Peroxisome proliferator-activated receptor-γ inhibits pancreatic cancer cell invasion and metastasis via regulating MMP-2 expression through PTEN

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Abstract. The invasive and metastatic behavior of pancreatic cancer is associated with a poor prognosis. Therefore, understanding the molecular mechanisms underlying the invasion and metastasis of pancreatic cancer has important application values theoretically and clinically. In previous years, with increasing studies focusing on tumor pathogenesis, it has been revealed that peroxisome proliferator-activated receptor-γ (PPARγ) and phosphatase and tensin homolog (PTEN) are closely associated with the occurrence and development of pancreatic cancer. Thus, in the present study, a scratch wound assay, western blotting and transwell assays were used to investigate their function. The scratch wound assay demonstrated that treatment with the PPARγ ligand rosiglitazone (RGZ) could reduce the movement and migration of pancreatic cancer cells. Western blotting results indicated that while RGZ inhibited the expression of matrix metalloproteinase (MMP)-2, PPARγ inhibitors promoted MMP-2 expression. However, PPARγ ligands and inhibitors did not affect the expression of MMP-9. Further investigation indicated that the regulation of MMP-2 by PPARγ activation occurred through PTEN. In addition, PPARγ activation promoted PTEN expression, thereby inhibiting the expression of MMP-2. Subsequent transwell experiments demonstrated that RGZ treatment significantly inhibited the invasiveness of pancreatic cancer cells and the inhibitory effect of RGZ was completely reversed by simultaneous transfection of the MMP-2-overexpressing vector, which increased the invasiveness of pancreatic cancer cells. Therefore, PPARγ activation can activate PTEN expression, thereby suppressing the expression of MMP-2 and hence inhibiting the invasion and metastasis of pancreatic cancer cells.

Introduction

Pancreatic carcinoma is a malignant tumor of the digestive system and has an extremely poor prognosis. The average survival time following diagnosis is <6 months and the 5-year survival rate is <5% (1). In China, the incidence of pancreatic cancer is increasing every year and only a small number of patients are able to undergo surgical resection. However, there remains a high recurrence rate and a poor prognosis (1,2). Invasion and metastasis are the key factors impacting the recurrence and mortality of patients with pancreatic carcinoma. Therefore, identifying effective molecular targets to prevent or reduce pancreatic cancer invasion and metastasis is critical for improving the treatment efficacy and prognosis.

Peroxisome proliferator-activated receptor-γ (PPARγ) belongs to a class of ligand-activated nuclear transcription factors and is a member of the type II nuclear receptor superfamily (3). PPARγ ligands are divided into two categories as natural ligands, including 15-deoxy prostaglandin J2 (15d-PGJ2), and synthetic ligands, including mainly thiazolidinedione compounds, such as rosiglitazone (RGZ), pioglitazone (PGZ) and troglitazone (TGZ). Among the synthetic ligands, RGZ has the highest bioavailability and efficacy and the least adverse effects. Previous *in vivo* experiments demonstrated that following activation with ligands, PPARγ can inhibit tumor cell growth, induce apoptosis of tumor cells and exert other biological effects (3,4). Sasaki *et al* (5) detected the expression of PPARγ in human pancreatic cancer cells and human pancreatic tissues using western blot analysis and reverse transcription polymerase chain reaction (RT-PCR) methods. The authors found that PPARγ was expressed in all human pancreatic cancer cells and 71% of human pancreatic cancer tissues, while no PPARγ expression was observed in the adjacent normal pancreatic tissues. Farrow *et al* (6) used RGZ to activate PPARγ in the pancreatic cancer cell line AsPC-1 and thereby suppressed the invasiveness of AsPC-1 cells via reducing the secretion of a tumor cell invasion factor i.e. tissue plasminogen activator. Thus, PPARγ activation can inhibit the

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invasion of pancreatic cancer, though the exact mechanism remains to be elucidated.

Hashimoto *et al* (7) found that the PPARγ natural ligand 15d-PGJ2 can suppress the expression of MMP-2 and MMP-9 and inhibit pancreatic cancer cell invasion. MMPs are a group of calcium- and zinc-dependent proteases, and together with their inhibitor, tissue inhibitor of metalloproteinases (TIMPs), regulate tumor invasion and metastasis. Among them, MMP-2 and MMP-9 were found to be overexpressed in pancreatic cancer tissue and closely associated with tumor invasion and metastasis (8,9). In addition, Farrow *et al* (10) reported that the selective PPARγ agonist RGZ enhanced the expression of phosphatase and tensin homolog (PTEN) in pancreatic cancer AsPC-1 cells and inhibited AKT phosphorylation, while application of the PPARγ inhibitor GW9662 suppressed PTEN expression, suggesting that PPARγ ligands attenuate pancreatic cancer cell growth via inhibiting phosphoinositide 3-kinase (PI3K) activity through upregulating the expression of the tumor suppressor gene PTEN. PTEN was identified in 1997 as a tumor suppressor gene closely associated with cancer (11) w. It is important in tumor cell apoptosis and migration and is the main component of signaling pathways that control cell cycle initiation and exit, promote differentiation, initiate repair and regulate apoptosis (11). Therefore, PPARγ activation may possibly regulate the expression of MMPs through PTEN and thereby inhibit the invasion of pancreatic cancer cells. These aspects were examined in the present study.

Materials and methods

Cell culture. Human pancreatic carcinoma PANC-1 cell lines were purchased from the American Type Culture Collection and were maintained in Dulbecco's modified Eagle's medium (Gibco-BRL, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco-BRL), 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Overexpression vector and small interfering RNA (siRNA) transfection. The following primers were used for PTEN and MMP-2 CDS amplification: PTEN-CDS, forward 5'-CG<u>GAATTC</u>G**CCACC**ATGACAGCCATCATCAAAGAG-3' and reverse 5'-CCG<u>CTCGAG</u>TCAGACTTTTGTAATTTGTGTATGC-3'; MMP2-CDS, forward 5'-CG<u>GAATTC</u>**GCCACC**ATGGAGGCGCTAATGGCCCG-3' and reverse 5'-CCG<u>CTCGAG</u>TCAGCAGCCTAGCCAGTCGG-3'. Underlined letters represent the restriction enzyme sites, and bold letters represent the Kozak sequence. The products were digested with *Eco*RI and *Xho*I (Takara Bio, Inc., Otsu, Japan), cloned into pcDNA3.1 vectors, sequenced and verified. PTEN and negative control (NC) siRNAs were obtained from Santa Cruz Biotechnology, Inc. (Shanghai, China). The cells were seeded into 6-well plates at 1x10^5 cells/ml and incubated for 24 h. When the cell confluence reached ~70%, plasmid and siRNA transfection were performed according to the manufacturer's instructions of Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA). The medium was changed within 4-6 h after transfection. After 24 h of transfection, either RGZ (20 μM) or GW9662 (20 μM) was added and the cells were cultured for 24-48 h.

Western blot analysis. Total cellular proteins were extracted by incubating cells in lysis buffer. The protein concentrations in the cell lysates were determined by bicinchoninic acid assay (Pierce Biotechnology Inc., Rockford, IL, USA). SDS-PAGE was performed in 8% glycine gels (Bio-Rad Laboratories Inc., Hercules, CA, USA) loading equal quantities of proteins per lane. Following electrophoresis, separated proteins were transferred onto nitrocellulose membranes and blocked with 5% non-fat milk in Tris-buffered saline and Tween 20 for 1 h. Following this, the membranes were incubated with rabbit anti-human polyclonal MMP-2 (cat. no. 4022; Cell Signaling Technology, Inc., Beverly, MA, USA; 1:1,000), rabbit anti-human polyclonal MMP-9 (cat. no. 3852; Cell Signaling Technology, Inc.; 1:1,000), rabbit anti-human monoclonal PTEN (cat. no. 9552; Cell Signaling Technology Inc.; 1:600) and mouse anti-human monoclonal GAPDH (cat. no. NBP1-47339; Novus Biologicals, San Diego, CA, USA; 1:1,000) overnight at 4°C, and then secondary antibodies conjugated with horseradish peroxidase (anti-rabbit IgG; cat. no. 7074; Cell Signaling Technology, Inc.) at 1:7,000-8,000 dilution were incubated with membranes for 1 h at room temperature. Protein bands were detected using the West Femto System (Pierce Biotechnology, Inc.).

Wound healing assay. Cells (1x10^6 per well) were seeded in 6-well plates and allowed to grow until 70% confluence. The cells were pretreated with Mitomycin C (Roche Diagnostics, Hong Kong, China), which inhibits cell division, so that the difference in motility was not affected by differences in cell proliferation rates. The cells were treated as above and monolayers were then wounded with a pipette tip. Following being washed with phosphate buffered-saline (PBS) three times, the Olympus IX-70 microscope (Olympus, Tokyo, Japan) was used to capture images immediately following scratching for 0, 6, 24 and 48 h in the same marked location of the dish. Data were obtained from three independent experiments.

Transwell matrigel invasion assay. Invasion of cells was evaluated by Transwell matrigel invasion assay. Briefly, 200 μl of cells following transfection (1x10^6 cells/ml) and 600 μl of the complete medium were added to the upper and lower compartments of the chamber, respectively. Following incubation for 48 h, cells migrating to the lower side of the filter were fixed with 4% paraformaldehyde for 15 min at room temperature, washed with PBS, stained with crystal violet and then observed under an Olympus Fluoview 500 IX71 confocal microscope (Olympus).

Statistical analysis. The experiments were performed at least in triplicate and results are expressed as the mean ± standard deviation. SPSS statistical package (SPSS 13.0 for Windows; SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. The difference between two groups was analyzed by two-tailed Student's t-test and that between three or more groups was analyzed by one-way analysis of variance multiple comparisons. $P<0.05$ and $P<0.01$ were considered to indicate a statistically significant difference.

Results

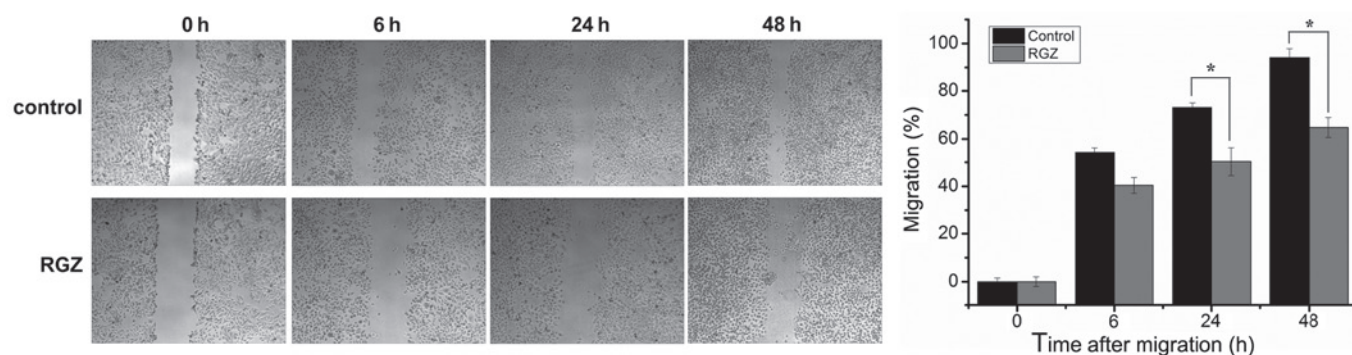*PPARγ activation by RGZ inhibits the migration of pancreatic cancer cells*. The majority of current studies suggest that

Figure 1. RGZ reduces pancreatic cancer cell migration *in vitro*. Each bar represents the mean ± standard deviation from three samples (*P<0.01). RGZ, rosiglitazone.

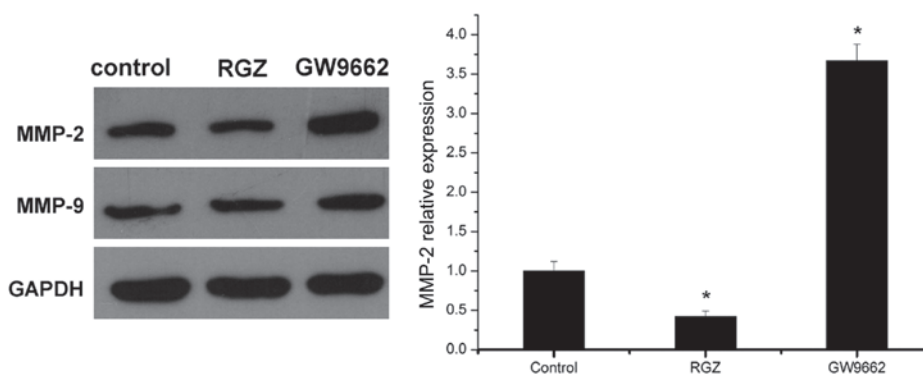Figure 2. Expression of MMP-2 and MMP-9 in PANC-1 cells. RGZ (20 μM) or GW9662 (20 μM) was added to PANC-1 cells and cultured for 48 h. Subsequently, the expression of MMP-2 and MMP-9 was measured by western blot analysis. The results are presented as the mean ± standard deviation (n=3). *P<0.01 vs. control. RGZ, rosiglitazone; MMP, matrix metalloproteinase.

PPARγ activation can inhibit the migration and invasion of tumor cells, however, few relevant studies have focused on pancreatic cancer. The present study initially demonstrated the effect of RGZ on the *in vitro* migration ability of the pancreatic cancer cell line Panc-1 using a scratch wound assay. The cells were subjected to scratch tests following RGZ treatment and the scratch wound was monitored following different periods of time. The results shown in Fig. 1 indicated that the migration of RGZ-treated cells was markedly slower than that of the control cells at the indicated time points. These results suggested that in pancreatic cancer cells, RGZ activation of PPARγ has the ability to inhibit migration.

PPARγ activation regulates MMP-2 expression in pancreatic cancer cells. During the process of invasion and metastasis, tumor cells must overcome a series of natural barriers, including the extracellular matrix (ECM). MMPs are the main direct factors involved in the destruction of the ECM; among them, type II collagenases, including MMP-2 and MMP-9, are most closely associated with tumor invasion and metastasis (12). Therefore, the present study further examined the association between PPARγ activation and the expression of MMP-2 and MMP-9. The results demonstrated that RGZ treatment significantly inhibited MMP-2 expression, whereas addition of the PPARγ-specific inhibitor GW9662 significantly upregulated the expression of MMP-2. However, neither RGZ nor GW9662 significantly altered the

expression of MMP-9 (Fig. 2), suggesting that in pancreatic cancer cells, PPARγ activation predominantly affected the expression of MMP-2, while MMP-9 expression may be regulated by other factors.

PPARγ activation regulates MMP-2 expression through PTEN. Existing studies have demonstrated that PPARγ activation exerts an inhibitory effect on tumor growth by activating the tumor suppressor gene PTEN (10,11). In addition, several studies have also indicated that PTEN has a tumor suppressor role via regulating a series of factors, including MMP-2 (11,13,14). Therefore, PPARγ, PTEN and MMP-2 are closely associated with each other. Following transfection of a PTEN-overexpressing vector into cells, the expression of MMP-2 was downregulated. When treating the pancreatic cancer cells with RGZ, upregulation of PTEN expression occurred and the expression of MMP-2 was simultaneously downregulated. However, following transfection of PTEN siRNA, the RGZ-induced upregulation of PTEN expression was inhibited and the inhibitory effect of RGZ on MMP-2 expression was also reversed (Fig. 3). These results indicated that PPARγ activation inhibits MMP-2 expression via activating PTEN.

PPARγ activation affects pancreatic cancer cell invasion by regulating the expression of MMP-2. PPARγ activation affects the invasion and metastasis of pancreatic cancer cells,
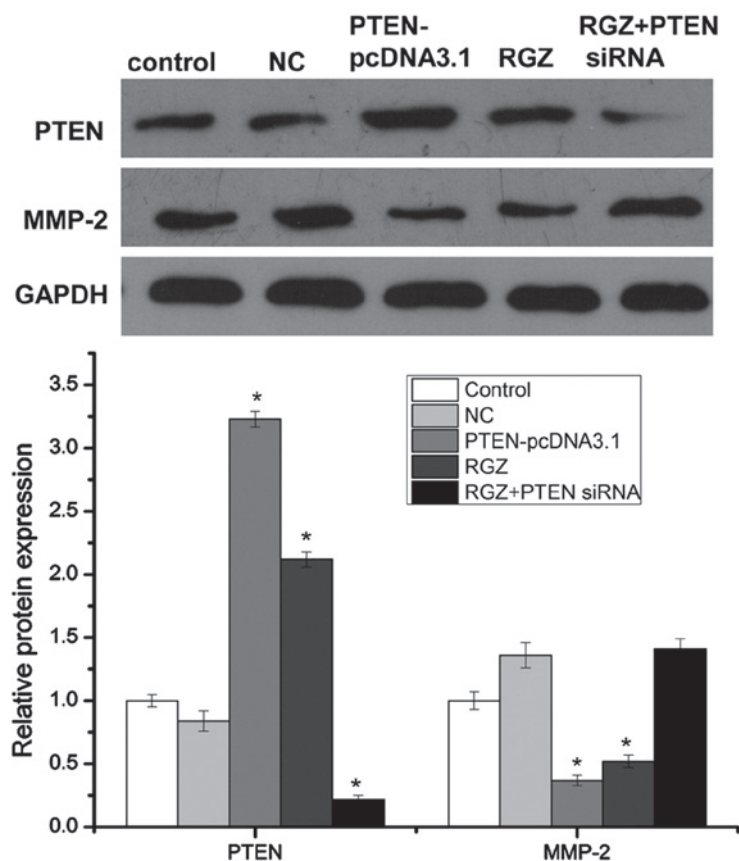
Figure 3. Expression of PTEN and MMP-2 in PANC-1 cells. PANC-1 cells were either transfected with a PTEN-overexpressing vector, treated with RGZ (20 μM) or transfected with PTEN siRNA and treated with RGZ (20 μM). The expression of PTEN and MMP-2 was then measured by western blot analysis. The results are presented as the mean ± standard deviation (n=3). *$P<0.01$ vs. control. RGZ, rosiglitazone; MMP, matrix metalloproteinase; PTEN, phosphatase and tensin homolog; siRNA, small interfering RNA; NC, negative control.
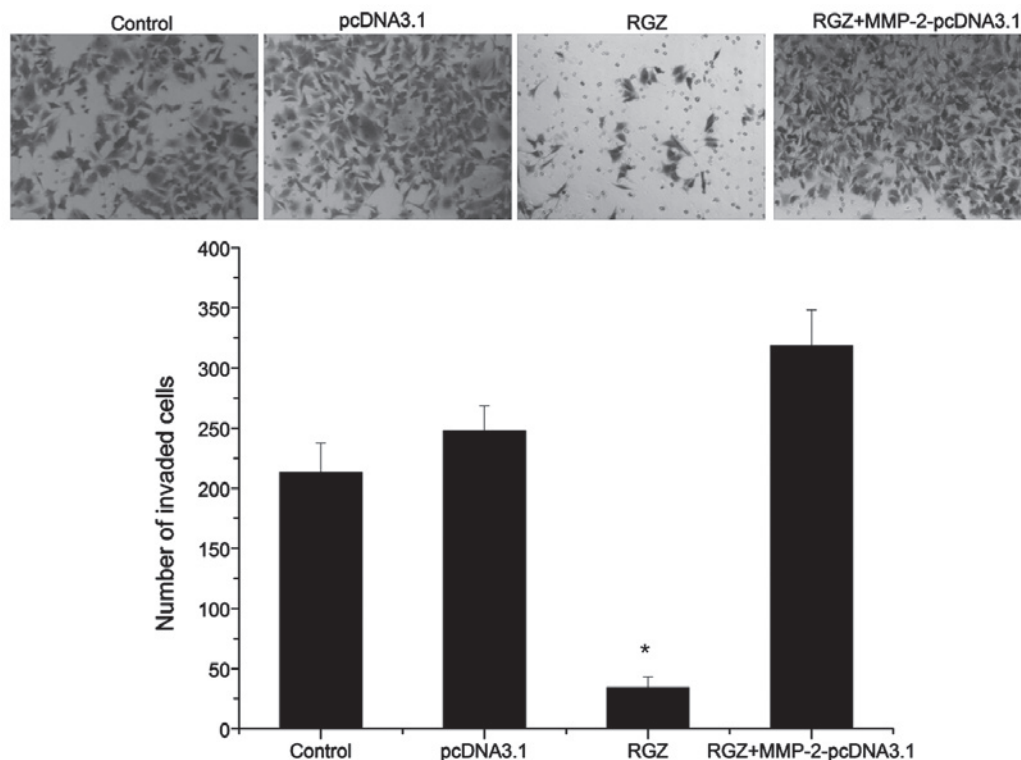

Figure 4. Effect of RGZ and MMP-2 on PANC-1 cell invasion. Transwell analysis of PANC-1 cells following different treatments. The relative ratios of invasive cells per field is shown. Each bar represents the mean ± standard deviation from three samples (*$P<0.01$ vs. control). RGZ, rosiglitazone; MMP, matrix metalloproteinase.

and MMP-2 is closely associated with tumor cell invasion and metastasis. Therefore, in order to determine whether PPARγ activation affects the invasion of pancreatic cancer cells via regulating MMP-2 expression, further investigation was conducted. The results of the transwell experiment demonstrated that RGZ significantly inhibited the invasiveness of pancreatic cancer cells. However, transfection with a vector overexpressing MMP-2 completely suppressed the inhibitory effect of RGZ on pancreatic cancer cell invasion, and the invasiveness of pancreatic cancer cells exhibited an increasing trend (Fig. 4), indicating that PPARγ activation can affect the invasion and metastasis of pancreatic cancer cells via regulating MMP-2 expression.

Discussion

In previous years, the incidence of pancreatic cancer has exhibited a rising trend globally. It has a latent onset and rapid progression, but there is are few effective methods for an early diagnosis and systemic treatment. Due to the late diagnosis and poor treatment level of pancreatic cancer there is an urgent requirement for new and effective molecular targets for the treatment of pancreatic cancer (15).

PPARs belong to the nuclear receptor gene superfamily, containing three subtypes: α, β (or δ) and γ. Among them, PPARγ is the most frequently investigated and is closely associated with tumors. Previous studies have demonstrated that upon activation with specific ligands, PPARγ can inhibit the growth of human tumor cells *in vitro* and *in vivo* (10,11,16). PPARγ and its ligands have thus become one of the main focuses of basic tumor research. Currently, the results of studies on PPARγ in pancreatic cancer have demonstrated that PPARγ is highly expressed in pancreatic tumor tissues (17) and PPARγ activation by ligands inhibits the growth and arrests the cell cycle of human pancreatic cancer cells at the G1 phase through various pathways (5,18-20). Itami *et al* (20) found that overexpression of PPARγ in the pancreatic cancer cell line KMP-3 led to lipid accumulation, while treatment with TGZ increased the level of p27Kip1 and thereby arrested the cell cycle at G1 phase. However, few studies have focused on the association between PPARγ and pancreatic cancer cell invasion and metastasis. A scratch wound assay in the present study demonstrated that following treatment with RGZ, pancreatic cancer cells exhibited decreased movement and migration, which is consistent with current findings in other cell lines. For example, the *in vitro* and *in vivo* experiments of Yu *et al* (21) identified that RGZ inhibited the invasion and metastasis of human hepatocellular carcinoma cells MHCC97-L and BEL-7404. Therefore, similarly, RGZ activation of PPARγ can inhibit the migration of pancreatic cancer cells.

The invasion and metastasis of pancreatic cancer cells is a continuous multi-step process, an important step of which is the detachment of tumor cells from the primary site by degrading the basal membrane (BM) and ECM through secretion of destructive enzymes; the tumor cells then migrate through the ECM, invade the circulation, and form metastatic lesions. The MMPs and TIMPs together regulate the integrity of the BM and ECM (12,22). Several studies have demonstrated that compared with normal pancreatic

tissue, subcutaneously-implanted hamster pancreatic ductal carcinoma cells overexpress MMP-2 and MMP-9, and the serum levels of MMP-2 and MMP-9 also increased significantly. The growth of pancreatic cancer was significantly correlated with serum MMP levels (23). Another study demonstrated that among 32 pancreatic resection specimens, 31 were found with venous invasion. Compared with patients without hepatic metastasis, cases with hepatic metastasis demonstrated a significantly increased invasion of large and medium vessels, which was correlated with the overexpression of MMP-2 and MMP-9 (24). Therefore, MMP-2 and MMP-9 has an important role in the invasion and metastatic process of pancreatic cancer. The present study found that RGZ significantly downregulated the expression of MMP-2 in pancreatic cancer cells, whereas the PPARγ specific inhibitor GW9662 was able to promote MMP-2 expression. However, the effect of RGZ and GW9662 on MMP-9 expression was not significant. These results suggested that in pancreatic cancer cells, PPARγ activation predominantly affects the expression of MMP-2, thereby affecting invasion and metastasis, which was further confirmed by a subsequent transwell test. The results of the transwell experiment demonstrated that RGZ significantly inhibited the invasiveness of pancreatic cancer cells. However, transfection with a vector overexpressing MMP-2 completely suppressed the inhibitory effect of RGZ on pancreatic cancer cell invasion, and the invasiveness of pancreatic cancer cells exhibited an increasing trend, indicating that PPARγ activation can affect the invasion and metastasis of pancreatic cancer cells via regulating MMP-2 expression. Using immunoblotting and RT-PCR methods, Sasaki *et al* (5) demonstrated that the PPARγ ligand 15d-PGJ2 significantly inhibited the infiltration of pancreatic cancer cells, which was associated with a reduced activity and expression of MMP-2 and MMP-9.

In addition, a previous study has also demonstrated that PPARγ activation increases the expression of PTEN, the first tumor suppressor gene identified with phosphatase activity, and can antagonize the activation of protein kinase B by PI3K, inhibit the Akt/PI3K signaling pathway, regulate cell proliferation and the cell cycle and inhibit cell growth, thus having a role in tumor cell metastasis (25). In previous years, with increasing studies on tumor pathogenesis, it has been revealed that PPARγ and PTEN are closely associated with the occurrence and development of pancreatic cancer (20,21). Therefore, whether PPARγ activation regulates the expression of MMP-2 via PTEN was investigated and the data confirmed that PPARγ activation inhibited the expression of MMP-2 through activating PTEN. Galli *et al* (26) found that thiazolidinediones inhibited the invasiveness of pancreatic cancer cells, affecting gelatinolytic and fibrinolytic activity with a mechanism involving MMP-2 and PAI-1 expression.

In conclusion, RGZ activates PPARγ leading to increased PTEN expression and consequently inhibits the expression of MMP-2, thereby inhibiting the invasion and metastasis of pancreatic cancer cells. The results further illustrated the mechanism underlying the inhibitory effect of PPARγ activation on the invasion and metastasis of pancreatic cancer cells, and hence provided sufficient scientific basis to establish the PPARγ signaling pathway as a new target for pancreatic cancer therapy.

Acknowledgements

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