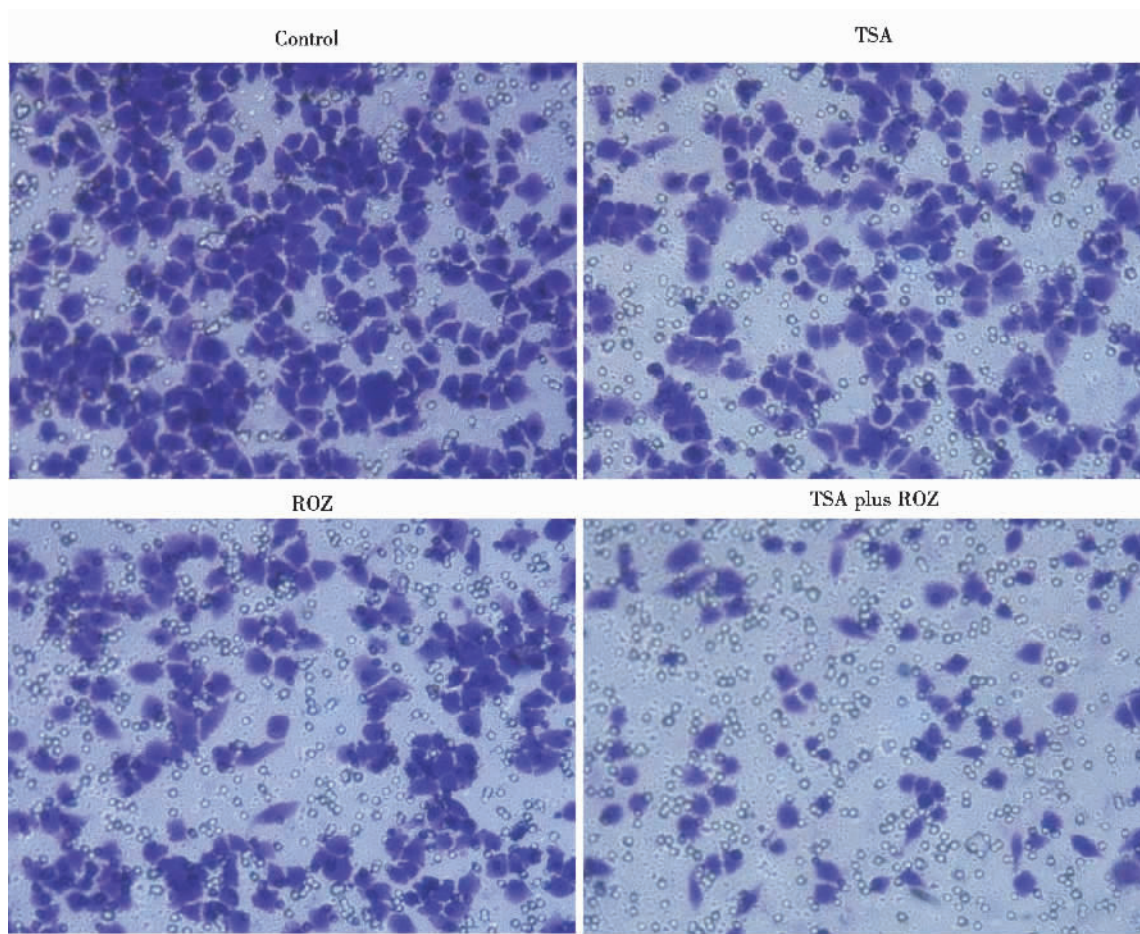


Figure 1 Effects of Trichostatin A (TSA) and Rosiglitazone (ROZ) on invasion of SGC-7901 cells (crystal purple staining  $\times 200$ )

statistically significant ( $P < 0.01$ ); the expression of PPAR $\gamma$ , MMP2 mRNA in ROZ group was of no significant change compared with the control group ( $P > 0.05$ ). The optical density ratios of MMP-2/ $\beta$ -actin were  $2.06 \pm 0.10$ ,  $2.10 \pm 0.10$ ,  $1.67 \pm 0.08$  and  $1.37 \pm 0.02$ . Compared with the control group, the expression of MMP2 mRNA in both ROZ group and TSA + ROZ group decreased significantly ( $P < 0.01$ ); the MMP2 mRNA expression in TSA + ROZ group decreased more than ROZ group, and the difference was significant ( $P < 0.01$ ); Compared with the control group, the expression of MMP2 mRNA in TSA group was of no significant difference ( $P > 0.05$ ). Figure 2.

**The influence of the combination of TSA and ROZ on MMP2 dealt with them.** The MMP2/ $\beta$ -actin optical density ratios of the control group, TSA group, ROZ group and TSA + ROZ group were  $0.70 \pm 0.01$ ,  $0.69 \pm 0.02$ ,  $0.42 \pm 0.00$ ,  $0.34 \pm 0.01$ . Compared with the

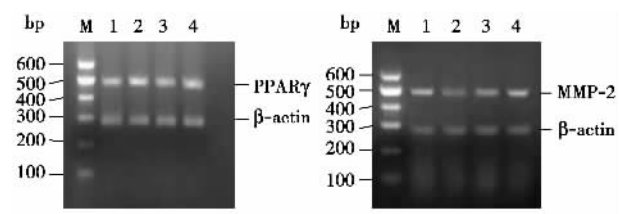


Figure 2 Effects of TSA and ROZ on mRNA expression of PPAR $\gamma$  and MMP-2 in SGC-7901 cells

Lane M: DNA marker; lane 1: untreated cells; lane 2: SGC-7901 cells treated with TSA and ROZ; lane 3: SGC-7901 cells treated with ROZ; lane 4: SGC-7901 cells treated with TSA.

control group, the expression of MMP2 protein of TSA group was observed with no statistically significant decrease ( $P > 0.05$ ). The expression of MMP2 protein of the ROZ group and TSA+ROZ group decreased, and the differences were statistically significant ( $P < 0.01$ ). The combination group decreased more significantly than ROZ group ( $P < 0.01$ ) (Fig. 3).

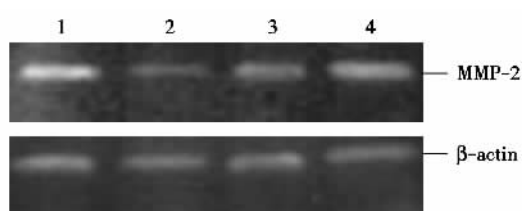


Figure 3 Effect of TSA and ROZ on protein expression of MMP-2 in SGC-7901 cells

Lane 1: untreated cells; lane 2: SGC-7901 cells treated with TSA and ROZ; lane 3: SGC-7901 cells treated with ROZ; lane 4: SGC-7901 cells treated with TSA.

## Discussion

The invasion and metastasis are the main causes of the gastric cancer patients' poor prognosis and death, therefore it becomes a key point to find new biological treatments and new ways to prevent the recurrence and metastasis of gastric cancer so as to raise the patients' survival.

The invasion and metastasis of tumor is a multi-factor and multi-step event, including the following major steps: (1) changes in tumor cell adhesion, (2) increase in extracellular matrix degradation, (3) migration of tumor cells, (4) changes in cell proliferation, (5) neovascularization.<sup>5</sup> The degradation of extracellular matrix is a particular important one in these steps, in which matrix metalloproteinases (MMP) and its inhibitor play a key role.<sup>6</sup> MMP2 is a member of matrix metalloproteinases family, and one of the most closely related members to tumor. Studies have shown that tumor cells complete degradation of matrix and the process of breaking matrix barriers to promote tumor invasion and metastasis through the secretion of MMP-2.<sup>7</sup> Therefore we selected MMP2 as the related gene to gastric cancer in this study.

PPAR $\gamma$  is expressed not only in gastric cancer tissues, but also in normal gastric musoca.<sup>8</sup> Although the physiological function of PPAR $\gamma$  in epithelial cells was poorly understood, studies found that activated PPAR $\gamma$  could not only inhibit the gastric cancer cell proliferation,<sup>9,10</sup> but also limit their ability to invasion and metastasis effectively.<sup>11,12</sup> In this study, we suggested that PPAR $\gamma$ 's ligand ROZ was able to inhibit

proliferation of human gastric cancer cell and invasion in vitro at a dose-dependent manner through reducing expression of MMP2, probably because of the inhibition mechanism of gastric cancer cell invasion by PPAR $\gamma$  ligands.

The state of histone lysine residues acetylation is regulated by two enzymes: HAT and HDAC. These enzymes regulate the dynamic equilibrium of histone acetylation and deacetylation in the nucleus accurately. When the histone acetylation is at a high degree, the combination of histone and genomic DNA relaxes, as more transcription factors can combine with the promoter region in order to activate gene transcription; in contrast, when the histone acetylation is at a low degree, the gene transcription is suppressed because of the close combination of histone and genomic DNA.<sup>13</sup> By inhibiting HDAC's activity, HDACi induces histone acetylation at a high degree, loosens the chromatin structure, and thus plays its biological effects. Acetyltransferase (TSA) is a typical inhibitor of HDAC. Recent studies showed that TSA could inhibit the growth of tumor cells.<sup>14</sup> After binding with its ligands, PPAR $\gamma$  also need combine with peroxisome proliferators responsive element (PPRE) to activate the transcription of target gene and play a role in regulation. It was reported that PPAR $\gamma$  could form co-immunoprecipitation complex with retinoblastoma protein (RB protein) in adipocytes. RB protein can mobilize histone deacetylase HDAC3 to combine this complex, and then inhibit the HDAC activity to promote the capacity of adipocyte differentiation.<sup>15</sup>

This experiment observed the invasive ability of gastric cancer cells dealt with the HDAC inhibitors and PPAR $\gamma$  agonists, and proved the role of HDAC played in the emotion process of PPAR $\gamma$ . We have chosen drug concentrations with no significant inhibition of cell proliferation in order to reduce the error, alone or jointly. After the cells were treated by the drugs, they were taken into invasion assay in vitro. The results showed that both TSA and ROZ could inhibit the invasion of gastric cancer cell alone in a dose-dependent manner. They had a good synergy. The detection of the expression of

MMP2 of cells in each group by RT-PCR and Western blot showed that: 5  $\mu$  mol/L of ROZ could up-regulate the transcription and protein expression of MMP2 significantly. When it jointed with 20 nmol/L of TSA, which had no significant influence to the expression of the MMP2, the expression of MMP2 mRNA and protein were significantly increased than that in the cells treated by ROZ alone. That may be one of the mechanisms by which the combination inhibited the gastric cancer cell invasion. In addition, the expression of PPAR $\gamma$  increased in the cells dealt with TSA, which suggested HDAC inhibitor can enhance the activity of PPAR $\gamma$ . And this may explain while the cells were treated by the combination, the expression of MMP2 increased. Also we proved that HDAC inhibited invasion of human gastric carcinoma cells by PPAR $\gamma$ -mediated pathway. Our experiments showed that the expression of PPAR $\gamma$  was not increased when the cells were treated by ROZ alone, which was not coincident with previous reports.<sup>16</sup>

In conclusion, when low doses of inhibitors inhibited the activity of HDAC, the effect of ROZ was enhanced greatly, that indirectly suggested that HDAC might inhibit the activity of PPAR $\gamma$  by some molecular mechanisms, and weaken the inhibition effect of ROZ on invasion of SGC-7901 cells.

It can be understood from the three levels about the regulation of PPAR $\gamma$ 's activity: changing the conformation of PPAR $\gamma$ , changing the expression of PPAR $\gamma$ , and affecting PPAR $\gamma$  target gene transcription and expression as a cofactor of PPAR $\gamma$ . The experimental results showed that ROZ primarily activated PPAR $\gamma$  by changing its conformation, but not up-regulated its expression. The mechanisms by which HDAC inhibits the activity of PPAR $\gamma$  can be assumed as follows: (1) HDAC inhibits the PPAR $\gamma$  gene transcription, and reduces its expression by histone acetylation of N-terminal and nucleosome tightening; (2) PPAR $\gamma$  protein regulates the transcription by acting with PPREs of the target gene in the promoter region, and the deacetylase of HDAC histone nucleosome

constraints the DNA structure, which is not conducive to the combination of PPAR $\gamma$  and DNA; (3) As an untypical adjuvant inhibitor, HDAC aggregates in the promoter region of PPAR $\gamma$  target gene so as to inhibit the transcriptional activity of PPAR $\gamma$ .

This study showed that, HDAC restricted activity of PPAR $\gamma$  by some molecular mechanisms, and weakened inhibition of ROZ on gastric cancer cell invasion. The inhibitory effect of PPAR $\gamma$  agonists on the invasion of SGC-7901 cells can be greatly enhanced while the PPAR $\gamma$  agonists combined with HDAC inhibitors. It may provide us new ideas for the treatment of gastric cancer.

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