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Emodin inhibits pancreatic cancer EMT and invasion by up-regulating microRNA-1271

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Abstract. Emodin has a direct inhibitory effect on the growth and metastasis of a variety of malignant tumor cells. MicroRNA-1271 (miR-1271) has an extensive tumor-suppression effect by inhibiting epithelial mesenchymal transition (EMT) in tumor cells and induces tumor cell apoptosis. Proceeding with the EMT regulatory mechanism of pancreatic carcinoma, the present study aimed to examine the inhibitory effect of miR-1271 and emodin against invasion and metastasis of pancreatic carcinoma. The expression of EMT-related markers (E-cadherin, ZEB1 and TWIST1) was analyzed by western blotting. mRNA levels of miR-1271, E-cadherin, ZEB1 and TWIST1 in pancreatic tumor cells (SW1990) were measured through reverse transcription-quantitative polymerase chain reaction and cell invasiveness was detected using Transwell assays. In addition, a liver metastatic model was established with an implantation of pancreatic tumor tissue into the spleens of nude mice to study the effect of emodin on pancreatic cancer liver metastasis. In the present study, it was demonstrated that miR-1271 significantly decreased in pancreatic cancer cells and tissues. Twist1 may be a target gene of miR-1271. Emodin could inhibit the proliferation ability of pancreatic cancer cells and increased miR-1271 expression level. Further, we found that miR-1271 significantly inhibited SW1990 cell EMT and invasive ability. We also provided the evidence that emodin inhibited SW1990 cell EMT by raising the level of miR-1271. Moreover, the *in vivo* experiments have verified the inhibiting effect of emodin against liver metastasis of pancreatic cancer. The data in the present study indicated that emodin inhibited pancreatic cancer EMT and invasion by increasing the content of miR-1271.

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Introduction

Pancreatic cancer is a common form of cancer of the highly malignant digestive system. The incidence of pancreatic cancer is high in western developed countries. According to statistics in 2012 in Europe and America, 137,286 cases of new pancreatic cancer were diagnosed, and pancreatic cancer mortality ranked fourth in all malignant tumors (1-3). Like most other countries, the health burden of pancreatic cancer in our country is also on the rise, with an annual mortality rate almost equal to the incidence (4). Because the clinical symptoms of pancreatic cancer are relatively vague and often diagnosed late, most patients with pancreatic cancer have obvious regional infiltration or distant metastasis at the time of diagnosis (5-7). Despite the joint efforts of clinicians and scientists for decades, pancreatic cancer remains a devastating disease with adverse consequences (8). Therefore, if we can find a way to inhibit the invasion and metastasis of pancreatic cancer, it has important clinical significance for the overall survival rate of pancreatic cancer patients and inhibition of the recurrence of pancreatic cancer.

In recent years, many studies found that microRNAs (miRNAs) play an important regulatory role in tumorigenesis and progression. A large number of oncogenes and tumor suppressor genes are regulated by miRNAs (9). miRNA is a single, non-coding 19-25 nucleotide RNA that regulates about 30% of the protein-coding mRNA in the human genome (10). miRNA can inhibit the translation of the target mRNA or degrade the target mRNA by binding with the 3' non-coding region (3'-UTR) of the target mRNA to exert post-transcriptional regulation (11). There are a wide range of abnormal miRNA expression in tumor tissues compared to normal tissues, some of which have oncogenic activity while others have tumor suppressor activity (12). In malignant cells, the majority of tumor suppressor miRNAs are down-regulated, while oncogenic miRNAs are up-regulated and involved in tumor pathology through multiple mechanisms (13,14). miRNA-661 was found to be over-expressed in the Snail-1-induced breast cancer EMT and its targets inhibits the intercellular adhesion protein Nectin-1 and the lipid transfer enzyme StarD 10 and leads to the down-regulation of epithelial markers (15). Another study found that miRNA-219-5p is an important part of the Twist-EMT regulatory pathway (16).

Among the miRNA families, miR-1271 is a newly discovered member (17). Recent studies show that the impact of

miR-1271 on the tumor is getting more and more attention. Many studies have found that miR-1271 has a wide range of tumor suppressor effects (18-20). MiR-1271 is down-regulated in many human tumors and is involved in the inhibition of tumor epithelial-mesenchymal transition (EMT), induction of apoptosis, and reversal of chemo-resistance (18-22). EMT refers to the cell under the physiological or pathological conditions into mesenchymal features similar to mesenchymal-like phenomenon. Cells lose their own cell polarity and cell-cell connections are the most important mechanism of induce tumor invasion and metastasis. The EMT of the tumor cells processed by a number of transcription factors, including ZEB1and Twist1, and these transcription factors can inhibit the expression of E-cadherin (23). Twist is an inhibitor of apoptosis protein found in recent ten years. It is an important regulatory transcription factor in the process of embryonic development. Twist is involved in the growth and development of normal organs, and it is also an important regulator of EMT in malignant tumor cells (24). E-cadherin, a member of the family of cadherin proteins in cell adhesion molecules, is an important intercellular adhesion molecule and epithelial phenotype that has been recognized as a suppressor of cancer metastasis. Tumors with reduced expression of E-cadherin are more likely to metastasize (25).

Based on the regulation mechanism of miRNA on EMT in pancreatic cancer, the present study aimed to investigate the inhibitory effect of miR-1271 and emodin against invasion and metastasis of pancreatic carcinoma, and further explored the relationship between miR-1271 and emodin. We provide theoretical and experimental evidence for the further development of miRNA-based targeted therapy of pancreatic cancer.

Materials and methods

Clinical specimens. A total of 58 paired pancreatic cancer and adjacent normal tissues were identified and collected during biopsies from 58 patients with pancreatic cancer who were diagnosed by clinical symptoms and imaging examination at the General Hospital of Tianjin Medical University (Tianjin, China). All tissue samples were immediately flash-frozen in liquid nitrogen and stored at -80˚C. The present study was approved by the Human Ethics Committee Review Board at the General Hospital of Tianjin Medical University (Tianjin, China). Informed consent was provided by every patient.

Cell culture and treatment. Human pancreatic cancer cell lines SW1990, BXPC3, PANC-1 and ASPC-1 as well as the normal human pancreatic ductal epithelial cell line HPDE6c7 were originally acquired from American Type Culture Collection ATCC (Manassas, VA, USA) and cultured in our institute. Cells were incubated in RPMI 1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum, and 1% penicillin-streptomycin solution (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at 37˚C with 5% CO_2. Cells were passaged every 2-3 days.

SW1990 cells ($3x10^4$ cells/well) were treated with various concentrations (0, 20, 40 μmol/l) of emodin for 24, 48 h at 37˚C, 5% CO_2.

Cell transfection. For cell transfection, SW1990 cells were seeded in 6-well plates (4x105 per well). Then, miRNA-1271 mimics, miRNA-1271 inhibitor or the negative control was transfected into SW1990 cells using Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) following with the manufacturer's protocol. 48 h after transfection, the cells were subjected to following experimental analysis. Transfection efficiency was detected using qRT-PCR.

Western blot analysis. After specific treatment, total cellular proteins from SW1990 cells were extracted by using RIPA Buffer (Auragene, Changsha, China). BCA protein quantitative kit (Thermo Fisher Scientific, Inc.) was carried out to measure the concentration of protein samples. Equal amount of protein samples were resolved by 12% SDS-PAGE and then transferred onto PVDF membranes. The membranes were blocked with 5% non-fat milk at room temperature for 1 h, followed by incubated with primary antibodies (anti-E-cadherin, cat no. 3195; anti-ZEB1, cat no. 3396; anti-TWIST1, cat no. 46702; dilution for all, 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA) at 4˚C overnight. Subsequently, membranes were incubated with a HRP-conjugated secondary antibody (anti-rabbit IgG, HRP-linked antibody, cat no. 7074; 1:5,000) at room temperature for 2 h. To visualize the protein blots, an ECL kit (Applygen Technologies, Inc., Beijing, China) was used to visualize the protein blots according the manufacturer's protocol.

MTT assay. The cells were evenly inoculated into 96-well plates (Corning Incorporated, Corning, NY, USA) with $2.0x10^3$ cells per well. 20 μl MTT solution was added in per well, incubated at 37˚C for 4 h. After removing the supernatant, 200 ul dimethyl sulfoxide (DMSO; Sigma-Aldrich; Merck KGaA) was added in per well. A microplate reader was used to determine the cell proliferation ability by detecting the OD value at 490 nm.

Reverse transcription-quantitative PCR (RT-qPCR). TRIZOL reagent (Takara, Japan) was used to extract the total RNA from the SW1990 cells. β-actin or U6 were acted as an internal control. cDNAs were generated by using the PrimeScript™ RT reagent kit (Takara, Japan) in line with the manufacturer's instructions. SYBR Premix Ex Taq (Takara, Japan) was carried out to analyze the synthesized cDNAs according to the manufacturer's instructions. Primer sequences used for real time PCR were listed as following: miRNA-1271 Forward: 5'-CAGCAC TTGGCACCTAGCA-3'. miRNA-1271 Reverse: 5'-TATGGT TGTTCTCCTCTCTGTCTC-3'. E-cadherin Forward: 5'-AAG TGCTGCAGCCAAAGACAGA-3'. E-cadherin Reverse: 5'-AGGTAGACCCACCTCAATCATCCTC-3'. ZEB1 Forward: 5'-GCACAACCAAGTGCAGAAGA-3'. ZEB1 Reverse: 5'-CATTTGCAGATTGAGGCTG-3'. Twist1 Forward: 5'-AGC TGAGCAAGATTCAGACCCTCA-3'. Twist1 Reverse: 5'-CTG CAGCTTGCCATCTTGGAGT-3'. U6 Forward: 5'-CTCGCT TCGGCAGCACA-3'. U6 Reverse: 5'-AACGCTTCACGAATT TGCGT-3'. β-actin Forward: 5'-CAGGGCGTGATGGTGGGC A-3'. β-actin Reverse: 5'-CAAACATCATCTGGGTCATCT TCTC-3'. Relative gene quantification was assessed using the $2^{-\Delta\Delta Cq}$ method (26).

Cell invasion assay. *In vitro* invasion assay was performed using transwell plates (BD Biosciences, Franklin Lakes,

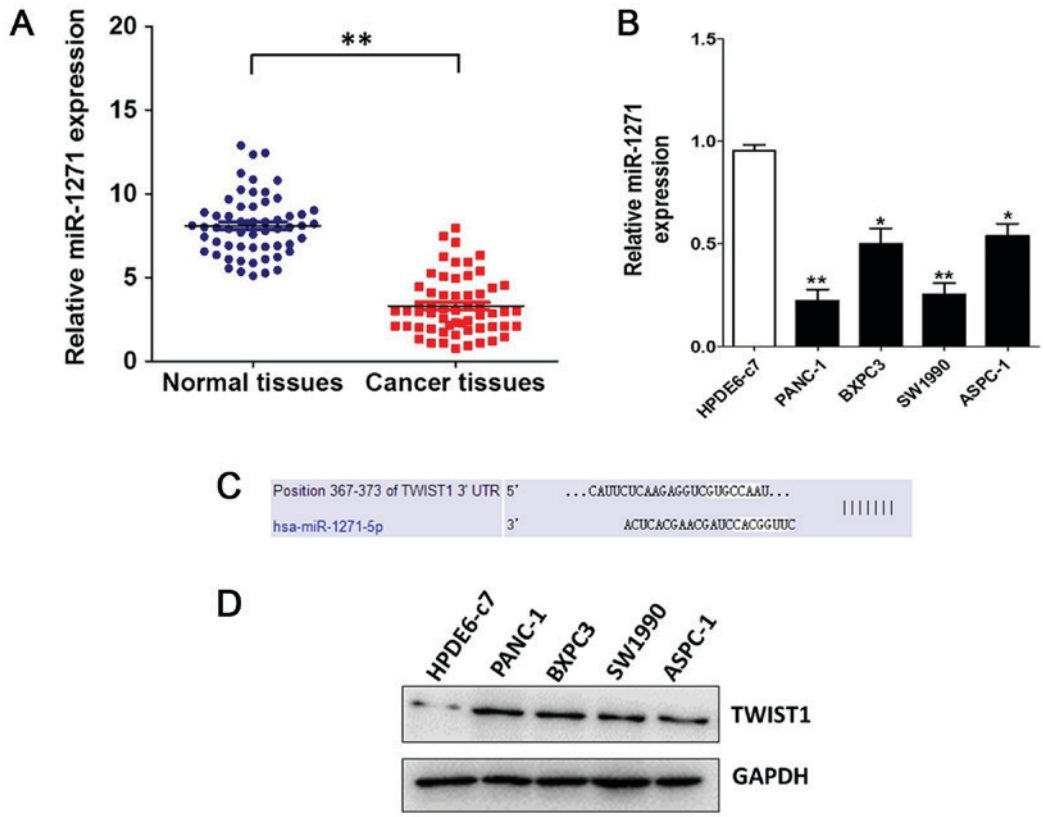

Figure 1. miRNA-1271 was down-regulated in pancreatic cancer. (A) The level of miR-1271 in pancreatic cancer tissues was detected by qRT-PCR $^{**}P<0.01$ vs. normal tissue. (B) The level of miR-1271 in pancreatic cancer cell lines was detected by qRT-PCR, $^{*}P<0.05$ and $^{**}P<0.01$ vs. HPDE6-c7. (C) Target Scan was used to predict the potential targets of miR-1271. (D) Protein level of TWIST1 in pancreatic cancer cell lines was measured by western blot.

NJ, USA) with 8 μm pores. The cells ($1x10^4$ cells) in contains (0, 20, 40 μmol/l) emodin-DMEM medium were added to the upper chamber pre-coated with Matrigel (BD Biosciences) of the Transwell plates. Then emodin-DMEM medium containing 20% FBS as a chemo-attractant was added to the lower chamber. After 48 h incubation, cells were removed using cotton wool which on the upper surface and the cells were fixed with methanol and stained with 0.5% crystal violet. Images were captured and the cells were counted using a photomicroscope (Olympus Corporation, Tokyo, Japan).

Animal models of pancreatic cancer cell metastasis. SW1990 cells were injected into the spleens of 45 nude mice to establish an animal model of pancreatic liver metastasis. Mice were divided into 3 groups: High dose emodin group (gavage administration; emodin, 50 mg/kg body weight/day; day 8 to day 35 after model establishment); low dose emodin group (gavage administration; emodin, 20 mg/kg body weight/day; day 8 to day 35 after model establishment), and the control group (gavage administration; 2 ml normal saline), each group of 15 mice. Six weeks later, the nude mice were sacrificed and the liver metastasis of pancreatic cancer in nude mice was observed. The number of tumor nodules, the proliferation inhibition rate and the liver metastasis inhibition rate were calculated in each group. The animal experiments performed in the present study were approved by the Animal Ethics Committee Review Board at Tianjin Medical University (Tianjin, China).

Immunohistochemistry. The paraffin-embedded tissue blocks were cut into 4 μm sections using a microtome. The sections were incubated for 1 h in 10% normal goat serum/PBS solution, then incubated overnight with the primary antibodies in 0.1% BSA/PBS solution in humid chambers at 4˚C. Primary antibodies used were TWIST1 and ZEB-1. Secondary antibodies were applied followed by Vectastain ABC complex according to manufacturer protocol. Immunostaining was visualized by 1xDAB/H_2O_2 solution, subsequently counter-stained with hematoxylin, and mounted with Permount (Sigma-Aldrich; Merck KGaA). Immunostaining without primary antibody or with the primary antiserum preabsorbed with its respective antigen was carried out as negative control. Alizarin Red S and Masson's Trichrome staining protocols were used for calcium and collagen detection, respectively.

Statistical Analysis. Results were expressed as mean values ± standard error (mean ± S.E.). Data were analyzed by one-way analysis of variance followed by a post hoc Tukey's test or a Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

MiR-1271 is down-regulated in pancreatic cancer. The level of miR-1271 in pancreatic cancer tissues and cells were detected by qRT-PCR. As shown in Fig. 1, the level of miR-1271 significantly down-regulated in both pancreatic cancer tissues and cell lines. According to the results of TargetScan, we found

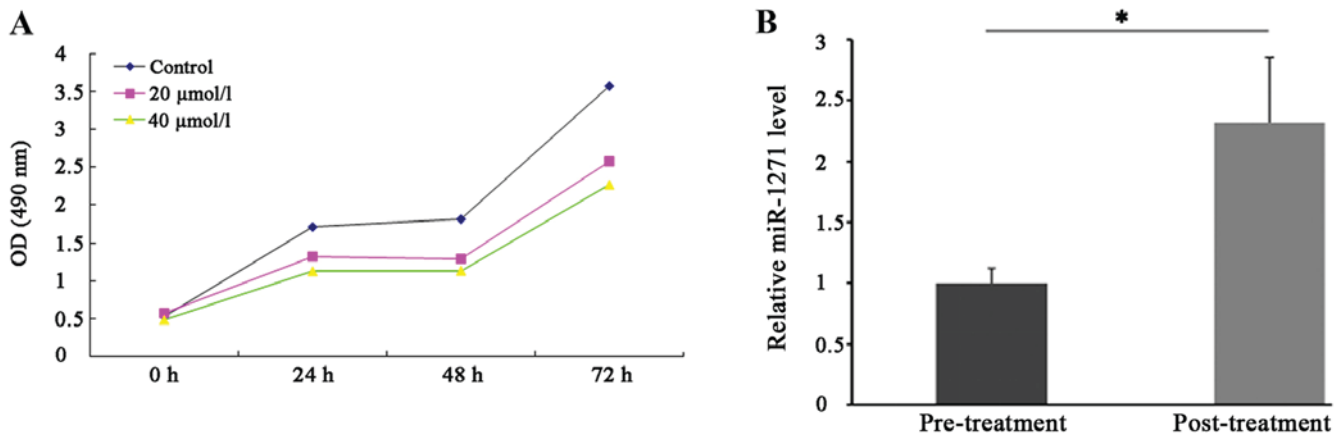

Figure 2. Emodin inhibited SW1990 cell proliferation ability. (A) After treatment, cell proliferation ability was detected using MTT assay. (B) The level of miR-1271 in SW1990 cells was detected using qRT-PCR before and after Emodin treatment. *P<0.05.

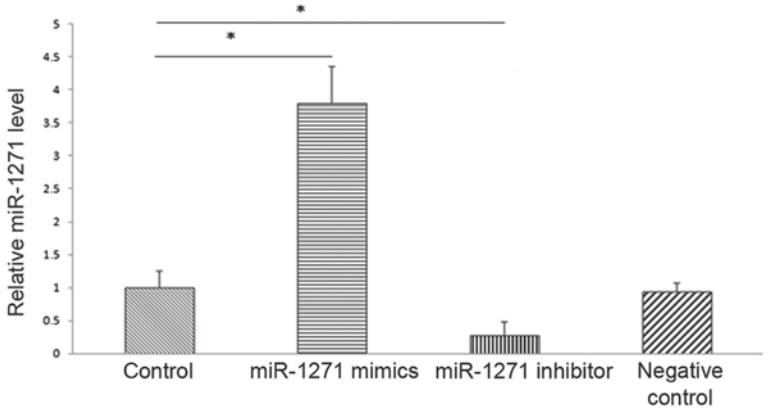

Figure 3. miRNA-1271 expression in different groups in SW1990 cells. 48 h after cell transfection, the expression of miRNA-1271 in pancreatic cancer cells were detected by qRT-PCR. *P<0.05.

that Twist1 may be a target gene of miR-1271. Compared with the human pancreatic ductal epithelial cell line HPDE6c7, Twist1 was up-regulated in human pancreatic cancer cell lines SW1990, BXPC3, PANC-1 and ASPC-1.

Emodin inhibits SW1990 cell proliferation ability. As shown in Fig. 2A, we found that emodin significantly inhibited SW1990 cell proliferation ability in a dose- and time-dependent manner. In addition, our findings suggested that emodin treatment markedly enhanced the expression level of miR-1271 in SW1990 cells (Fig. 2B).

Effect of miRNA-1271 on EMT in SW1990 cells. SW1990 cells were transiently transfected with miR-1271 mimics, miR-1271 inhibitor and empty plasmid as control. 48 h after transfection, transfection efficiency was detected using qRT-PCR, and the results indicated that compared with the control group, miRNA-1271 mimics significantly enhanced miR-1271 expression, while miR-1271 inhibitor significantly inhibited miR-1271 expression (Fig. 3). qRT-PCR also showed that compared with other experimental groups, miR-1271 and E-cadherin mRNA expression levels were increased in transfected miR-1271 mimics group, and mRNA levels of ZEB1 and TWIST1 in each experimental group had no significant difference (Fig. 4A). Western blot assay showed that the expression of E-cadherin protein was increased while the expression of ZEB1 and TWIST1 protein was decreased in the miR-1271 mimics group. In the miR-1271 inhibitor group, the opposite result was found, the E-cadherin protein level was the lowest, while the protein expression levels of ZEB1 and TWIST1 were significantly higher than the other groups (Fig. 4B). Transwell chamber model experiments showed that the invasion ability of SW1990 cells in miR-1271 mimic group was significantly lower than the other groups (Fig. 4C). The results indicated that miR-1271 can significantly inhibit pancreatic cancer EMT process. MiR-1271 may act on the post-transcriptional phase of ZEB1 and TWIST1 gene, but not the mRNA expression level.

Effect of emodin on the level of miR-1271 and EMT marker in SW1990 cells. SW1990 cells were treated with various concentrations (0, 20, 40 μmol/l) of emodin for 48 h. Then we found that the mRNA expression levels of miRNA-1271 and E-cadherin in SW1990 cells were increased at 20 and 40 μmol of emodin treatment, while the mRNA levels of ZEB1 and TWIST1 were not significantly different (Fig. 5A). When emodin was added, the expression of E-cadherin protein increased, while the expression of ZEB1 and TWIST1 protein

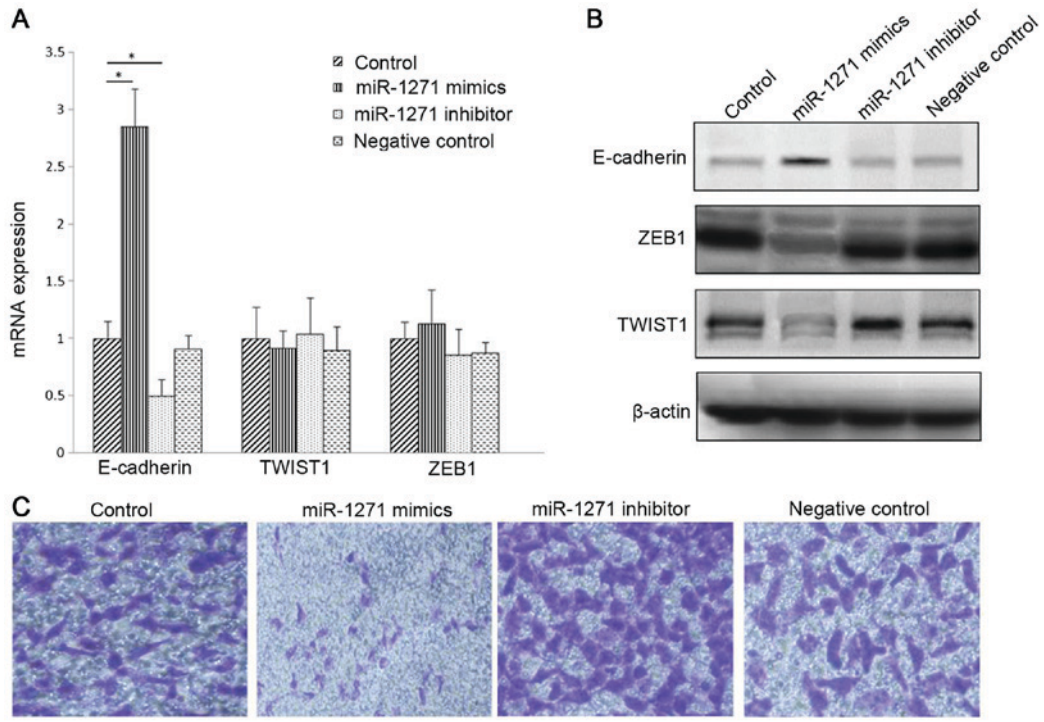

Figure 4. Effect of miRNA-1271 on EMT in SW1990 cells. (A) The expression of E-cadherin, ZEB1 and TWIST1 in pancreatic cancer cells were detected by qRT-PCR. (B) The protein content of EMT-related markers E-cadherin, ZEB1 and TWIST1 in pancreatic cancer cells was detected by western-blot assay. (C) Transwell chamber model was used to detect the change of invasion ability of pancreatic cancer cells in each experimental group. Magnification, x100. *P<0.05.

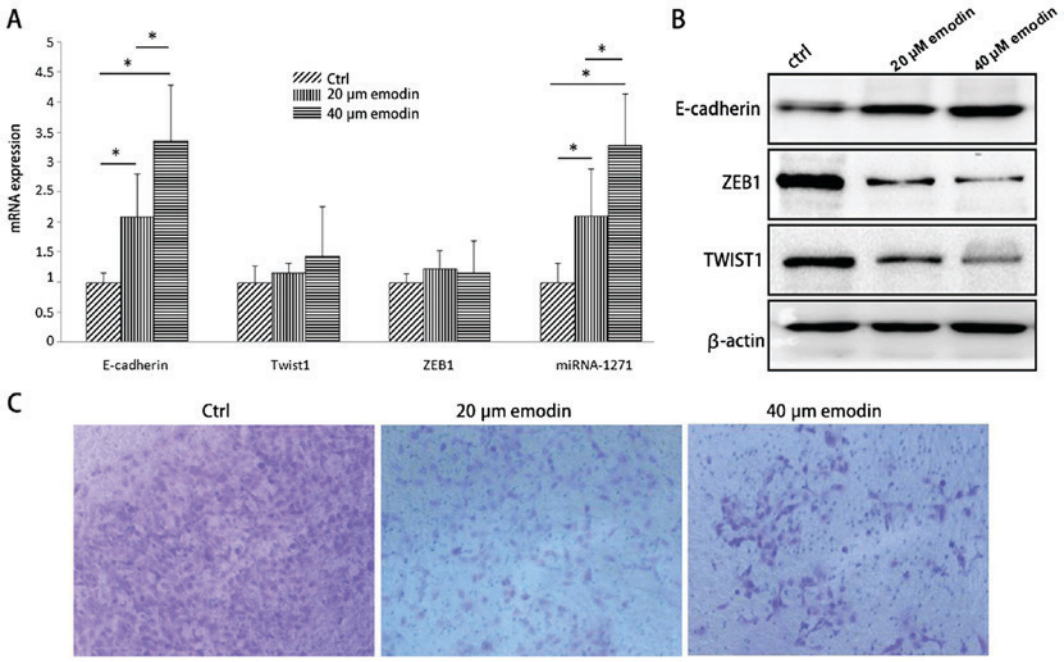

Figure 5. Effect of Emodin on the level of miR-1271 and EMT marker in SW1990 cells. (A) The expression of miRNA-1271, E-cadherin, ZEB1 and TWIST1 in pancreatic cancer cells were detected by qRT-PCR. (B) The protein content of EMT-related markers E-cadherin, ZEB1 and TWIST1 in pancreatic cancer cells was detected by western-blot assay. (C) Transwell chamber model was used to detect the change of invasion ability of pancreatic cancer cells in each experimental group. Magnification, x100. *P<0.05.

decreased (Fig. 5B). Transwell chamber model experiments showed that after emodin treatment, cell invasion of SW1990 cells significantly decreased compared with the control group, while the EMT inhibition effect is more significant in 40 μmol/l emodin treatment group (Fig. 5C). The experimental results indicated that emodin can inhibit the EMT of pancreatic cancer cells by increasing the level of miR-1271 in pancreatic cancer cells.

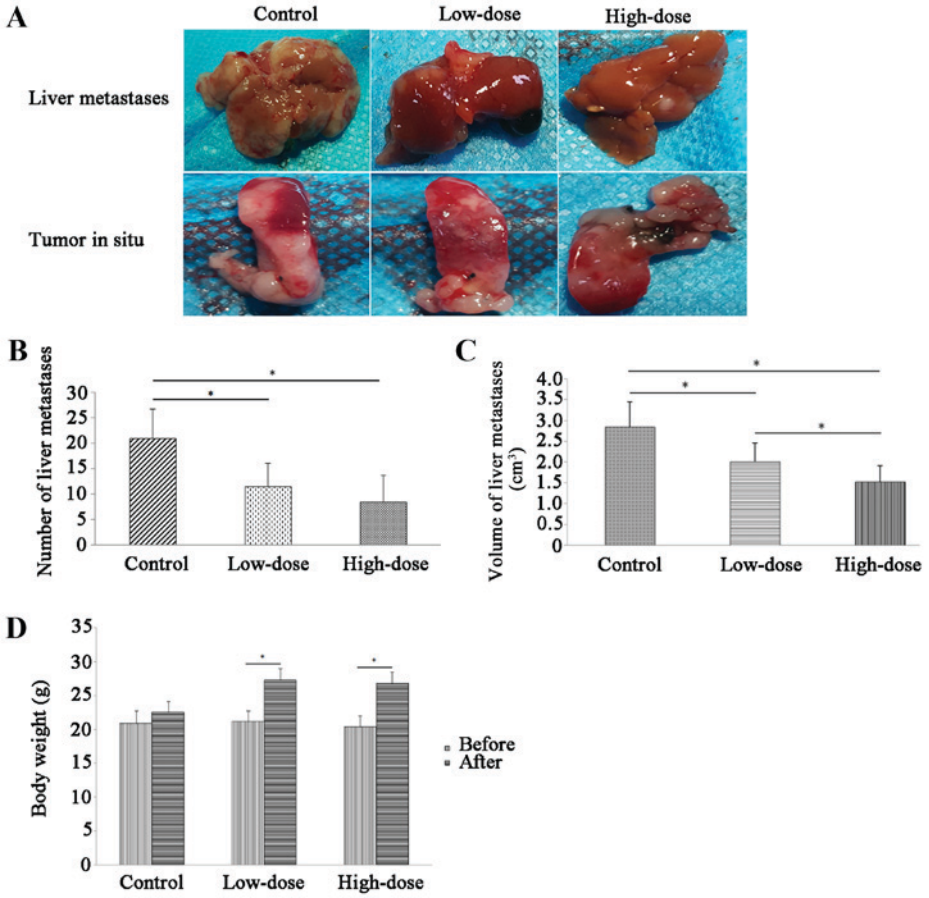

Figure 6. Inhibitory effect of emodin on hepatic metastasis of pancreatic cancer *in vivo*. Forty-five nude mice were injected with spleen into pancreatic cancer cells to establish animal model of hepatic metastasis of pancreatic cancer. The experiment was divided into 3 groups: High dose emodin group (High-dose, 50 mg/kg body weight/day, gavage), low dose emodin group (Low-dose, 20 mg/kg body weight/day, gavage), the control group (Control, 2 ml normal saline, gavage). (A) Representative tumor photos were present. The (B) number and (C) volume (of liver metastases, and the (D) nude mice weight was measured. *P<0.05.

Inhibitory effect of emodin on hepatic metastasis of pancreatic cancer in vivo. In 20 and 50 mg/kg emodin administration group, the number of metastatic nodules, the proliferation inhibition rate and the inhibition rate of hepatic metastasis in nude mice were better than those in the control group (Fig. 6). Emodin administration significantly increased the level of miR-1271 and mRNA level of E-cadherin in pancreatic cancer hepatic metastasis tissues (Fig. 7B). The results of western blot analysis (Fig. 7A) and immunohistochemistry (Fig. 7C) showed that emodin administration significantly increased E-cadherin protein level, however, the expression of ZEB1 and Twist1 protein significantly decreased. *In vivo* experiments showed that emodin can inhibit the EMT of pancreatic cancer cells by increasing the content of miR-1271 in pancreatic cancer, and then exert the therapeutic and preventive effects on the metastasis of pancreatic cancer.

Discussion

Pancreatic cancer is a malignant digestive system tumor with extremely high mortality rate. Currently, therapies such as surgery, radiotherapy and chemotherapy for pancreatic cancer are not effective (27). Therefore, methods that can effectively treat pancreatic cancer and inhibit its metastasis are the focus of current pancreatic cancer related research. A large number of studies have indicated that a variety of miRNAs are closely related to tumor development. MiR-1271, a kind of miRNA that has tumor suppressor effect, is involved in the inhibition of EMT, and induces the apoptosis of tumor cells (18-22). However, there are few researches on the effect of miR-1271 on pancreatic cancer cells and its regulation mechanism. The present study found that miR-1271 was significantly down-regulated in pancreatic cancer, and it can inhibit EMT in pancreatic cancer cells.

Emodin (6-methyl-1,3,8-trihydroxyanthraquinone) is a naturally occurring pupae present in various plants, especially in roots and barks (28). Emodin has many pharmacological effects, such as antibacterial, antiviral effects, anti-inflammatory effects as well as anticancer effects (29-31). The anti-tumor drug emodin has been shown to affect many different tumor cell lines and inhibit the growth of leukemia, breast cancer, colon cancer and lung cancer cells, etc (31-34). Moreover, although studies have indicated the anti-cancer effect of emodin on pancreatic carcinoma (35,36), the mechanism of action of emodin in inhibiting the development of pancreatic cancer remains incompletely elucidated. Therefore, we conducted the current study. And we found that emodin could inhibit the proliferation ability of pancreatic cancer cells and increased miR-1271 expression level in pancreatic cancer

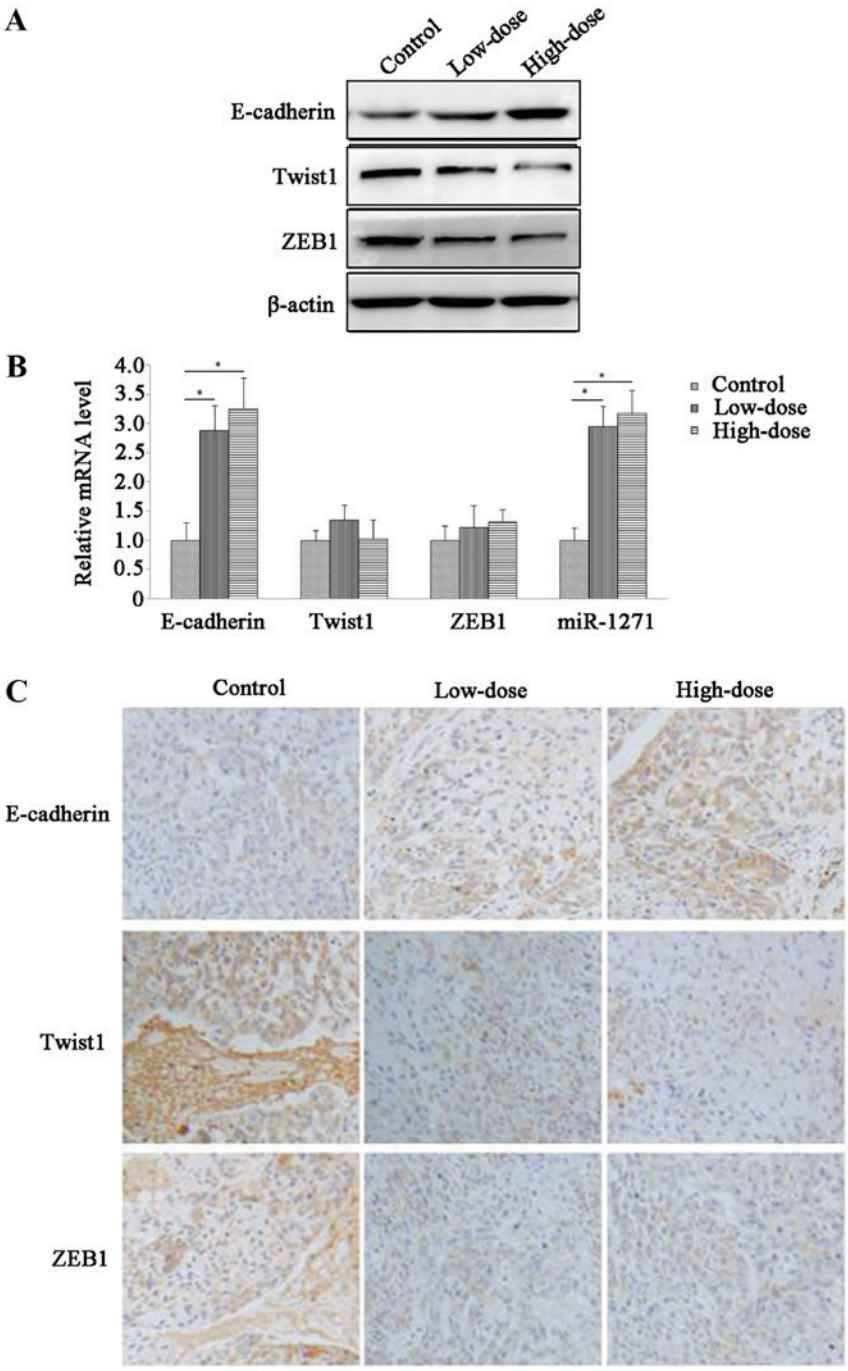

Figure 7. Inhibitory effect of emodin on the level of miR-1271 and EMT marker *in vivo*. The protein level of E-cadherin, ZEB1 and TWIST1 in pancreatic cancer hepatic metastasis tissues were detected by (A) western blot and (C) immunohistochemistry respectively. (B) The mRNA expression of miRNA-1271, E-cadherin, ZEB1 and TWIST1 in pancreatic cancer hepatic metastasis tissues were detected by qRT-PCR. Magnification, x200. *P<0.05.

cells. Moreover, we found that emodin inhibited pancreatic cancer cell EMT by raising the level of miR-1271.

E-cadherin, a member of the cadherin family of cell adhesion molecules, is an important intercellular adhesion molecule and is now recognized as a cancer metastasis suppressor molecule (when E-cadherin expression is reduced, tumors are more likely to metastasize) (25). Twist1, a apoptosis inhibitor protein found in recent ten years, is an important regulatory factor in the development of malignant tumor cells and EMT (24). ZEB (zinc finger E-boxbinding homeobox) is an important transcription factor whose family members include ZEB1 and ZEB2, which are important condition factors for EMT (37). In our study, we showed that miR-1271 may act on ZEB1 and TWIST1 at the post-transcriptional phase, but not the mRNA level. In addition, we found that emodin can inhibit the EMT of pancreatic cancer cells by increasing the level of miR-1271 in pancreatic cancer cells.

In vivo experiments showed that emodin can inhibit the EMT of pancreatic cancer cells by increasing the content of miR-1271 in pancreatic cancer, and then exert the therapeutic and preventive effects on the metastasis of pancreatic cancer.

The data of our research suggested that miR-1271 significantly decreased in pancreatic cancer cells and tissues. Twist1 may be a target gene of miR-1271. MiR-1271 significantly

inhibited pancreatic cancer cell SW1990 EMT and invasive ability, and emodin could inhibit SW1990 cell EMT by raising the level of miR-1271. In addition, we found that emodin inhibited the liver metastasis of pancreatic cancer by inhibiting EMT *in vivo*. We provide theoretical and experimental evidence for the further development of miRNA-based targeted therapy of pancreatic cancer.

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Availability of data and materials

The analyzed data sets generated during the present study are available from the corresponding author on reasonable request.

Authors' contributions

NL designed the study. CW was responsible for data access and analysis. PZ performed literature analysis. NL, CW, PZ and SY interpreted the results and developed the manuscript.

Ethics approval and consent to participate

The present study was approved by the Human Ethics Committee Review Board at The General Hospital of Tianjin Medical University (Tianjin, China). Written informed consent was provided by every patient. The animal experiments performed in the present study were approved by the Animal Ethics Committee Review Board at Tianjin Medical University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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