Effects of emodin on the demethylation of tumor-suppressor genes in pancreatic cancer PANC-1 cells

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Abstract. Emodin, a natural anthraquinone derivative isolated from Rheum palmatum, has been reported to inhibit the growth of pancreatic cancer cells through different modes of action; yet, the detailed mechanism remains unclear. In the present study, we hypothesized that emodin exerts its anti-tumor effect by participating in the regulation of the DNA methylation level. Our research showed that emodin inhibited the growth of pancreatic cancer PANC-1 cells in a dose- and time-dependent manner. Dot-blot results showed that 40 µM emodin significantly inhibited genomic 5mC expression in the PANC-1 cells, and mRNA-Seq showed that different concentrations of emodin could alter the gene expression profile in the PANC-1 cells. BSP confirmed that the methylation levels of P16, RASSF1A and ppENK were decreased, while concomitantly the unmethylated status was increased. RT-PCR and western blotting results confirmed that the low expression or absence of expression of mRNA and protein in the PANC-1 cells was re-expressed following treatment with emodin. In conclusion, our study for the first time suggests that emodin inhibits pancreatic cancer cell growth, which may be related to the demethylation of tumor-suppressor genes. The related mechanism may be through the inhibition of methyltransferase expression.

Introduction

Pancreatic cancer is a malignant tumor caused by the mutation of normal pancreatic tissues, and 95% of pancreatic cancers are adenocarcinomas (1). In the US, pancreatic cancer is the fourth highest cause of cancer-related mortality each year, and the eighth highest in the world (2). In China, pancreatic cancer death ranks sixth among cancer-related deaths each year (3). Since the pathogenesis is not clear, there are no effective drugs for the treatment of pancreatic cancer. Currently, surgical resection is the most common treatment for pancreatic cancer. However, there are no convincing results concerning clinically relevant improvements in the quality of life and survival of these patients. Therefore, it is imperative to identify drugs that exhibit low toxicity and are relatively inexpensive.

Epigenetic changes in cancer have gained great attention. Epigenetic modifications mainly include DNA methylation and hydroxymethylation, histone methylation and acetylation, chromatin remodeling, genomic imprinting and RNA interference. DNA methylation is catalyzed by a group of DNA methyltransferase (DNMT) enzymes: mainly DNMT1, DNMT3a and DNMT3b (4). Studies have shown that DNA methylation plays an important role in the occurrence and development of many malignant tumors. In recent years, it has been discovered that under the catalysis of the Tets family, 5mC is transformed into 5hmC and 5fmc. The content of 5hmC in many types of cancer is low, and is closely related to the occurrence and development of melanoma (5).

Studies have found that many tumor-suppressor genes have abnormal methylation in pancreatic cancer, including CDKN1C (7), SPARC (8), P16 (9), RASSF1A (10) and ppENK (11). Methylated tumor-suppressor genes have a different degree of methylation and are not able to express the corresponding mRNAs. Among these tumor-suppressor genes, P16, RASSF1A and ppENK have been extensively studied. Ueki et al (11) and Fukushima et al (12) found an increase of more than 90% of ppENK gene methylation level in pancreatic cancer. Schutte et al (13) reported that 95% of P16 gene inactivation, 15% was related with methylation in
pancreatic cancer. Moore et al (14) reported that the P16 gene was methylated in 27% of pancreatic cancer cells. Dammann et al (10) reported that after culturing various pancreatic cells with the demethylation drug 5-Aza-CdR, the RASSF1A gene methylation level was increased in 64% of primary pancreatic ductal cell carcinoma, 83% of pancreatic endocrine tumors and 88% of pancreatic cancer cell lines. P16 and RASSF1A were re-expressed at various degrees to play an antitumor function.

Currently, the most commonly used demethylation drugs are 5-azacytidine (5-AzaC) and decitabine (5-aza-2-deoxycytidine; 5-Aza-CdR), which are two different types of nucleoside analogs (15). In the US, 5-AzaC and 5-aza-2'-deoxycytidine have been approved by the Food and Drug Administration (FDA). These drugs are mainly used for the treatment of hematological malignancies. Other approved demethylation drugs include zebularine (16), DHAC (17), hydralazine (18), selenite (19), RG108 (20) and arsenic trioxide (21). However, clinical trials have reported that the demethylation effects of certain drugs are not specific. Genomic hypomethylation was found to occur when the drug concentration was too high, and side effects, such as drug toxicity and inhibition of bone marrow, limit its application in the clinical treatment of tumors. The development of a demethylation drug with strong specificity, high safety and low toxicity has become one of the important tasks in tumor treatment.

In recent years, Chinese herbal medicines have been recognized as having antitumor efficacy. Emodin (1,3,8-trihydroxy-6-methyl anthraquinone), one such Chinese herbal medicine, has extensive pharmacological effects such as immune regulation (22), and antibacterial (23), anti-inflammatory and antitumor activities (24). Liu et al (25) reported that emodin inhibits pancreatic cancer cell growth through different modes of action, but the detailed mechanism remains unclear. Studies have found that curcumin (26) and epidermal catechins (EGCG) (27) exert similar antitumor efficacy through demethylation. Therefore, we hypothesized that emodin may exert its antitumor effect though participating in the regulation of the DNA methylation level.

Materials and methods

Chemicals and reagents. Emodin (purity ≥98%) and 5-Aza-CdR were both purchased from Sigma (St. Louis, MO, USA). Emodin was dissolved in dimethyl sulfoxide (DMSO) to create a stock solution at a concentration of 10 mmol/l and was stored at -70 °C. The DMSO concentration was maintained below 0.1% in all of the cell cultures and did not exert any detectable effect on cell growth or cell death. The Cell Counting Kit-8 (CCK-8) was purchased from Gibco. A cell and tissue genomic DNA extraction kit was purchased from Fastagen Biotech (Shanghai, China). The EpiTect® Bisulfite and EpiTect® MSP kits were purchased from Qiagen. The RNA extraction kit was purchased from Tiangen (Beijing, China). The antibodies, anti-RASSF1a and anti-ppENK were purchased from Abcam. The anti-P16/INK4a antibody and anti-β-actin were purchased from Epitomics.

Cell line and culture. Human pancreatic cancer cell line PANC-1 was obtained from the American Type Culture Collection (ATCC: Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO₂. The medium was changed every 2-3 days, and the cells were subcultured when confluency reached 70-80% in 0.25% trypsin at 37 °C.

Cell proliferation assay. Cell survival was determined using CCK-8. Briefly, logarithmic phase PANC-1 cells were plated in 96-well culture plates (~5x10³ cells/well). After 24 h of incubation, the cells were treated with the vehicle alone (0.1% DMSO) and various concentrations (10, 20, 40 and 80 µM) of emodin, followed by a 24-, 48- and 72-h cell culture. Each group had 6 wells. A total of 10 µl CCK-8 was added to each well 1 h before the end of the incubation period. The absorbance at 450 nm was read using the Bio-Tek ELx800 absorbance microplate reader. The experiment was repeated 3 times. The degree of cellular inhibition by each drug was calculated by the following formula: Relative % inhibition = 1 - (dosing absorbance - blank absorbance)/(control absorbance - blank absorbance) x 100%.

Dot-blot assay. The PANC-1 cells were treated with emodin (0, 10, 20 and 40 µM) and 5-Aza-CdR (1 µM) for 72 h. The control cells were treated with 0.1% DMSO only. The total DNA was isolated from the cultured cells using the cell/tissue genomic DNA extraction kit according to the manufacturer's instructions. The concentrations of DNA were determined by fluorometry using the Qubit® dsDNA HS kit and fluorometer (both from Invitrogen). The procedure for the dot-blot assay was performed with reference to a previous study (28).

mRNA-sequence. The PANC-1 cells were treated with emodin (0, 10, 20 and 40 µM) and 5-Aza-CdR (1 µM) for 72 h. The control cells were treated with 0.1% DMSO only. The cells were collected and sent to Major Biotechnology Company (Shanghai, China), where the mRNA-seq was analyzed.

Bisulfite sequencing PCR (BSP). The PANC-1 cells were treated with emodin (0, 10, 20 and 40 µM) and 5-Aza-CdR (1 µM) for 72 h. The control cells were treated with 0.1% DMSO only. Genomic DNA was extracted using the cell/tissue genomic DNA extraction kit according to the manufacturer's instructions. When the DNA was concentrated to 1 µg, bisulfite modification of genomic DNA was performed using the EpiTect® Bisulfite kit. The sequences of the methylation-specific primers for P16, RASSF1A and ppENK are shown in Table I. Bisulfite-modified DNA (4 µl), methylation-specific primers (3 µl), 2X Taq PCR Master Mix (12.5 µl) and DEPC-H₂O (5.5 µl) were added to achieve a final volume of 25 µl. PCR amplification conditions were as follows: 95 °C for 5 min, 94 °C for 30 sec, annealing for 45 sec, and extension at 72 °C for 45 sec; a total of 40 cycles; followed by a final extension at 72 °C for 10 min. A total of 10 µl of the PCR product was separated on a 2% agarose gel electrophoresis, and the results were photographed. BSP products were extracted from agarose gel, then purified and sequenced (ShangHai Maipu Biotechnology Co., Ltd., China). The methylation of the sample was analyzed using BiQ Analyzer software.
Real-time PCR. The PANC-1 cells were treated with emodin (0, 10, 20 and 40 µM) and 5-Aza-CdR (1 µM) for 72 h. The control cells were treated with 0.1% DMSO only. Total RNA was isolated from the cells using the TRizol reagent according to the manufacturer's instructions. For reverse transcriptase analysis, 1 µg of total RNA was reversely transcribed in a 20 µl volume using RevertAid™ First Strand cDNA Synthesis kit (Fermentas). Real-time PCR amplification with 1 µl of the reverse transcriptase reaction mixture was performed with SYBR Green Real-Time PCR Master Mix-Plus (Toyobo, Japan). The initial denaturation step was 95˚C for 60 sec followed by 40 cycles of amplification at 95˚C for 15 sec, 60˚C for 15 sec, and 72˚C for 45 sec. All samples were performed in triplicate, and the relative amount of the target gene was normalized to GAPDH. The primers are listed in Table I.

Western blot analysis. The PANC-1 cells were treated with emodin (0, 10, 20 and 40 µM) and 5-Aza-CdR (1 µM) for 72 h. The control cells were treated with 0.1% DMSO only. Total proteins were extracted from the cells using cell lysis buffer (20 mmol/l Tris-HCl pH 7.5, 150 mmol/l NaCl, 1 mmol/l Na2EDTA, 1 mmol/l EGTA, 1% Triton, 2.5 mmol/l sodium pyrophosphate, 1 mmol/l β-glycerophosphate, 1 mmol/l Na3VO4, 1 µg/ml leupeptin, and 1 mmol/l PMSF). After centrifugation at 14,000 x g for 10 min at 4˚C, the supernatant was collected and the protein concentration was determined using the BCA protein assay kit according to the manufacturer's instructions. The protein lysates (20-µg -lane) were separated on 12% SDS polyacrylamide gel and transferred onto a nitrocellulose membrane. Each membrane was blocked with 5% skim milk and then incubated with the indicated primary antibodies against P16, RASSF1A, ppENK and β-actin over-night at 4˚C. Subsequently, the membrane was incubated with the secondary antibodies, goat anti-rabbit and anti-mouse IgG conjugated with HRP, for 1 h at room temperature, and the formed immunocomplex was visualized by enhanced chemiluminescence reagent and exposed to X-ray film. Quantitative data are expressed as a percentage of the mean ± standard deviation (SD) of the relative levels of the objective protein and control β-actin of each group of cells from three independent experiments.

Statistical analysis. All results were repeated in at least three separate experiments. The data are expressed as the mean ± SD. Statistical comparisons were conducted using one-way analysis of variance, which revealed significant differences between groups, and the Student's t-test which revealed significant differences between two sample means. Statistical analyses were carried out using SPSS version 17.0 software (SPSS Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of emodin on PANC-1 cell proliferation. To investigate the effect of emodin on cell growth, PANC-1 cells were cultured with 0, 10, 20, 40 and 80 µM emodin for 24, 48 and 72 h. Cell proliferation was determined by the CCK-8 assay. As demonstrated in Fig. 1, emodin was shown to inhibit the growth of the cells in a dose- and time-dependent manner. This result was similar with a previous study (29). The inhibition rate of emodin at a concentration of 40 µM for 72 h was 49.4%, while the inhibition rate of emodin at a concentration of 80 µM for 72 h reached 64.4%. Since the methylation efficiency of drugs is usually exerted under the appropriate low
drug concentration, emodin at a concentration of 10, 20 and 40 µM was used for the following experimental research.

Emodin decreases the level of genome 5mC, but has no significant effect on 5hmC. To determine the effects of emodin on the genomic DNA methylation level in the PANC-1 cells, a dot-blot assay was performed. As shown in Fig. 2, emodin at a concentration of 40 µM and 1 µM 5Aza-CdR significantly reduced the 5mC level when compared with the level in the control group. However, there were no significant differences in 5hmC levels. The levels of 5hmC were slightly decreased by emodin at concentrations of 10 and 20 µM, but the effect was not obvious. To further confirm this result, the effects of emodin on gene expression profiles were detected by mRNA-Seq. As shown in Fig. 3, different concentrations of emodin promoted differences between changes in the gene expression profiles. The number of differentially expressed genes was also increased in a dose-dependent manner. In each group, the number of downregulated genes was higher than the upregulated genes. For example, in the emodin (40 µM) vs the control group, the expressed genes were mainly concentrated in the KEGG pathway which was closely related to tumor pathways such as cell cycle, p53 signaling, pathways in cancer and apoptosis, cell division, DNA metabolic processes and regulation of cell death. The data suggest that emodin treatment has significant effects on gene expression, and plays an important role in regulating metabolic processes such as cell differentiation and proliferation.

Pyrophosphate sequencing PCR (BSP). In order to verify the differential expression of gene methylation after emodin treatment, BSP was used to detect the methylation levels of P16, RASSF1A and ppENK in the promoter region. As shown in
Fig. 4, treatment with emodin led to different degree of demethylation levels of P16, ppENK and RASSF1A in the promoter regions. Scopes of P16, ppENK, RASSF1A gene sequencing were respectively composed of 17, 11 and 36 CpG islands, 10 of which were randomly selected to clone and sequence. After treatment of PANC-1 cells with various concentrations of emodin (0, 10, 20 and 40 μM) and 5-Aza-CdR (1 μM) for 72 h, the methylation rates of the P16 gene were 93.5, 71.8, 59.4 and 33.5%, respectively. While the methylation rate in the 5-Aza-CdR group was decreased to 25%. The effect in the 5-Aza-CdR group was stronger than that in the emodin-treated group. The methylation rates of the ppENK gene were 86.4, 82.7, 67.3 and 52.7%, respectively, while that in the 5-Aza-CdR group was 39.1%. The methylation rates of the RASSF1A gene were 65.3, 60, 27.2 and 16.4%, respectively, while that in the 5-Aza-CdR group was 15.3%. These results demonstrated that emodin reduced the methylation levels of P16, RASSF1A and ppENK in the promoter region in pancreatic cancer cells.

Effects of emodin on mRNA and protein expression of P16, RASSF1A, ppENK and DNMTs. As shown in Fig. 5, emodin increased the mRNA expression levels of P16, RASSF1A and ppENK in a dose-dependent manner. 5-Aza-CdR also increased the mRNA expression levels of P16, RASSF1A and ppENK. The effect was stronger than that of emodin. As shown in Fig. 6, emodin increased the protein expression levels of P16, RASSF1A and ppENK in a dose-dependent manner. 5-Aza-CdR also increased the protein expression levels of P16, RASSF1A and ppENK. The effect was stronger than that of emodin. In Figs. 5B and 6, while emodin decreased the mRNA and protein expression of methyltransferase DNMT1 and DNMT3a, it had no effect on DNMT3b (data not shown). The results suggest that emodin may decrease overall genomic methylation levels by the downregulation of DNMT1 and DNMT3a at the transcription and protein levels, thereby affecting the transcriptional level of the whole genome, upregulating the mRNA and protein levels of the tumor-suppressor genes, P16, RASSF1A and ppENK, and consequently inhibiting tumor growth.

Discussion
The incidence of pancreatic cancer is increasing yearly, and the fatality rate is high. According to reports in 2008, 37,680
new patients in the US were diagnosed with pancreatic cancer and 34,290 of them (91%) succumbed to the disease (30). The pathogenesis of pancreatic cancer remains unclear and discovering a treatment for this cancer has been a challenge. Emodin has shown curative effects on pancreatic cancer in the clinic. Emodin was found to trigger apoptosis in cancer pancreatic cells (31), to inhibit the formation of new vessels (29) and to improve the resistance to gemcitabine in pancreatic cancer cells (30). However, the specific mechanisms of these curative effects remain unclear. Our data showed that emodin affected the whole genome expression by demethylation, which especially decreased methylation levels of the tumor-suppressor genes P16, RASSF1A and ppENK, thus playing an important role in cancer treatment.

In the present study, the data indicated that varying concentrations of emodin effectively inhibited the proliferation of the PANC-1 cell line at different time points. When PANC-1 cells were treated with 80 µM emodin for 72 h, there were significant changes in the growth inhibition rate and the cell morphology, which is consistent with previous literature (29). Notably, when PANC-1 cells were treated with 40 µM emodin for 72 h, a decrease in the growth inhibition rate was noted, but no significant changes in the cell morphology were noted. Therefore, emodin at concentrations of 0, 10, 20 and 40 µM were selected for the experiments. Our results revealed that emodin caused various degrees of demethylation of tumor-suppressor genes P16, RASSF1A and ppENK in PANC-1 cells. Methylation is often the cause of tumor-suppressor gene inactivation, and its expression was found to be inversely proportional to the density of CpG island methylation. Low levels of methylation caused a 67-90% inactivation of gene expression, while methylation of high density CpG islands completely extinguished gene expression (32). PCR also confirmed that emodin caused the re-expression of P16, RASSF1A and ppENK inactivated...
Figure 5. Effects of emodin on the mRNA expression of P16, RASSF1A, ppENK, DNMT1 and DNMT3a in the PANC-1 cells. (A) RT-PCR analysis showed that mRNA expression levels of P16, RASSF1A and ppENK were upregulated. (B) RT-PCR analysis showed that mRNA expression levels of DNMT1 and DNMT3a were downregulated. The effect of emodin was weaker than that of 5-Aza-CdR in the above experiments. *P<0.05, **P<0.01.

Figure 6. Effects of emodin on the protein expression levels of P16, RASSF1A, ppENK, DNMT1 and DNMT3a in the PANC-1 cells. (A) Protein expression levels were detected by western blot analysis. Protein expression levels of P16, RASSF1A and ppENK were upregulated, while protein expression levels of DNMT1 and DNMT3a were downregulated. The effect of emodin was weaker than that of 5-Aza-CdR in the above experiments. (B and C) The quantification was performed assigning a value of 1 to the control group. The results obtained from three separate experiments are expressed as mean ± SD. *P<0.05, **P<0.01.
by methylation, and whose expression levels increased in a dose- and time-dependent manner. The results of the western blot analysis further confirmed our research, which provides further evidence that emodin causes the demethylation of tumor-suppressor genes in PANC-1 cells.

In order to further investigate the role of emodin in the demethylation at the epigenetic level in PANC-1 cells, 5-Aza-CdR, which was proven to have demethylation effects in clinical trials, was used in the present study. The results showed that 5-Aza-CdR significantly reduced the methylation levels of the tumor-suppressor genes P16, RASSF1A and ppENK by enhancing the expression of mRNA and protein. Although emodin also plays a demethylation role in PANC-1 cells, the effect was weaker than that of 5-Aza-CdR. In vivo, the methylation was mainly catalyzed by methyltransferases (DNMT1, DNMT3a and DNMT3b). There are two ways to demethylate tumor-suppressor genes: inhibition of methyltransferase activity and reduction in methyltransferase expression.

RT-PCR and western blot analysis showed that both 40 µM emodin and 1 µM 5-Aza-CdR significantly reduced the expression of DNMT1 and DNMT3a, suggesting that demethylation of emodin may be linked with the inhibition of methyltransferase expression. Our study confirmed that emodin caused a certain degree of demethylation in the tumor-suppressor genes P16, RASSF1A, ppENK in the PANC-1 cells. However, it remains to be discovered whether emodin plays a role in the demethylation of different suppressor genes in other pancreatic cancer cell lines. In addition, whether emodin enhances the demethylation of 5-Aza-CdR warrants further research.

The methylation of DNA CpG islands (5mC) plays an integral role in gene transcriptional silencing, genomic imprinting and X chromosome inactivation. Currently, 5mC is the main focus of research. There are 10 to 11 translocation enzymes which catalyze the oxidation of 5mC to produce 5hmC; demethylases: a structural perspective. Structure 16: 341-350, 2008.

These results are consistent with the above results from PCR and western blotting. However, 5mC is catalyzed by TET to generate 5hmC, emodin may have no effect on the expression and activity of TET enzymes.

In summary, BSP demonstrated that emodin, to a certain degree, affected the demethylation of tumor-suppressor genes P16, RASSF1A, and ppENK in the pancreatic cancer cell line PANC-1. RT-PCR and western blot results showed that emodin caused the re-expression of P16, RASSF1A and ppENK which were previously not expressed or weakly expressed in the PANC-1 cells. Emodin also reduced the expression of DNMT1 and DNMT3a. The dot-blot results confirmed that emodin reduced the expression of the 5mC genome, likely by inhibiting the expression of DNMT. This discovery provides a novel strategy for the treatment of pancreatic cancer. Clinical treatment of emodin in pancreatic cancer occurs not only through apoptosis or inhibition of angiogenesis but also through demethylation in epigenetics.

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**References**


