

Emodin inhibits angiogenesis in pancreatic cancer by regulating the transforming growth factor- β /drosophila mothers against decapentaplegic pathway and angiogenesis-associated microRNAs

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Abstract. Emodin is a traditional Chinese medicine, which has been demonstrated to inhibit the growth of pancreatic cancer cells. However, the underlying molecular mechanisms remain to be elucidated. The present study investigated whether emodin suppresses angiogenesis in pancreatic cancer. A nude mouse pancreatic cancer xenograft model was established using SW1990 human pancreatic cancer cells by surgical orthotopic implantation. Different doses of emodin were injected into the abdominal cavities of the tumor-bearing mouse models and controls three times each week for 2 weeks. The tumors were measured and weighed, the expression of cluster of differentiation 34 was detected using immunochemistry, and microvessel densities were calculated. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blotting were performed to determine the mRNA and protein expression levels of transforming growth factor (TGF)- β and drosophila mothers against decapentaplegic (Smad) homologs. The angiogenesis-associated microRNAs (miR), miR-20, miR-155 and miR-210 were assessed by RT-qPCR. A negative dose-dependent association was revealed between treatment with emodin and the volume and weight of tumors and microvessel density. Emodin was associated with lower mRNA and protein expression levels of TGF- β 1 and its downstream target, angiopoietin-like 4, and higher mRNA and

protein expression levels of TGF- β receptor (T β R)I, T β RII and Smad4. Notably, treatment with emodin was associated with lower expression levels of miR-155 and miR-210 and higher expression levels of miR-20b. The present study suggested that treatment with emodin may repress angiogenesis in pancreatic cancer by altering the activities of the TGF- β /Smad pathway and angiogenesis-associated miR-20b, miR-155, and miR-210.

Introduction

Pancreatic cancer is an exocrine gland neoplasm of the pancreas. It is the most prevalent type of malignant tumor of the digestive system and is the fourth most prevalent of all types of malignant tumor (1). Due to the depth and concealed position of the pancreas in the retroperitoneum, the majority of cases of pancreatic cancer are diagnosed at late stages with distant metastases. Significant advances have been made in understanding the biology and therapeutics of pancreatic cancer, however the 5-year survival rate remains <6% (1,2). Surgery increases the survival rate, however, surgery is only suitable for 10-15% of patients as metastasis is usually already present at diagnosis (3).

Systemic chemotherapy is the major treatment approach for 85% of patients with advanced pancreatic cancer (3). Gemcitabine is the standard chemotherapeutic agent for late stage pancreatic cancer (4), however, its curative effect is limited. The median survival rate following radical surgery with adjunct gemcitabine treatment is only 22.1 months (5). In addition, a number of patients with pancreatic cancer develop resistance to gemcitabine. Gemcitabine, in combination with fluorouracil, cisplatin, oxaliplatin and capecitabine, can somewhat improve the survival rates of patients with advanced disease, however, the median survival rate remains between 6.5 and 9.4 months (6-10). Therefore, novel and effective therapeutic strategies are urgently required for the treatment of advanced pancreatic cancer.

Emodin is an anthraquinone drug, which has been demonstrated to induce apoptosis and inhibit the growth

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and metastasis of various types of malignant tumor. Its low toxicity has been demonstrated *in vivo* and *in vitro* (11-13). A previous study indicated that emodin may inhibit angiogenesis in pancreatic cancer (14), however, the underlying molecular mechanisms remain to be elucidated. In the present study, the anti-angiogenesis effects of emodin were investigated in a mouse xenograft pancreatic cancer model. Changes in the tumor angiogenesis-associated transforming growth factor (TGF)- β /drosophila mothers against decapentaplegic (Smad) pathways and in the expression levels of microRNAs (miR), including miR-20b, miR-155 and miR-210, were examined.

Materials and methods

Reagents. Emodin was purchased from Sigma-Aldrich (St. Louis, MO, USA), dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) at 0.2 mmol/l as a stock solution and stored at -20°C. The final concentration of DMSO was <0.1%. A mouse polyclonal anti-cluster of differentiation (CD)34 antibody (cat. no. sc-74499), mouse polyclonal anti-TGF β receptor (T β R)II antibody (cat. no. sc-17799), mouse monoclonal anti-Smad4 antibody (cat. no. sc-7966) and goat polyclonal anti-angiopoietin-like (Angptl)4 antibody (cat. no. sc-34113) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). A rabbit polyclonal anti-TGF- β 1 antibody (cat. no. ab92486) and rabbit polyclonal anti-T β RI antibody (cat. no. ab31013) were purchased from Abcam (Cambridge, MA, USA). TRIzol[®] reagent was purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). The RevertAid First Strand cDNA Synthesis kit was purchased from Fermentas (Burlington, ON, Canada). The Takara One Step PrimeScript miRNA cDNA Synthesis kit (Perfect Real Time) and Takara SYBR Premix ExTaq[™] II (Perfect Real Time) were purchased from Takara Bio, Inc. (Otsu, Japan). The study was approved by the Ethics Committee of First Affiliated Hospital, Zhejiang University School of Medicine (Hangzhou, China).

Cell line. The SW1990 human pancreatic cancer cell line was purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI-1640 medium (Invitrogen Life Technologies), supplemented with 10% heat-incubated fetal bovine serum (Invitrogen Life Technologies), 100 U/ml penicillin and 100 mg/ml streptomycin (Beyotime Institute of Biotechnology, Shanghai, China) at 37°C in a humidified 5% CO₂ atmosphere. The cells were passaged at 70-80% confluence.

Animals. Female athymic BALB/c nu/nu mice (4-6-weeks old, weighing 20-22 g) were purchased from the Shanghai Cancer Institute for Tumor Implantation (Shanghai, China). The animals were maintained in a sterile environment maintained at room temperature (25°C) and 60-65% relative humidity, with *ad libitum* provision of water at the Animal Experiment Center of Zhejiang University School of Medicine (Zhejiang, China).

Orthotopic implantation of pancreatic cancer and treatment. A total of 2x10⁶ SW1990 cells were subcutaneously injected into the flanks of donor nude mice. At a volume of 1 cm³, the subcutaneous tumor was removed under sterile conditions. The central necrotic tissues in the tumor were removed and

the healthy peripheral tissue from the subcutaneous tumor was cut into tissue blocks of 1 mm³ for orthotopic transplantation. Recipient nude mice (n=40) were anesthetized with pentobarbitalum natricum (50 mg/kg; Sigma-Aldrich) and opened via a left longitudinal laparotomy. The spleen and the pancreatic tail were gently exteriorized, and a tissue pocket in the pancreatic parenchyma was created using micro-scissors (Santa Cruz Biotechnology, Inc.). A tumor fragment (~1 mm³) was placed into the tissue pocket, ensuring that it was entirely surrounded by normal pancreatic tissue. Following careful relocation of the pancreas and spleen into the abdominal cavity, the cavity was closed in two layers using 4-0 silk sutures for the muscular layer and nonabsorbable stainless steel wound clips for the skin.

Following a recovery period of 3 weeks, the *in situ* xenograft model of pancreatic cancer was complete and the mice were randomly divided into four groups, termed the control, E20, E40 and E80 group, each containing 10 mice. The mice in the control group were each injected with 0.2 ml 0.9% sodium chloride (Shanghai Chemical Reagent Plant, Shanghai, China) into the abdominal cavity. The mice in the E20, E40 and E80 groups were injected with 20, 40 and 80 mg/kg emodin, respectively, using the same method as the control (15). Each group was treated three times each week for 2 weeks. The mice were euthanized 7 weeks after implantation by cervical dislocation and the pancreatic tumors were carefully separated and weighed (scales from Mettler-Toledo International, Inc., Zurich, Switzerland). The orthotopic tumor tissues were then either stored in 4% paraformaldehyde (Shanghai Chemical Reagent Plant) for immunohistochemistry or were frozen in liquid nitrogen.

CD34 immunohistochemistry and calculation of microvessel density. The tissue sections were cut from the paraffin-embedded pancreatic cancer tissues, blocked with goat serum (Zhejiang Tianhang Biological Technology Co., Ltd., Hangzhou, China) and immunostained following deparaffinization with xylene for 10 min and rehydration with ethanol for 5 min 3 times. Immunostaining was performed using primary antibodies specific for CD34 (1:50) at room temperature for 60 min, followed by staining with horseradish peroxidase-conjugated secondary antibodies (1:1,500) at room temperature for 30 min. The slides were developed in diaminobenzidine (Beyotime Institute of Biotechnology) and counterstained with hematoxylin (Beyotime Institute of Biotechnology). The stained slides were dehydrated and mounted in Permount (Beyotime Institute of Biotechnology) and visualized under a light microscope (Olympus, Tokyo, Japan). Images were captured using an attached camera linked to a computer. The slides were visualized for the identification of clusters of microvessels under low power (magnification, x100). Random visual fields (n=5) were selected for inspection under high power (magnification, x400). The number of CD34-positive cells in each visual field was defined as the microvessel density (16-18).

Detecting the mRNA expression levels of TGF- β 1, T β RI, T β RII, Smad4 and Angptl4 via reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The total RNA in the tumor tissues was extracted using TRIzol reagent, according to the manufacturer's instructions, and quantified

Table I. Primers used in the quantitative polymerase chain reaction.

Gene	Primer (5'-3')	Product length (bp)
TGF- β 1	Forward: CAATTCCTGGCGATACCTCAG Reverse: GCACAACTCCGGTGACATCAA	86
T β RI	Forward: GCTGTATTGCAGACTTAGGACTG Reverse: TTTTGTTCCTCCACTCTGTGGTT	90
T β RII	Forward: AAGATGACCGCTCTGACATCA Reverse: CTTATAGACCTCAGCAAAGCGAC	119
Smad4	Forward: CCACCAAGTAATCGTGCATCG Reverse: TGGTAGCATTAGACTCAGATGGG	76
Angptl4	Forward: GGCTCAGTGGACTTCAACCG Reverse: CCGTGATGCTATGCACCTTCT	103
β -actin	Forward: CATTGCCGACAGGATGCAG Reverse: CTCGTCATACTCTGCTTGCTG	169

TGF, transforming growth factor; T β R, TGF- β receptor; Smad, drosophila mothers against decapentaplegic; Angptl, angiopoietin-like; bp, base pairs.

by spectrophotometric analysis (RF-5301PC; Shimadzu Corporation, Kyoto, Japan). The RNA integrity was verified using agarose gel electrophoresis. cDNA was synthesized using a RevertAid First Strand cDNA Synthesis kit (Fermentas), a random primer (β -actin) and 1 mg RNA. The cDNA product was diluted 1:4 into deionized water. The cDNA, primers specific for TGF- β 1, T β RI, T β RII, Smad4, Angptl4 and β -actin (Table I), and Sybr Green I mix were added to a 20 μ l reaction volume, supplemented with deionized water, according to the manufacturer's instructions. The PCR was performed using a Roche real-time PCR system (Roche Diagnostics, Basel, Switzerland) and the data were analyzed using LightCycler 480 software (Roche Diagnostics). β -actin was used as an internal control. The conditions for the PCR reactions were as follows: Denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 60 sec for up to 40 cycles.

microRNA RT-qPCR. To determine the expression levels of miR, the cDNA was synthesized using a Takara One Step PrimeScript miRNA cDNA Synthesis kit (Takara Bio, Inc.), using an miRPrimeScript RT Enzyme mix (Takara Bio, Inc.) and 1 mg RNA. The cDNA product was diluted to 1:4 in deionized water and the cDNA, primers specific to miR-20b, miR-155 and miR-210, or the U6B control, and the SYBR Premix Ex TaqII were added to a 20 μ l reaction volume, supplemented with deionized water, according to the manufacturer's instructions. The PCR was performed using a LightCycler Real Time PCR 480 system. The primers used were as follows: miR-20b, 5'-CAAAGUGCUCAUAGUGCAGGUAG-3', miR-210, 5'-AGCCCCUGCCCACCGCACACUG-3' and miR-155, 5'-UUAAGUCUAAUCGUGAUAGGGGU-3'.

Western blotting. The total proteins were routinely extracted from the tumor tissues using radioimmunoprecipitation lysis buffer (containing 50 mM Tris, pH 7.4; 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS, sodium orthovanadate, sodium fluoride, EDTA and leupeptin;

Beyotime Institute of Biotechnology) and the protein concentrations were determined using a bicinchoninic acid assay (Thermo Fisher Scientific, Waltham, MA, USA). The proteins were separated on 12% SDS-PAGE gels (Bio-Rad Laboratories, Inc., Shanghai, China), electrotransferred onto polyvinylidene fluoride membranes (Invitrogen Life Technologies), blocked with 5% non-fat milk (Yili, Inc., Inner Mongolia, China) and subsequently probed with primary antibodies (anti-TGF- β 1, anti-T β RI and anti-GAPDH at a dilution of 1:1,000, anti-Smad4 at a dilution of 1:800, and anti-T β RII and anti-Angptl4 at a dilution of 1:500) and horseradish peroxidase-conjugated secondary antibody (at a dilution of 1:10,000). Following washing with Tris-buffered saline with Tween 20 (Beyotime Institute of Biotechnology), the bound antibody complexes were analyzed using an enhanced chemiluminescence reagent (Amersham Pharmacia Biotech., Piscataway, NJ, USA). A GAPDH antibody (anti-rabbit GAPDH; cat. no. AB-P-R 001; Hangzhou Goodhere Biotechnology Co., Ltd., Hangzhou, China) was used as the control.

Statistical analysis. TotalLab 2.1 software (TotalLab, Ltd., Newcastle, UK) was used to analyze the western blots, and LightCycler 480 software V1.5.0 was used to analyze the RT-qPCR data. The images presented are representative and data are expressed as the mean \pm standard error of the mean of each group. Differences between groups of cells or mice were analyzed using analysis of variance and Student's post-hoc t-test using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Emodin inhibits the growth of orthotopically transplanted pancreatic cancer. The tumor weights were measured 7 days after the final treatment of different doses of emodin (Fig. 1 and Table II). The tumor weights of the E40 and

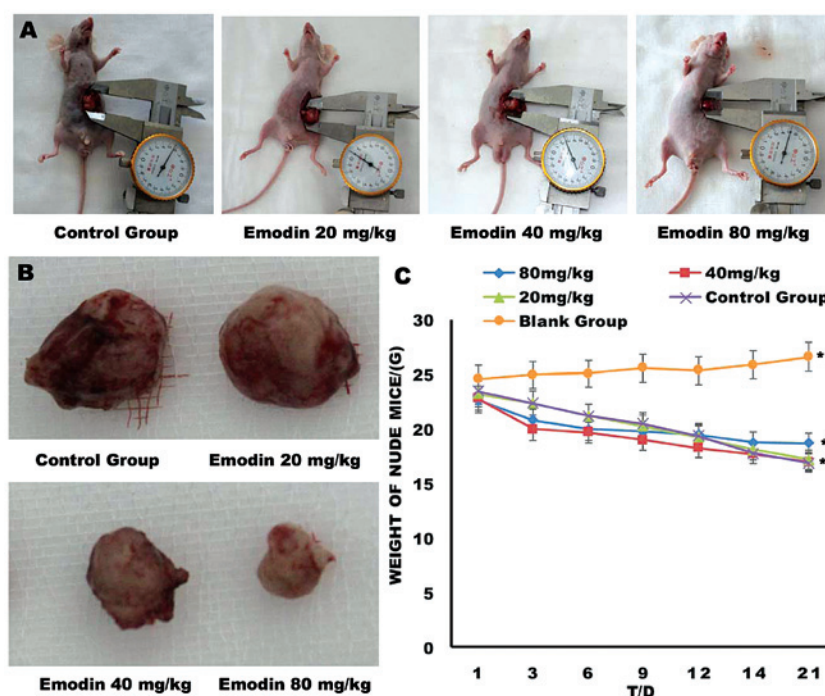


Figure 1. Pancreatic tumors. (A) Representative tumors from a mouse from each of the four treatment groups. (B) Orthotopically implanted pancreatic tumors on day 21 following treatment. The tumor diameters in the E40 and E80 groups were significantly lower compared with those from the untreated control. (C) Weights of the mice during the treatment period. Blank group contained healthy mice, which did not undergo transplant surgery. Data are expressed as the mean \pm standard error of the mean. * $P < 0.05$, ** $P < 0.01$ vs. control group. T/D, time (days).

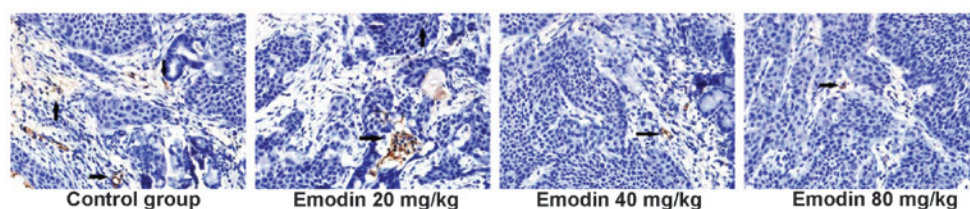


Figure 2. Expression of CD34 detected by immunohistochemistry (magnification, $\times 400$). The mean optical densities of CD34 were determined in the orthotopically transplanted tumor tissues from the mice following treatment without (control) or with different doses of Emodin. CD, cluster of differentiation.

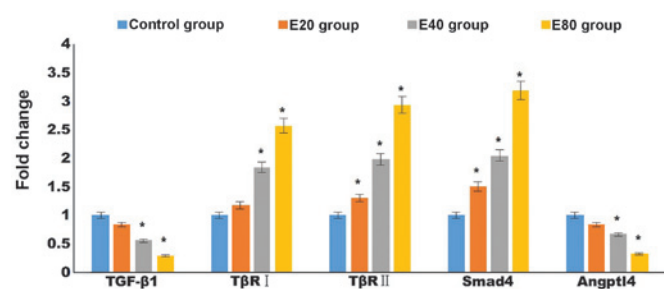


Figure 3. Treatment with emodin led to changes in the mRNA expression levels of angiogenesis-associated TGF- β 1, T β RI, T β RII, Smad4 and Angptl4. Data are expressed as the mean \pm standard error of the mean. * $P < 0.05$ vs. control group. TGF, transforming growth factor; T β R, TGF- β receptor; Smad, drosophila mothers against decapentaplegic; Angptl, angiopoietin-like.

E80 groups were significantly lower compared with those of the control and E20 groups ($P < 0.05$). No significant difference in mean tumor weight was observed between the control and E20 groups ($P > 0.05$). Therefore, tumor growth was effectively inhibited in the tumor-bearing mice following treatment with 40 mg/kg emodin.

Emodin significantly reduces the angiogenesis of orthotopically transplanted pancreatic cancer. To determine whether treatment with emodin inhibited the angiogenesis of pancreatic cancer, the numbers of CD34-positive cells and the microvessel density were calculated by immunohistochemistry (Fig. 2 and Table III). The microvessels were significantly less dense in the E20, E40 and E80 groups compared with the control group. Additionally, treatment of tumor-bearing mice with 20 mg/kg emodin significantly reduced microvessel density.

Emodin decreases the mRNA expression levels of TGF- β 1 and Angptl4 and increases the mRNA expression levels of T β RI, T β RII and Smad4. To examine the mechanisms by which emodin inhibits the angiogenesis of pancreatic cancer, the gene expression levels of the TGF- β 1, T β RI, T β RII, Smad4 and Angptl4 angiogenesis-associated genes were determined. Treatment with emodin decreased the mRNA expression levels of TGF- β 1 and Angptl4 in a dose-dependent manner, and the expression levels were significantly lower in the E40 and E80 groups compared with those of the control group ($P < 0.05$). In addition, treatment with emodin increased the mRNA expression levels of T β RI, T β RII and Smad4. The expression levels

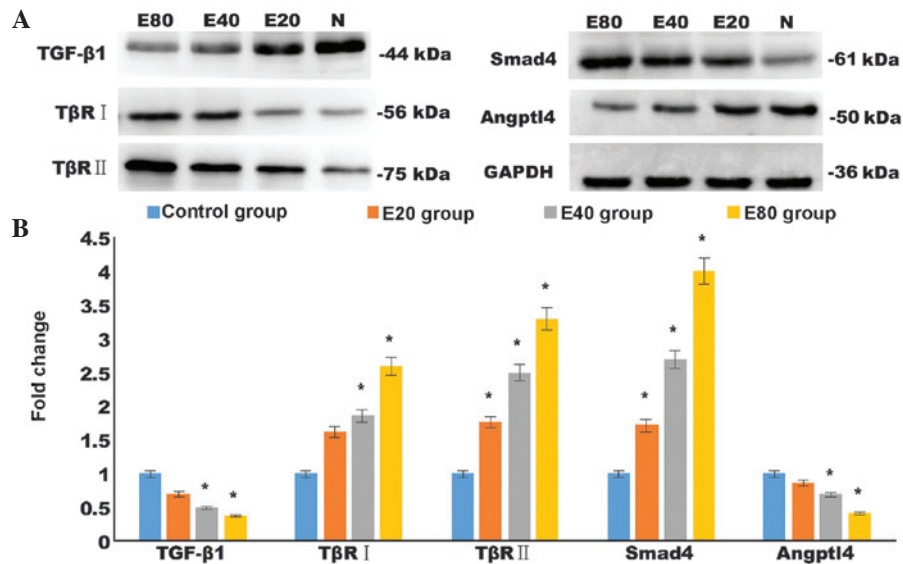


Figure 4. Emodin altered the protein expression levels of angiogenesis-associated TGF-β1, TβRI, TβRII, Smad4, and Angptl4. (A) Protein expression levels of TGF-β1, TβRI, TβRII, Smad4 and Angptl4 in tumor tissues was detected by western blotting. (B) Quantified data from the western blotting. Data are expressed as the mean ± standard error of the mean. *P<0.05, compared with the control group. TGF, transforming growth factor; TβR, TGF-β receptor; Smad, drosophila mothers against decapentaplegic; Angptl, angiopoietin-like.

Table II. Weight of orthotopically implanted pancreatic tumors on day 21 following treatment with emodin.

Group	Tumor weight (g)	Inhibition rate ^a (%)
Control	1.671±0.289	0
E20	1.487±0.248	11.0
E40	1.172±0.205 ^b	29.8
E80	0.741±0.210 ^b	55.6

^aInhibition rate of tumor = (1 - average weight of emodin group/average weight of the control group) × 100%; ^bstatistical significant (P<0.05).

Table III. Emodin reduced microvessel density of orthotopically transplanted pancreatic cancer.

Group	Microvessel density
Control	16.5±1.1
E20	12.3±1.8 ^a
E40	5.4±1.6 ^a
E80	4.8±1.6 ^a

^aP<0.05.

of TβRI were significantly higher in the E40 and E80 groups compared with the control group (P<0.05) and the expression levels of TβRII and Smad4 were significantly higher in the E20, E40 and E80 groups (P<0.05 and Fig. 3).

Emodin decreases the protein expression levels of TGF-β1 and Angptl4, and increases the expression levels of TβRI, TβRII

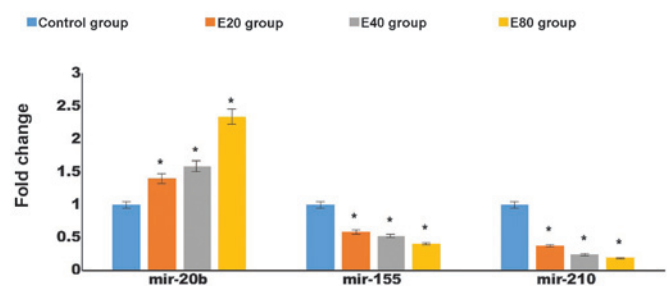


Figure 5. Emodin altered the expression levels of angiogenesis-associated mir-155, mir-210 and mir-20b. Quantitative results from reverse transcription-quantitative polymerase chain reaction. Data are expressed as the mean ± standard error of the mean. *P<0.05, compared with the control group). mir, microRNA.

and Smad4. Consistent with the differences in the mRNA expression levels, the results of the western blotting demonstrated that different doses of emodin upregulated the protein expression levels of TβRII and Smad4 compared with control group in the pancreatic cancer tissues (P<0.05). Similarly, emodin increased the protein expression of TβRI compared with the control group with significant differences between the E40 and E80 group, and the control group (P<0.05). Treatment with emodin also downregulated the protein expression levels of TGF-β1 and Angptl4 compared with the control group in the pancreatic cancer tissues, with significant differences between the E40 and E80 group, and the control group (P<0.05; Fig. 4).

Effect of emodin on the expression levels of angiogenesis-associated miR-155, miR-210 and miR-20b in pancreatic cancer tissues. miRNAs have been found to be important in angiogenesis. To determine whether emodin affects the expression levels of angiogenesis-associated miRNAs in pancreatic cancer tissues, the expression levels of miR-155, miR-210 and miR-20b were assessed by RT-qPCR. The levels of miR-20b in the pancreatic cancer tissues were higher in the groups treated

with emodin ($P < 0.05$), whereas those of miR-155 and miR-210 were lower compared with the control group. These changes occurred in a dose-dependent manner ($P < 0.05$; Fig. 5).

Discussion

The tumor microenvironment is important during tumorigenesis. Sustained angiogenesis is a hallmark of cancer and is key to the metastasis of cancer cells. Initial tumor growth depends on the original blood vascular system, however, when the tumor is $>1\text{--}2\text{ mm}^3$, the nutrients supplied by the existing blood vessels are not sufficient to sustain further tumor growth, requiring the production of new vessels through the process of angiogenesis (19). In as early as in 1971, Folkman (18) suggested that the growth of tumor cells was dependent on angiogenesis.

In the present study, treatment with emodin was observed to inhibit the growth of pancreatic cancer in a dose-dependent manner. Multiple previous studies have revealed that emodin can inhibit the proliferation of vascular endothelial cells (21,22). To assess the anti-angiogenic properties of emodin in a pancreatic xenograft tumor model, changes in microvascular density following treatment with emodin were determined. The microvessel densities of the emodin-treated groups were significantly lower compared with the control group, consistent with a previous report that emodin inhibited pancreatic cancer-associated angiogenesis (14). These data suggested that the suppression of angiogenesis may be a mechanism by which emodin inhibits tumor growth.

The TGF- β /Smad pathway is an antitumor signaling pathway, which is deregulated in several types of cancer (23,24). In the early stages of tumorigenesis, TGF- β inhibits cell proliferation, promotes cell differentiation and induces cell apoptosis and, as tumorigenesis progresses, TGF- β inhibits immunologic function, increases angiogenesis and promotes invasion of the tumor (25). TGF- β binds to and activates T β RII and T β RI, which in turn phosphorylates the Smad transcription factors. The phosphorylated Smads translocate to the cell nucleus to regulate a series of gene transcription events (24). Our previous study demonstrated that mutations in T β RI and T β RII promoted tumor angiogenesis and cancer cell proliferation in several types of cancer, including pancreatic, gall bladder, breast and rectal cancer (26). The Smad protein family is the key component of the TGF- β signaling pathway. Deregulation of the TGF- β /Smad signaling pathways has been observed in $>50\%$ of pancreatic cancer cases, via mutations in the Smad genes, predominantly Smad4, or in genes of the TGF- β superfamily and its receptor (27). Similar to mutations in Kirsten rat sarcoma viral oncogene, tumor protein p53 and cyclin-dependent kinase inhibitor 2A, $\sim 50\%$ of the cases of pancreatic cancer exhibit mutated Smad4 (28), however, inactivation mutation in the Smad4 gene usually occurs at a later stage of tumorigenesis. It has been observed that TGF- β activates the expression of Angptl4 in tumor cells in the circulation through the Smad signaling pathways (29). Angptl4 is secreted from tumor cells, breaks the junction of endothelial cells and subsequently increases capillary permeability (29). The present study revealed that treatment with emodin downregulated the expression levels of TGF- β 1

and Angptl4, and upregulated the expression levels of the T β RI and T β RII TGF- β 1 receptors and Smad4 in the orthotopically implanted pancreatic tumor mouse models. These results suggested that emodin may inhibit angiogenesis by inhibiting the expression levels of TGF- β 1 and, therefore, inhibiting the secretion of Angptl4 via Smad4.

MicroRNAs are small endogenous non-coding single-chain RNAs. The miRNA gene family occupies $\sim 1\%$ of the human genome and regulates the expression levels of around one third of human genes. MicroRNAs can also function as oncogenes or tumor suppressor genes. It has been demonstrated that the angiogenesis, which is associated with pancreatic cancer is regulated by microRNAs (30), and miR-20b, miR-155 and miR-210 are among the most extensively investigated of the angiogenesis-associated miRNAs. miR-155 and miR-210 function as oncogenes in various types of tumor, including breast (31), lung (32) and pancreatic (33) cancer, and clear cell carcinoma of the kidney (34). In the present study, treatment with emodin increased the expression of miR-20b and inhibited the expression levels of miR-155 and miR-210. These results suggested that treatment with emodin inhibited the growth of tumors by suppressing tumor-associated angiogenesis.

In conclusion, using an orthotopically transplanted pancreatic cancer model, it was observed that treatment with emodin inhibited angiogenesis by regulating the TGF- β /Smad signaling pathway and the expression levels of miRNA. These results revealed that emodin inhibits the growth of pancreatic cancer through a variety of different mechanisms (15), suggesting emodin as a promising natural drug for the treatment of pancreatic cancer.

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