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Original Article

Anti-tumor effect of piceatannol through induction of cell apoptosis via up-regulation of microRNA-125b expression on pancreatic cancer

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Abstract: Accumulating evidence has revealed that a natural compound piceatannol exerts its anti-tumor activity in a variety of tumors cancer. In the current study, we explored the potential anti-pancreatic cancer activity of piceatannol in vitro, and studied the underlying mechanisms. Cells viability was evaluated using cell counting Kit-8. Apoptosis were analyzed by flow cytometry. Bcl-2, Bax and caspase-3 levels were analyzed by Western blot and miR-125b levels were determined by real-time RT-PCR. The effects of miR-125b on pancreatic cancer cells were assessed by silencing and over-expressing the miRNA in vitro. Our results indicated that piceatannol significantly inhibited PC cells growth while inducing considerable cell apoptosisthrough up-regulating the expression of miR-125b, and inhibiting expression of Bcl-2. Moreover, overexpression of miR-125b in pancreatic cancer could promote the cell apoptosis of PANC-1 and SW1990 cells. Downregulation of miR-125b not only alleviates the reduction of piceatannol on PANC-1 and SW1990 cells, but also promoted expression of Bcl-2 that was suppressed by piceatannol. Therefore, our findings would provide a new insight into the use of piceatannol against pancreatic cancer in future.

Keywords: Piceatannol, pancreatic cancer, miR-125b, apoptosis

Introduction

Pancreatic cancer is a highly lethal malignancy and fourth leading cause of cancer-related death in the United States [1]. With the enhanced treatment options available worldwide, pancreatic cancer treatment effect has improved to a certain degree. However, the overall 5-year survival rate of PC still is less than 6% over the last decades [2]. Thus, it is a significant challenge to improve the efficacy of PC treatment and enhance the quality of life of patients. From this perspective, to discover and develop novel and highly effective treatment methods is particularly important.

Piceatannol (PIC; 3, 5, 3', 4'-tetrahydroxytrans-stilbene), is a phenolic compound and an analogue of resveratrol naturally occurring in grapes and red wine [3]. Experimental studies have shown that resveratrol inhibits the growth of various cancer cells and induces apoptotic cell death [4, 5]. Recent studies have demonstrated that PIC induces anticancer activity in several cancer cell lines, such as breast cancer, prostate cancer, bladder cancer and so on [6-8]. Extensive studies have demonstrated that PIC has also been shown to possess anti-inflammatory activity via down-regulation of nuclear transcription factor (NF-κB) [9, 10]. Although accumulating evidence indicated piceatannol's anti-tumor potential, the underlying mechanism of PIC on pancreatic cancer has not been fully elucidated.

MicroRNAs (miRNAs) are a class of non-coding endogenous single-strand RNAs (20-23 nt) that mediate post-transcriptional inhibition of target gene expression by inducing transcript degradation, translational repression, and gene silencing through binding to the 3'-untranslated region (3'UTR) of target mRNA [11, 12]. Researchers have indicated that miRNAs are involved in cell growth, differentiation, and dea-

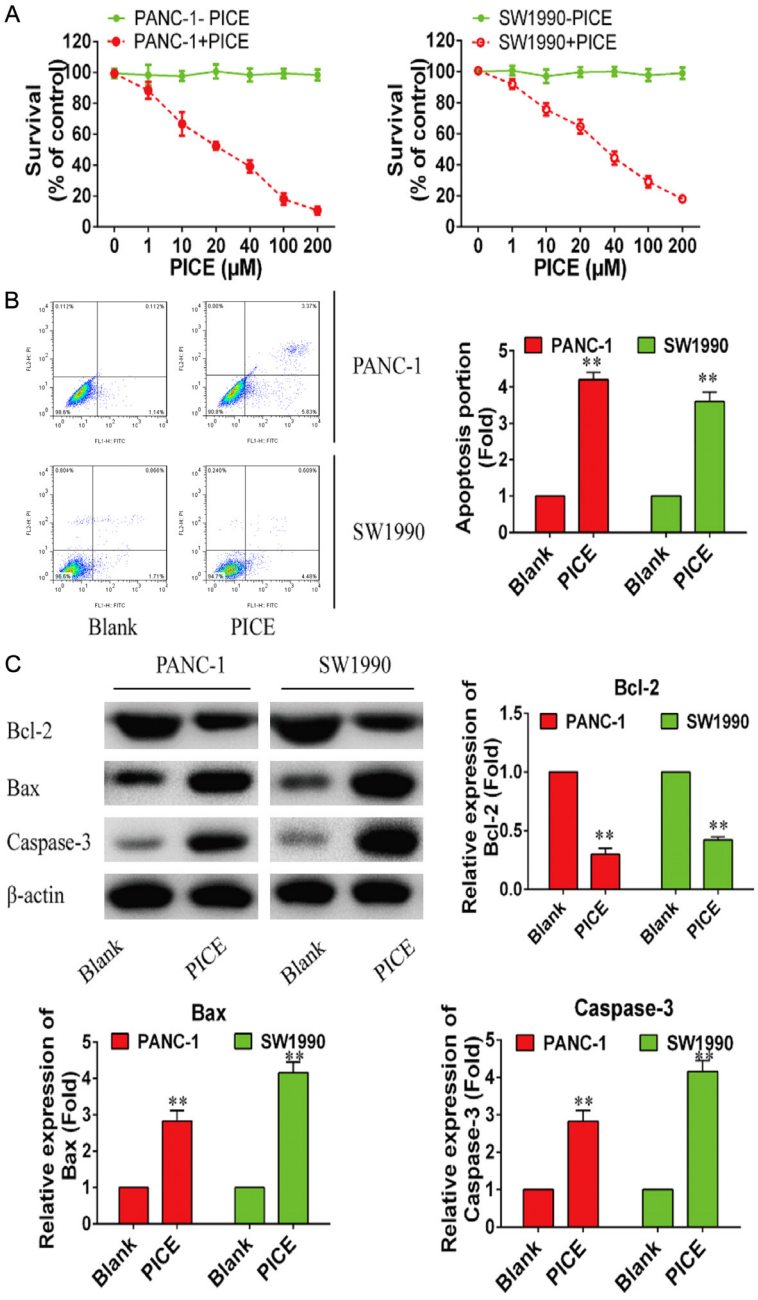

Figure 1. The effect of Piceatannol on PC cell growth and apoptosis. A. Effects of different concentration of piceatannol on cell viability of SW1990 and PANC-1 cells. B. Piceatannol increases apoptosis of SW1990 and PANC-1 cells, as assessed by flow cytometry. C. Piceatannol causes dysregulation of Bcl-2 and Bax protein, and induces activation of caspase-3 in SW1990 and PANC-1 cells, as determined by Western blot. N = 5, **P < 0.01 vs. Blank.

th; they regulate the initiation, development, and progression of human cancers, including tumor growth, apoptosis, invasion, and metastasis [13]. Studies have revealed that miR-125b was critically involved in tumorigenesis and the progression of many types of cancer including PC [14-17]. For example, miR-125b acted as a tumor suppressor in hepatic tumor development by targeting Bcl-2 and inducing cancer cell apoptosis [18]. Furthermore, miR-125b was proposed to function as a metastasis promoter through targeting STARD13 in breast cancer cells [19].

In the current study, we explore whether PIC could regulate miR-125b expression and subsequently control the expression of Bcl-2, one of miR-125b targets, in PC cells. Our results demonstrated that PIC exerts its anti-tumor activity via up-regulation of miR-125b and subsequent down-regulation of Bcl-2 expression in PC cells.

Material and methods

Patient samples

All samples were handled and made anonymous according to the ethical and legal standards. Paired tissue specimens (tumor and adjacent normal tissues) from 20 patients with PC were obtained and histologically confirmed by a pathologist at Rui Jin Hospital affiliated with Shanghai Jiaotong University, China.

Reagents and antibodies

Piceatannol (purity > 99%) was purchased from Sigma-Aldrich, and was dissolved in ethanol to prepare a 100 mM stock solution which was stored at -20°C in the dark. Antibodies against Caspase-3, Bax, Bcl-2 and β-actin were purchased from Cell Signaling technologies (Danvers, MA). Rabbit antibodies conjugated with horseradish peroxidase (HRP) and sheep anti-mouse-HRP were purchased from Zhong San Jin Qiao (Beijing, China). All others chemical reagents

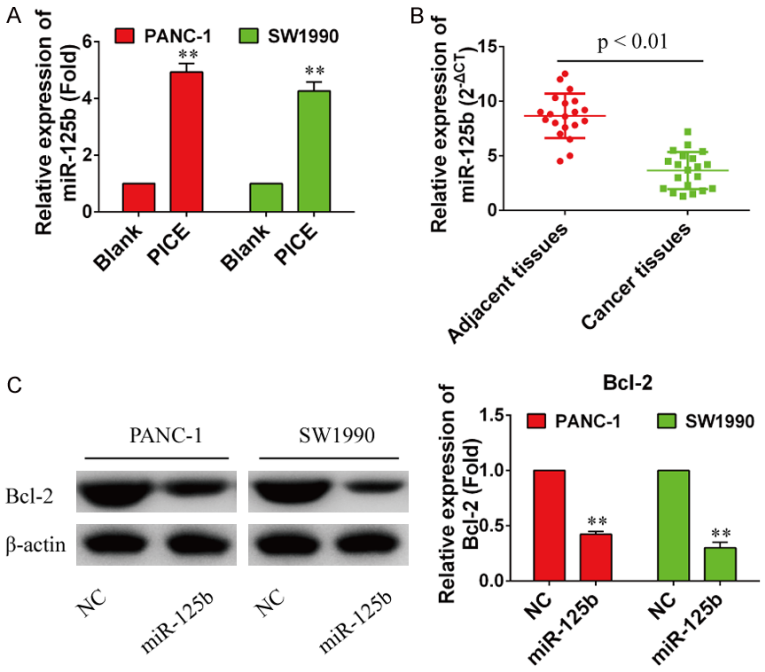

Figure 2. Piceatannol treatment increased miR-125b in PC cells. A. Piceatannol increases miR-125b expression in SW1990 or PANC-1 cells. N = 5, **P < 0.01 vs. Blank. B. miR-125b is down-regulated in pancreatic cancer samples compared with adjacent samples, as measured by real-time PCR. N = 20, **P < 0.01 vs. adjacent samples. C. Effects of miR-125b on the expression of Bcl-2 protein in SW1990 or PANC-1 cells. n = 5, **P < 0.01 vs. NC. NC: negative control.

were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China).

Cell culture and treatments

Human pancreatic cancer cell lines SW1990 and PANC-1 were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China), and cultured in Dulbecco's modified eagle's medium (DMEM) (Hyclone, Logan, UT, USA) containing 10% fetal bovine serum (FBS) (Biowest, Nuaillé, France), 100 U/mL penicil-lin, and 100 mg/mL streptomycin (Hyclone). All cell lines were maintained in a humidified atmosphere of 5% CO_2/air at 37°C. The cells treated with the indicated concentrations of PIC were collected at 24 h and 48 h for further measurements.

MiRNA transfection

The DNA fragment encoding miR-125b mimics and miR-125b inhibitor were purchased from GenePharma (Shanghai, China). Cells were transfected with miR-125b inhibitor, miR-125b mimic or the nonspecific control using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction.

Cell proliferation assay

Cells were seeded in 96-well plates at 0.8-1×10^3 per well. Cell proliferation was evaluated using Cell Counting Kit-8 (Beyotime, China) according to the manufacturer's instructions. Briefly, 10 μl of CCK-8 solution was added to culture medium, and incubated for 2 h. The absorbance at 450 nm wavelength was determined with a reference wavelength of 570 nm.

Quantitative real-time RT-PCR analysis

Total mRNA was extracted from pancreatic cancer tissues and cultured cell lines using RNeasy Mini Kit (Qiagen, Valencia, CA, USA). The expression of mature miR-125b was quantified by real-time PCR using the miScript SYBR Green PCR Kit which contained 10× miScript Universal Primer (Qiagen) and was performed according to the manufacturer's protocol. Quantization of U6 was used to normalize miRNA expression level. Real-time PCR was carried out in the BioRad IQ5 amplification system (Bio-Rad, USA) and the results were calculated using ΔCT method. Primer sequences were: U6 forward, 5'-gcttcggcagcacatatactaaaa-3', U6 reverse, 5'-cgcttcacgaatttgcgtgtcat-3'; miR-125b RT primer 5'-gtcgtatccagtgcagggtccgaggtattcgcactggatacgactcacaa-3', MiR-125b ST forward primer: 5'-gccctccctgagacctcaa-3'; ST reverse primer: 5'-gtgcagggtccgaggt-3'.

Cell apoptosis assay

Cell apoptosis was analyzed using an Annexin V-FITC/PI apoptosis detection kit (Beyotime Institute of Biotechnology China). The SW1990 and PANC-1 cells were seeded in 6-well plates overnight and then treated with PIC at indicated concentration for 48 h. The following steps were conducted as the manufacturer's

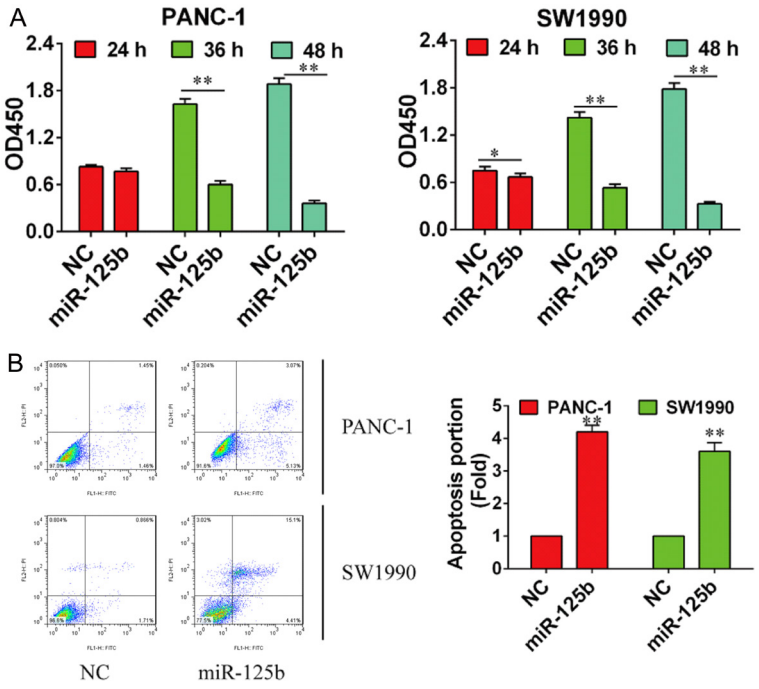

Figure 3. Overexpression of miR-125b induces apoptosis in PC cells. A. Forced expression of miR-125b inhibits cell viability in SW1990 or PANC-1 cells. B. Overexpression of miR-125b increases apoptosis of SW1990 and PANC-1 cells, as assessed by flow cytometry. N = 5, **P < 0.01 vs. NC.

instruction. The cells were subjected to Annexin V-FITC/PI staining and analyzed using flow cytometer [20].

Western blot analysis

Protein extracts from SW1990 and PANC-1 cells were subjected to 10% SDS-PAGE and subsequently transferred to a PVDF membrane. This was followed by probing with mouse primary antibodies against Caspase-3, Bax and Bcl-2. Anti-β-actin antibody was used as an internal control. The immunoreactivity was detected using Odyssey Infrared Imaging System and analyzed using Odyssey software (Infrared Imaging System LI-COR Biosciences) [21].

Statistical analysis

Statistical analyses were performed with SPSS 13.0 software. The results were evaluated by χ^2 test and the other data were evaluated by Student's t-test and expressed as the mean ± SD from three independent experiments. A *P*-value of less than 0.05 was considered statistically significant.

Results

Piceatannol inhibited cell proliferation in PC cells

To investigate the effect of piceatannol on the growth of PC cells, first, we conducted CCK-8assaytomeasurecellsurvival of SW1990 and PANC-1 cells after piceatannol treatment for 72 h. Our results showed piceatannol treatment caused cell proliferation inhibition in a dose-dependent manner in both SW1990 and PANC-1 cells (**Figure 1A**). The IC_{50} value of piceatannol after 72 h of incubation was 30.69 μM and 21.82 μM for SW1990 and PANC-1 cells, respectively. In the following studies, we chose piceatannol with the concentration of 30 μM for further investigation.

Piceatannol induced cell apoptosis in PC cells

To determine whether apoptosis contributes to cell growth inhibition, flow cytometry assay was applied to detect apoptotic rate of SW1990 and PANC-1 cells treated with piceatannol. As shown in **Figure 1B**, the percentage of apoptosis cells was increased by piceatannol treatment in both SW1990 and PANC-1 cells compared with the Blank group. Furthermore, we also examined the expression of certain genes involved in apoptosis regulation. As shown in **Figure 1C**, piceatannol decreased the expression of the anti-apoptotic protein Bcl-2, increased the expression of the pro-apoptotic proteins caspase-3 and Bax. These results suggest that the piceatannol could induce apoptosis in PC cells by caspase-dependent process.

Piceatannol promotes expression of miR-125b

Studies have revealed that miR-125b is critically involved in tumorigenesis and the progression of many types of cancer including PC. To investigate whether piceatannol treatment could regulate the expression of miR-125b in PC cells, we performed miRNA RT-PCR assay to

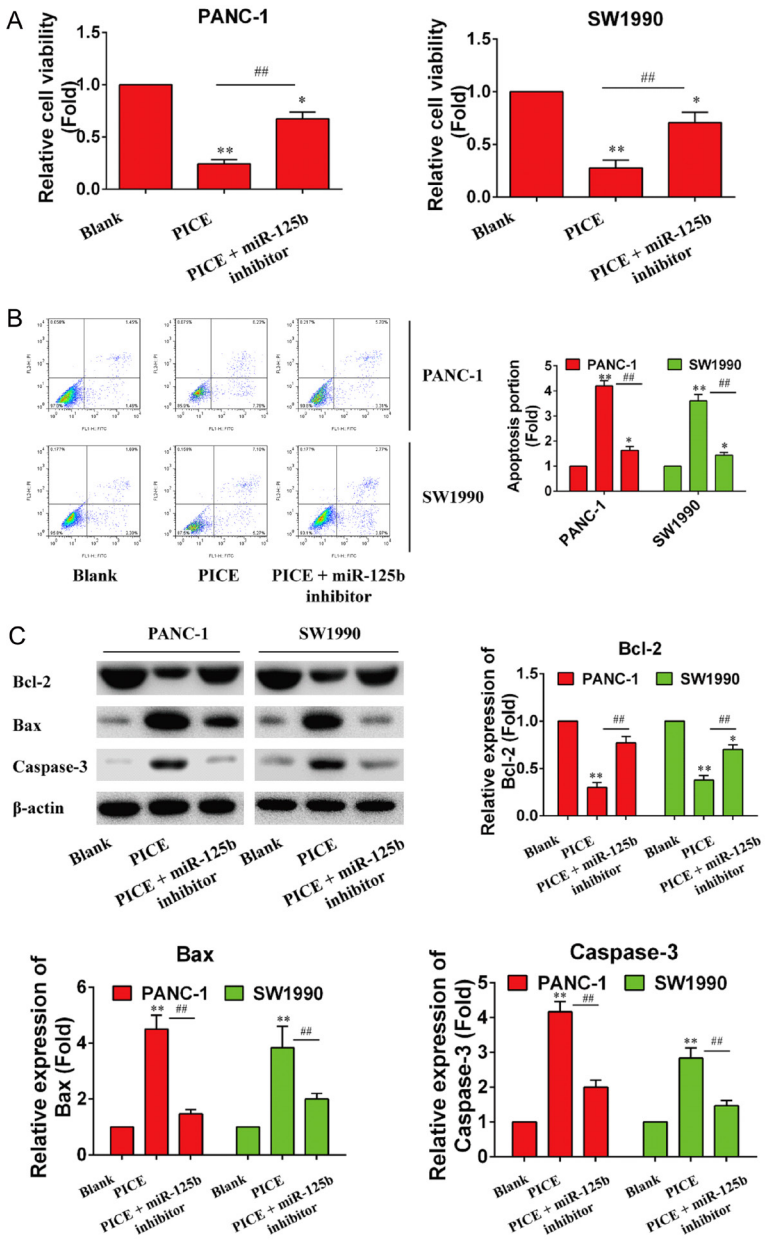

Figure 4. Knock down of miR-125b alleviates the pro-apoptotic effects of piceatannol in PC cells. A. Knock down of miR-125b mitigates the reduction of cell viability induced by piceatannol in SW1990 and PANC-1 cell. B. Knock down of miR-125b alleviates the raise of cell apoptosis induced by piceatannol in SW1990 and PANC-1 cell. C. Inhibition of miR-125b reverses dysregulation of Bcl-2, Bax and caspase-3 protein caused by piceatannol in SW1990 and PANC-1 cells. n = 5, *P < 0.05, **P < 0.01 vs. Control; ##P < 0.01 vs. Pice.

detect the level of miR-125b after treating with piceatannol in SW1990 and PANC-1 cells for 48 h. As shown in **Figure 2A**, qRT-PCR analysis indicated that piceatannol treatment caused a significant elevation of miR-125b expression in both SW1990 and PANC-1 cells, as compared to untreated cells (P < 0.05). Further experiments confirmed that miR-125b was decreased in the patients of PC (**Figure 2B**). And, forced expression of miR-125b by transfection markedly reduced the level of Bcl-2 protein, a known target for miR-125b (**Figure 2C**).

Enforced expression of miR-125b triggered apoptosis in PC cells

To examine the effect of miR-125b on apoptosis, we transfected miR-125b mimics into PC cells. Indeed, we observed that miR-125b mimics decreased the cell viability and increased percentage of apoptotic cells in SW1990 and PANC-1 cells (**Figure 3A**, **3B**). These data indicating that miR-125b could induce apoptosis in PC cells.

Knockdown of miR-125b alleviates apoptosis induced by piceatannol

To examine whether the upregulation of miR-125b contributes to the piceatannol-induced apoptosis in PC cells, we knocked down the expression of miR-125b by transfection of its specific inhibitor, miR-125b inhibitor, and then observed the alteration of piceatannol-induced apoptosis in PC cells. As shown in **Figure 4A**, the combination of miR-125b inhibitor transfection and piceatannol treatment restored the reduction of cell viability induced by piceatannol alone. And, the combination of miR-125b inhibitor transfection and piceatannol treatment alleviated the raise of cell apoptosis induced by piceatannol alone (**Figure 4B**). Moreover, the combination of miR-125b inhibitor transfection and piceatannol treatment

reversed the dysregulation of Bcl-2 and Bax caused by piceatannolalone in SW1990 and PANC-1 cells (**Figure 4C**). These results suggested that piceatannol induced cell apoptosis partly due to up-regulation of miR-125b in PC cells.

Discussion

In this present study, we demonstrated that piceatannol could inhibit the growth of PC cell in a dose-dependent manner. Furthermore, we provide the evidence showing that piceatannol induced cell apoptosis through up-regulation of miR-125b in PC cells. Our findings suggest that piceatannol might be of benefit in the treatment of pancreatic cancer.

Recent studies have demonstrated that piceatannol, a natural analog of the anti-cancer stilbene resveratrol, exerts its inhibitory effects on the cancer development and progression [4]. For example, it has been reported that piceatannol inhibits growth and induces apoptosis in the human prostate cancer lines DU145 and NRP-154, which indicate piceatannol has potential as an anti-prostate cancer agent [22, 23]. Moreover, piceatannol was found to block cell cycle progression in the G0/G1 phase and induces apoptosis in T24 and HT1376 human bladder cancer cells [7]. In addition, piceatannol has been shown to inhibit the metastasis in leukemia cells [24, 25]. However, little attention has been paid to the effects of piceatannol in PC. Our data reinforce this anti-tumor action of piceatannol by showing that the ability of piceatannol to inhibit PC cells growth by at least partially inducing a caspase-3-dependent apoptosis, suggesting that piceatannol could be used for the treatment of pancreatic cancer.

Several reports have demonstrated that kinds of miRNAs have been confirmed to participate in the initiation and progression of pancreatic cancer [26, 27]. For example, Liu et al identified that miR-34b was downregulated in pancreatic cancer and its restoration significantly inhibited pancreatic cancer progression in vitro and in vivo [28]. Similarly, Another study has shown that miR-27a, an oncogenic miRNA, is significantly overexpressed in pancreatic cancer and its inhibition diminishes growth and malignant behavior of pancreatic cancer cells by inducing tumor suppressor Spry2 [29]. Interestingly, it has been reported that miRNAs could be regulated by natural chemopreventive agents (natural agents), leading to the inhibition of cancer cell growth, EMT, drug resistance, and metastasis [30-34]. Therefore, modulation of miRNA expression may be an important mechanism underlying the biological effects of piceatannol.

Recent studies have demonstrated the crucial role of miR-125b in tumorigenesis and metastasis [35-38]. Aihua Zhao et al found that miR-125b acted as a tumor suppressor in hepatic tumor development by targeting Bcl-2 and inducing cancer cell apoptosis [18]. It was also reported that miR-125b suppressed liver cancer proliferation and metastasis by targeting the oncogene LIN28B [39]. These findings argued that up-regulation of miR-125b could represent a new treatment approach for human cancer. To further support the role of miR-125b, we observed that up-regulation of miR-125b inhibited cell proliferation, caused down-regulation of Bcl-2, up-regulation of Bax and activation of caspase-3, and finally induced the apoptosis in PC cells. More importantly, we found that piceatannol significantly increased the expression of miR-125b, suggesting that piceatannol could be a potential agent to treat PC through modulating miR-125b expression.

In conclusion, our findings demonstrated that piceatannol suppressed cell proliferation, induced cell apoptosis partly through up-regulation of miR-125b. Because insensitivity to growth inhibitory signals is a hallmark of cancer, piceatannol has potential as an anti-pancreatic cancer agent. Future studies will be warranted to evaluate the effects of piceatannol on the development of PC in animal cancer models.

Disclosure of conflict of interest

None.

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