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ORIGINAL ARTICLE

Clobenpropit enhances anti-tumor effect of gemcitabine in pancreatic cancer

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Abstract

AIM: To evaluate the anti-tumor effect of clobenpropit, which is a specific H_3 antagonist and H_4 agonist, in combination with gemcitabine in a pancreatic cancer cell line.

METHODS: Three kinds of human pancreatic cancer cell lines (Panc-1, MiaPaCa-2, and AsPC-1) were used in this study. Expression of H_3 and H_4 receptors in pancreatic cancer cells was identified with Western blotting. Effects of clobenpropit on cell proliferation, migration and apoptosis were evaluated. Alteration of epithelial and mesenchymal markers after administration of clobenpropit was analyzed. An *in vivo* study with a Panc-1 xenograft mouse model was also performed.

RESULTS: H_4 receptors were present as 2 subunits in human pancreatic cancer cells, while there was no expression of H_3 receptor. Clobenpropit inhibited cell migration and increased apoptosis of pancreatic cancer cells in combination with gemcitabine. Clobenpropit up-regulated E-cadherin, but down-regulated vimentin and matrix metalloproteinase 9 in real-time polymerase chain reaction. Also, clobenpropit inhibited tumor growth (gemcitabine 294 ± 46 mg *vs* combination 154 ± 54 mg, $P = 0.02$) and enhanced apoptosis in combination with gemcitabine (control 2.5%, gemcitabine 25.8%, clobenpropit 9.7% and combination 40.9%, $P = 0.001$) by up-regulation of E-cadherin and down-regulation of Zeb1 in Panc-1 xenograft mouse.

CONCLUSION: Clobenpropit enhanced the anti-tumor effect of gemcitabine in pancreatic cancer cells through inhibition of the epithelial-mesenchymal transition process.

Key words: Clobenpropit; Epithelial-mesenchymal transition; Histamine; Histamine receptors; Pancreatic neoplasm

Core tip: Histamine is associated with carcinogenesis through activation of its 4 membrane-specific receptors. Clobenpropit, which is an agonist of H_4 receptor, inhibited cell migration and increased apoptosis of pancreatic cancer cells in combination with gemcitabine. Clobenpropit up-regulated an epithelial marker, but down-regulated mesenchymal markers in real-time polymerase chain reaction. In addition, clobenpropit inhibited tumor growth and enhanced apoptosis in combination with gemcitabine by up-regulation of E-cadherin and down-regulation of Zeb1 in Panc-1 xenograft mouse. In conclusion, clobenpropit enhanced anti-tumor effects of gemcitabine in pancreatic cancer cells through inhibition of epithelial-mesenchymal transition process.

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INTRODUCTION

Pancreatic cancer is a very aggressive human cancer and has dismal prognosis with only 6% of patients surviving 5 years after diagnosis[1]. In spite of the progress of treatments, attempts at survival prolongation, especially in the advanced stage, have failed and resulted in no significant improvement[2]. Surgical resection is the only potentially curative treatment in pancreatic cancer, but only 15% of patients are candidates for resection[3,4]. Some chemotherapeutic agents are used in pancreatic cancer, and gemcitabine became the standard chemotherapeutic agent in pancreatic cancer after a randomized trial in 1997[5]. Gemcitabine is a nucleoside pyrimidine analogue which exerts its cytotoxic actions primarily by the incorporation of gemcitabine triphosphate into DNA, leading to masked chain termination[6]. However, pancreatic cancer is highly resistant to chemotherapy including gemcitabine[7], and the disappointing circumstance of pancreatic cancer is mainly due to late diagnosis at which chemoresistance in patients is a critical issue[8]. Resistance to gemcitabine has been increasing and the effectiveness of gemcitabine has been reduced to less than 20%[9]. Hence, new therapeutic targets and chemotherapeutic agents of pancreatic cancer are desperately required.

A high concentration of histamine has been shown in melanoma[10], small cell lung carcinoma[11], breast carcinoma[12] and colorectal carcinoma[13]. Histamine participates in tumor proliferation or apoptosis through activation of its four membrane-specific receptors, H_1, H_2, H_3 and H_4[14]. As in other human cancers, expression of histidine decarboxylase and histamine contents have been reported in pancreatic cancer[15,16]. Histamine inhibits cell proliferation through the H_1 and H_2 receptor, which is associated with a partial differentiation in pancreatic cancer[17]. Through the H_2 receptor, histamine induces G_0/G_1 phase arrest and modulation of mitogen activated protein kinase and Bcl-2 family proteins[18-20]. Furthermore, a previous study suggests that H_3 and H_4 receptors are involved in pancreatic cancer cell growth, with proliferation increased through H_3 receptor and diminished *via* H_4 receptor[21]. However, the mechanism of the anti-cancer effect through the histamine receptor still remains unclear.

Clobenpropit, which is a specific H_3 antagonist and H_4 agonist, inhibits the spread of mammary adenocarcinoma by decreasing invasion potential[22]. A recent study suggested that the modulation of H_4 receptor by clobenpropit disrupts epithelial-mesenchymal transition (EMT) processes, extracellular matrix (ECM) breakdown, and invasion potential and decreases tumor growth in cholangiocarcinoma[23]. Similarly, EMT plays a crucial role in tumor progression and metastasis of pancreatic cancer[24]. Furthermore, EMT regulators including Zeb1 are known to induce chemoresistance of human pancreatic cancer cells[25]. Thus, therapeutic agents targeting the EMT process could restore the chemoresistance of pancreatic cancer. Therefore, we aimed to investigate the anticancer efficacy of clobenpropit with gemcitabine combination in human pancreatic cancer cells. Additionally, we evaluated the alteration of EMT markers after administration of clobenpropit with *in vitro* and *in vivo* studies.

MATERIALS AND METHODS

Pancreatic cancer cell lines

Human pancreatic cancer cell lines, Panc-1, MiaPaCa-2 and AsPC-1 were obtained from Korea Cell Line Bank and maintained in Dulbecco modified Eagle medium containing 10% fetal bovine serum. They were incubated at 37 ℃ and 5% CO_2.

Western blotting

After washing with PBS, three kinds of pancreatic cancer cells (Panc-1, MiaPaCa-2 and AsPC-1) at 70-80% confluence were processed and lysed in NP-40 buffer (20 mmol Tris-HCl, pH 7.4, 100 mmol NaCl, 1% NP-40, 0.5% sodium deoxycholate, 5 mmol $MgCl_2$, 0.1 mmol phenylmethylsulfonyl fluoride, and 10 mg/mL protease inhibitor mixture). Then, proteins were quantified with a BCA protein assay kit (Pierce, Rockford, IL). Proteins were separated by 10% to 15% SDS-polyacrylamide denaturing gels, transblotted onto nitrocellulose membranes and probed with rabbit antihuman H_3 and H_4 receptor antibodies (Millipore, Billerica, MA). Immunoreactivity was developed using a peroxidase conjugate antiserum (Sigma-Aldrich, St Louis, MO) and detected by enhanced chemiluminescence reagents (Amersham Biosciences, Baied'Urfe, Quebec, Canada). Western blotting of Panc-1, MiaPaCa-2 and AsPC-1 treated with 50 or 100 μmol/L of clobenpropit (Sigma-Aldrich) alone or in combination with 5 μmol/L of gemcitabine (Yuhan, Seoul, South Korea) was also performed.

Wound healing assay

Three kinds of pancreatic cancer cells (5×10^5) were seeded in 6-well plates and cultured until reaching 70%-80% confluence as a monolayer. A straight scratch was made on cell plates, and then cell plates were gently rinsed to remove the detached cells. After adding 0, 10 and 50 μmol/L of clobenpropit to each plate, cells were grown for an additional 48 h. After washing the cells with PBS twice, photos were taken on a confocal microscope (Leica, Wetzlar, Germany). Wound healing ranges were measured by Aperio ImageScope V11.1.2.752 (Aperio Technologies, Vista, CA). Additional wound healing assays after administration of gemcitabine (5 μmol/L) and/or clobenpropit (50 μmol/L) in Panc-1 and gemcitabine (15 μmol/L) and/or clobenpropit (50 μmol/L)

Table 1 Sequences of primers used for real-time polymerase chain reaction

Gene	Sequences
H_4 receptor	Forward: 5′-GTGGTTAGCATAGGTTATAC-3′
	Reverse: 5′-ATGCCACTGCACTCCTGC-3′
GAPDH	Forward: 5′-ACGGATTTGGTCGTATTGGG-3′
	Reverse: 5′-TGATTTTGGAGGGATCTCGC-3′
E-cadherin	Forward: 5′-GCCTCCTGAAAAGAGAGTGGAAG-3′
	Reverse: 5′-TGGCAGTGTCTCTCCAAATCCG-3′
Vimentin	Forward: 5′-AGGCAAAGCAGGAGTCCACTGA-3′
	Reverse: 5′-ATCTGGCGTTCCAGGGACTCAT-3′
Fibronectin	Forward: 5′-ACAACACCGAGGTGACTGAGAC-3′
	Reverse: 5′-GGACACAACGATGCTTCCTGAG-3′
MMP-9	Forward: 5′-TTCTGCCCCAGCGAGAGA-3′
	Reverse: 5′-GTGCAGGCGGAGTAGGATTG-3′
CK-19	Forward: 5′-GAAGAACCATGAGGAGGAAATCA-3′
	Reverse: 5′-ACCTCATATTGGCTTCGCATGT-3′

were performed to evaluate the change of cell migration after gemcitabine and clobenpropit combination treatment.

Real-time polymerase chain reaction

Gene expression was evaluated in mRNA from all pancreatic cancer cell lines. Total RNA was extracted using Trizol Reagent (Invitrogen, Carlsbad, CA). RNA samples were diluted to a final concentration of 0.5 mg/mL in RNase-free water and stored at -80 ℃ until use. Synthesis of the cDNA was performed with 1 mg of total RNA with M-MLV reverse transcription reagents (Invitrogen), and real-time polymerase chain reaction (PCR) reaction was carried out on the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) in 20 μL TaqMan Gene Expression Master Mix (Applied Biosystems) using 200 ng cDNA.

Human primers sets were ordered and used according to their protocols. The specific primers were described in Table 1[26-29]. The human β-actin gene was used as an endogenous reference to control for the independent expression of sample-to-sample variability. The relative expression of target genes was normalized by dividing the target Ct value by the endogenous Ct values. All equipment was purchased from Applied Biosystems and used according to manufacturer's protocols.

MTS proliferation assay

Cells were plated into 96-well plates at a density of 4 × 10^3 cells/well and stimulated with clobenpropit (1 to 100 μmol/L) for up to 48 h to determine optimal dose and stimulation time. 3-(4,5-Demethylthiazole-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS; Promega, Madison, WI) was added to the cells, and the numbers of live cells were counted after allowing development for 1 h. The plates were read on a Wallac-1420 plate reader (Perkin-Elmer, Boston, MA) at an absorbance of 490 nm. Data are expressed as fold change of treated cells as compared with basal treated controls.

Apoptosis determination

Pancreatic cancer cells were cultured and divided into 4 groups according to the treatment: (1) control; (2) gemcitabine (5 μmol/L) alone; (3) clobenpropit (50 μmol/L) alone; and (4) gemcitabine (5 μmol/L) and clobenpropit (50 μmol/L) combination. After trypsinization, cells were incubated with annexin V-fluorescein isothiocyanate and propidium iodide (BD Biosciences, Franklin Lakes, NJ) for 15 min at room temperature in the dark. The degree of apoptosis was analyzed by fluorescence activated cell sorting. The proportion of stained cells in each quadrant was quantified with CellQuest software (BD Biosciences).

Animal experiments

Five-week-old male BALB/c nude mice were purchased from Orient (Gyeonggi-do, Korea). Mice were housed under specific pathogen-free conditions, and a γ-ray-irradiated laboratory rodent diet (Purina Korea, Gyeonggi-do, South Korea) and autoclaved water were provided ad libitum. All the protocols for the animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee at Seoul National University Hospital (IACUC No. 12-0213). All animal procedures were consistent with the "Guide for the Care and Use of Laboratory Animals" issued by the Institute of Laboratory Animal Resources Commission on Life Science, United States National Research Council.

To generate tumors, Panc-1 was subcutaneously inoculated with 1 × 10^6 cells suspended in 0.15 mL of Matrigel. All mice were divided into 4 groups randomly, and each group consisted of 5 mice: (1) control (vehicle alone); (2) gemcitabine (twice-a-week intraperitoneal injection at 125 mg/kg for 40 d); (3) clobenpropit (every other day intraperitoneal injection at 20 μmol/L per kilogram for 40 d); and (4) gemcitabine (twice-a-week intraperitoneal injection at 125 mg/kg for 40 d) and clobenpropit (every other day intraperitoneal injection at 20 μmol/L per kilogram for 40 d)[23,30]. The body weight of each mouse was measured weekly with an electronic scale. Tumor size was measured every week with electronic calipers and the volume was calculated by the following formula: tumor volume = (length × width^2) × π/6[31]. One week later, after finishing the treatment schedule, mice were anesthetized with isoflurane and tissue, organs and tumors were harvested for analysis.

The expression of EMT markers was investigated by real-time PCR in whole tumor mRNA. Tumor samples were fixed in 10% buffered formalin, embedded in low-temperature fusion paraffin, and sectioned for terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) staining and immunohistochemical staining. Western blots were conducted for the assessment of E-cadherin, vimentin, MMP-9 and Zeb1 expressions. Antibodies of E-cadherin, vimentin, MMP-9 and Zeb1 were purchased from Santa Cruz.

Statistical analysis

All experimental results represent at least 3 indepen-

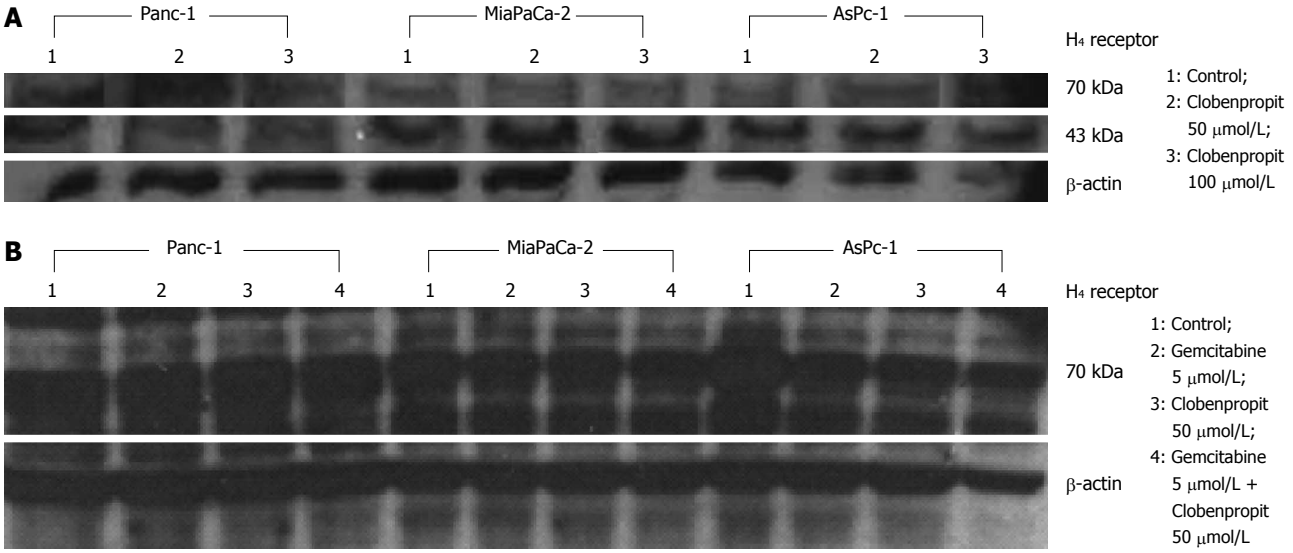


Figure 1 Western blotting of histamine receptors in pancreatic cancer cells. Cellular extracts of Panc-1, MiaPaCa-2 and AsPC-1 were separated by SDS-PAGE, transblotted onto nitrocellulose membranes and detected using anti-H_4 receptor antibodies. Pancreatic cancer cell lines were treated with different concentrations of clobenpropit and/or gemcitabine, and Western blotting was performed. The specific antibody to H_4 receptor showed immunoreactivity mainly as a band at 70000 and 43000 Da. Expression of H_4 receptor was still present after administration of clobenpropit alone (A) or combination with gemcitabine (B).

dent experiments using cells from a minimum of three separate isolations. Results for continuous variables are expressed as mean ± SE of mean and compared with the Kruskal-Wallis ANOVA followed by Dunn's multiple comparison test or repeated measures ANOVA. $P < 0.05$ was considered statistically significant. Analysis was performed with GraphPad Prism version 5.04 (GraphPad Software Inc., La Jolla, CA).

RESULTS

H_4 receptor was present in pancreatic cancer cells

The specific antibody to H_4 receptor showed immunoreactivity mainly as a band at 70000 and 43000 Da in Panc-1, MiaPaCa-2 and AsPC-1 (Figure 1A). However, there was no H_3 receptor expression in all cell lines (data not shown). When clobenpropit (50 μmol/L) and/or gemcitabine (5 μmol/L) were added, H_4 receptor expression was also seen but was not significantly changed in all cell lines (Figure 1B).

Clobenpropit inhibited cell migration by inhibition of EMT process

The inhibition of cell migration was assessed by a wound healing assay. Clobenpropit inhibited the migration of pancreatic cancer cells in a wound healing assay (Figure 2). Wound healing ranges were decreased when clobenpropit concentration was higher. In addition, the migration rate was also inhibited after treatment with gemcitabine and clobenpropit combination compared with control and gemcitabine or clobenpropit alone (Figure 3).

To investigate the mechanism of clobenpropit on cell migration, real-time PCR for epithelial and mesenchymal markers was performed. E-cadherin was about 4-fold increased after treatment of clobenpropit in Panc-1. MMP-9 was reduced in MiaPaCa-2, and vimentin and MMP-9 were reduced to about half after treatment of clobenpropit in AsPC-1 (Figure 4). Therefore, clobenpropit down-regulated epithelial markers, while up-regulating mesenchymal markers; this means that clobenpropit might disrupt EMT process of pancreatic cancer cells.

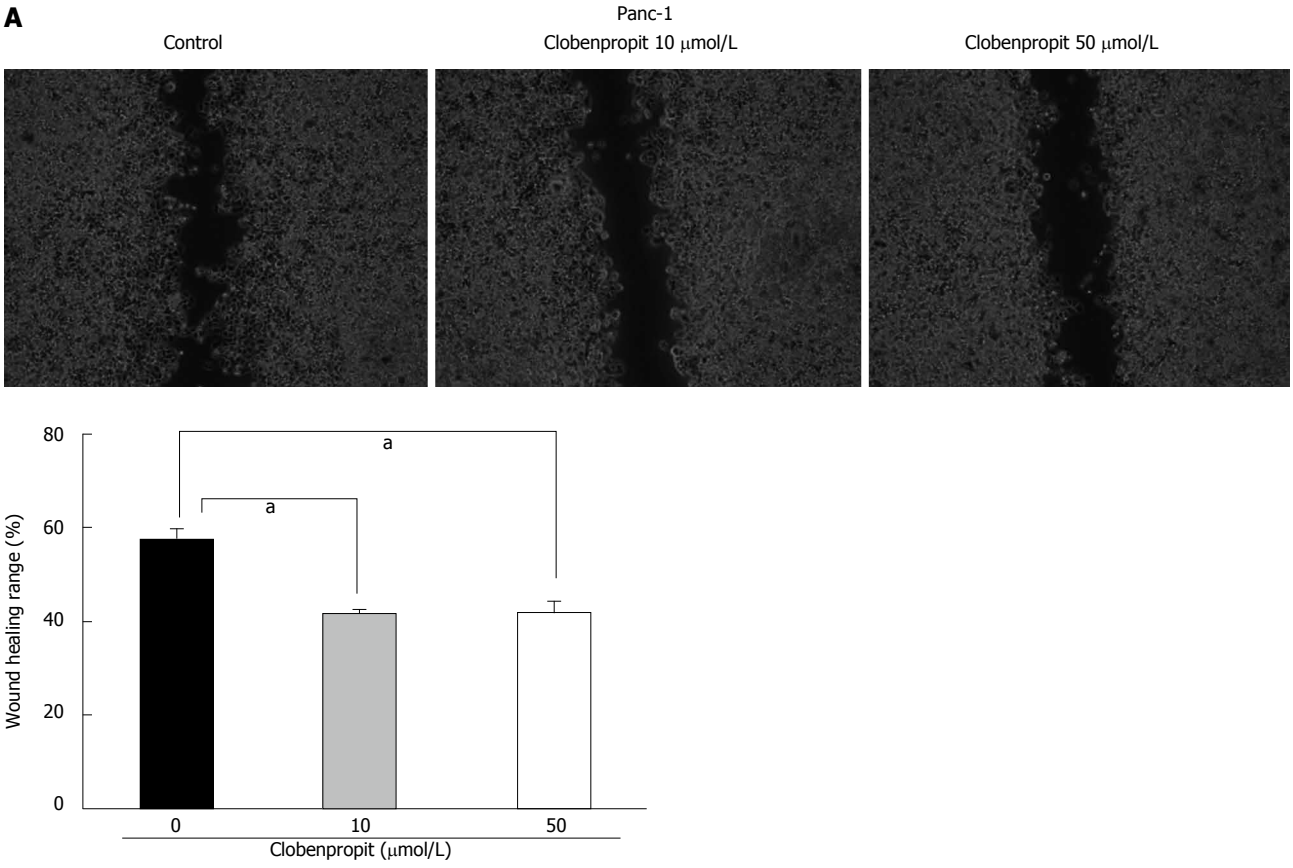
Clobenpropit enhanced gemcitabine-induced apoptosis

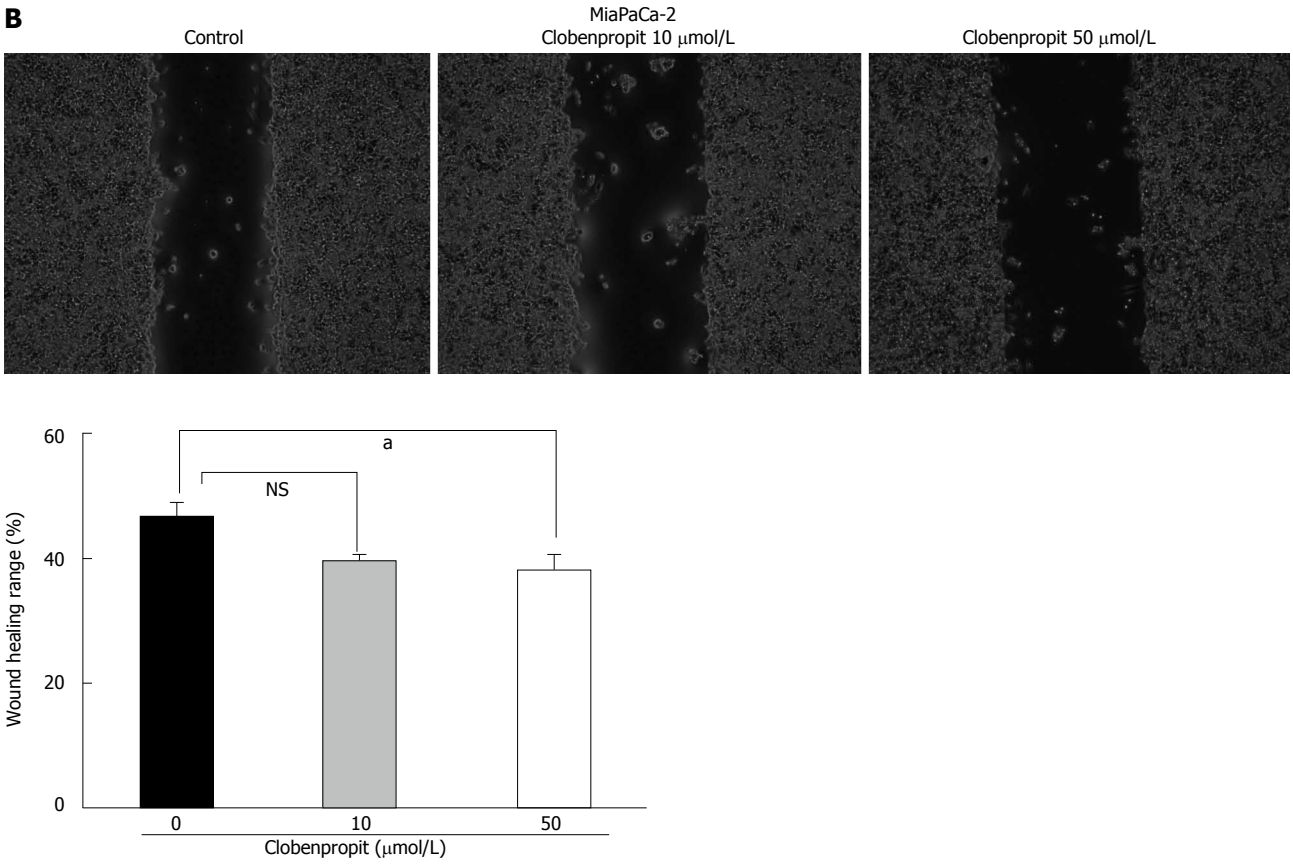
Clobenpropit alone did not affect the proliferation of pancreatic cancer cells (Figure 5). However, the exposure to gemcitabine (5 μmol/L) and/or clobenpropit (50 μmol/L) induced apoptosis of pancreatic cancer cells. Gemcitabine and clobenpropit combination therapy significantly increased apoptosis of Panc-1, MiaPCa-2 and AsPC-1 compared with control (Figure 6). However, gemcitabine alone did not increase apoptosis of pancreatic cancer cells significantly compared with control.

Xenograft mouse model

In the Panc-1 xenograft mouse model, the body weight of mice was checked every week (Figure 7A). There was no significant difference in mean body weight between the groups. There was no mortality of mice until the end of treatment. The combination treatment of gemcitabine with clobenpropit showed significant tumor growth inhibition compared with other treatment groups (control 501 ± 92 mg, gemcitabine 294 ± 46 mg, clobenpropit 444 ± 167 mg, and combination 154 ± 54 mg, Figure 7B and C).

E-cadherin was up-regulated after clobenpropit administration in real-time PCR (Figure 8A). Immunohistochemical staining also showed up-regulation of E-cadherin in clobenpropit alone and combination group (Figure 8C). To quantify apoptosis of tumors, TUNEL staining was performed. The percentage of TUNEL-


A
Panc-1
Control
Clobenpropit 10 μmol/L
Clobenpropit 50 μmol/L
Wound healing range (%)
80
60
40
20
0
a
a
0
10
50
Clobenpropit (μmol/L)


B
MiaPaCa-2
Control
Clobenpropit 10 μmol/L
Clobenpropit 50 μmol/L
Wound healing range (%)
60
40
20
0
a
NS
0
10
50
Clobenpropit (μmol/L)

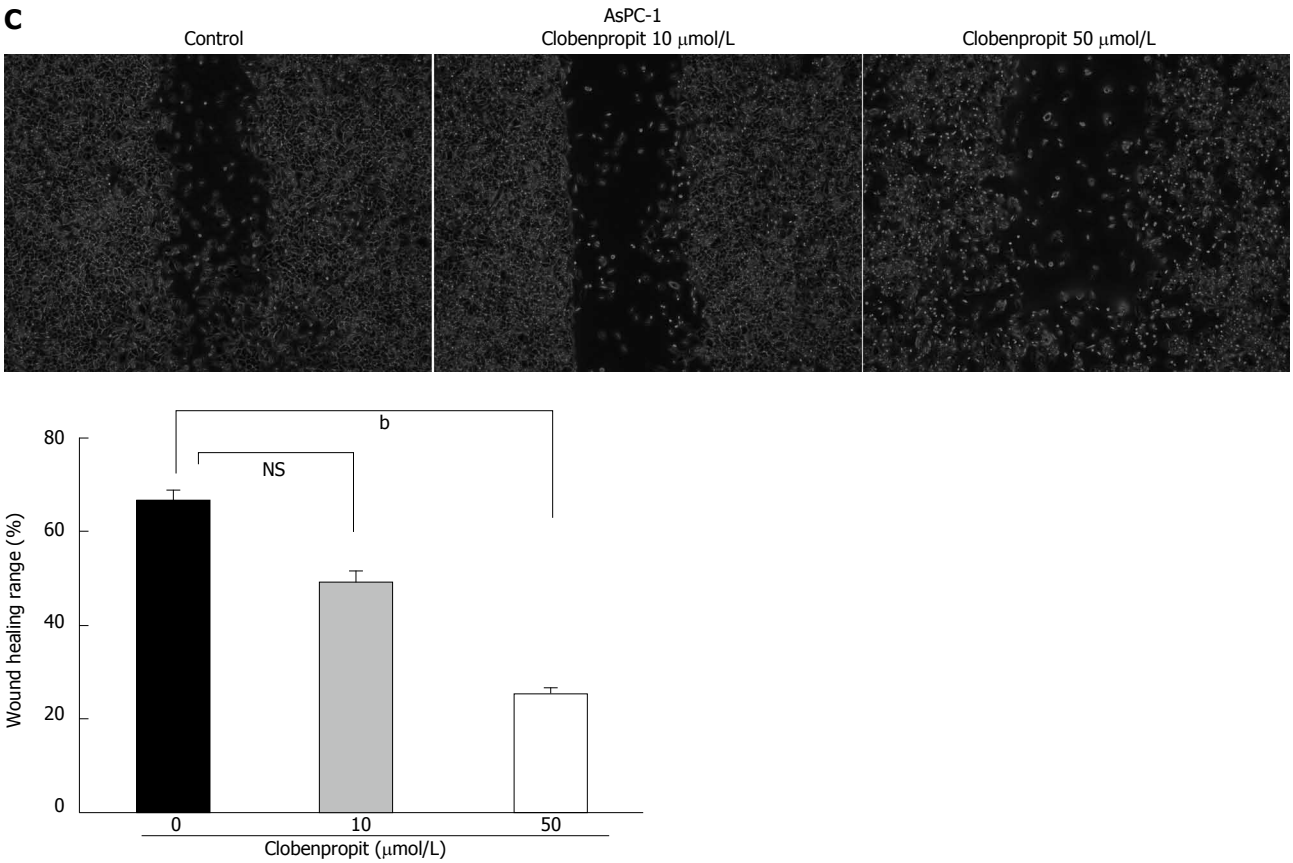


Figure 2 Effect of clobenpropit on cell migration. Clobenpropit inhibits the migration of Panc-1 (A), MiaPaCa-2 (B) and AsPC-1 (C) in wound healing assay. Wound healing range was inversely correlated with clobenpropit concentration in MiaPaCa-2 and AsPC-1. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control.

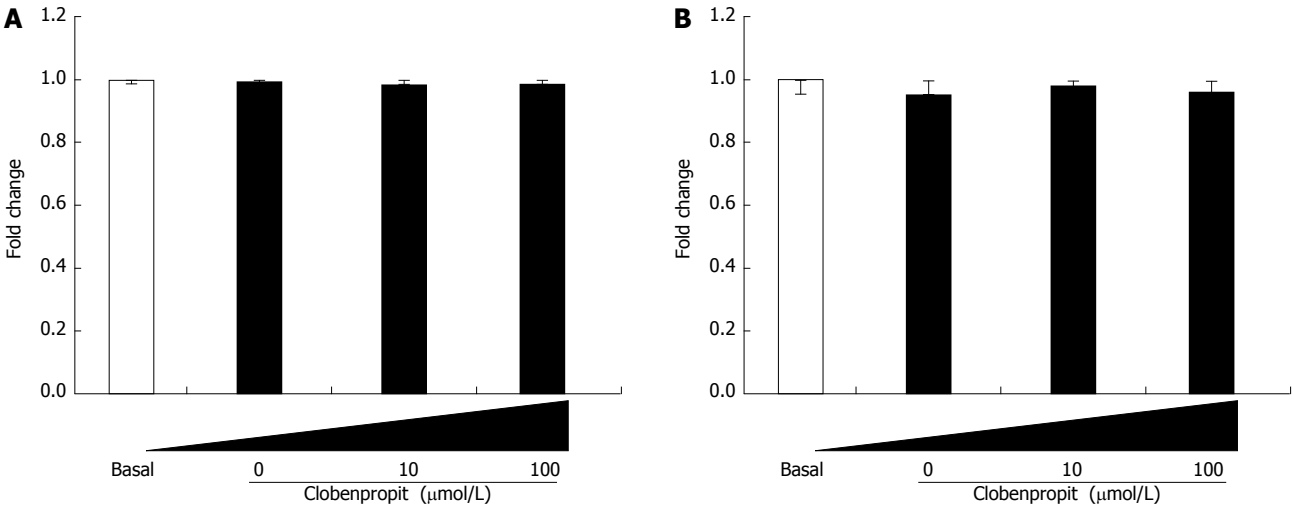


Figure 3 Effect of clobenpropit on pancreatic cancer cell proliferation was evaluated by MTS assay. A: Panc-1, B: AsPC-1. Clobenpropit showed no cytotoxicity in pancreatic cancer cells.

stained cells was increased more in the gemcitabine and clobenpropit combination group compared with other groups (control 2.5%, gemcitabine 25.8%, clobenpropit 9.7% and combination 40.9%, Figure 8B). The expression of E-cadherin was also increased in clobenpropit alone and combination group by Western blotting, whereas Zeb1 expression, a repressor of E-cadherin, was decreased in combination group (Figure 8D).

DISCUSSION

The role of histamine and its receptors in carcinogenesis is complex and somehow confusing. We evaluated the effect of a specific histamine receptor and its agonist against human pancreatic cancer cells in this study. The significant findings in the present study are that clobenpropit emphasized gemcitabine-induced apoptosis of hu-

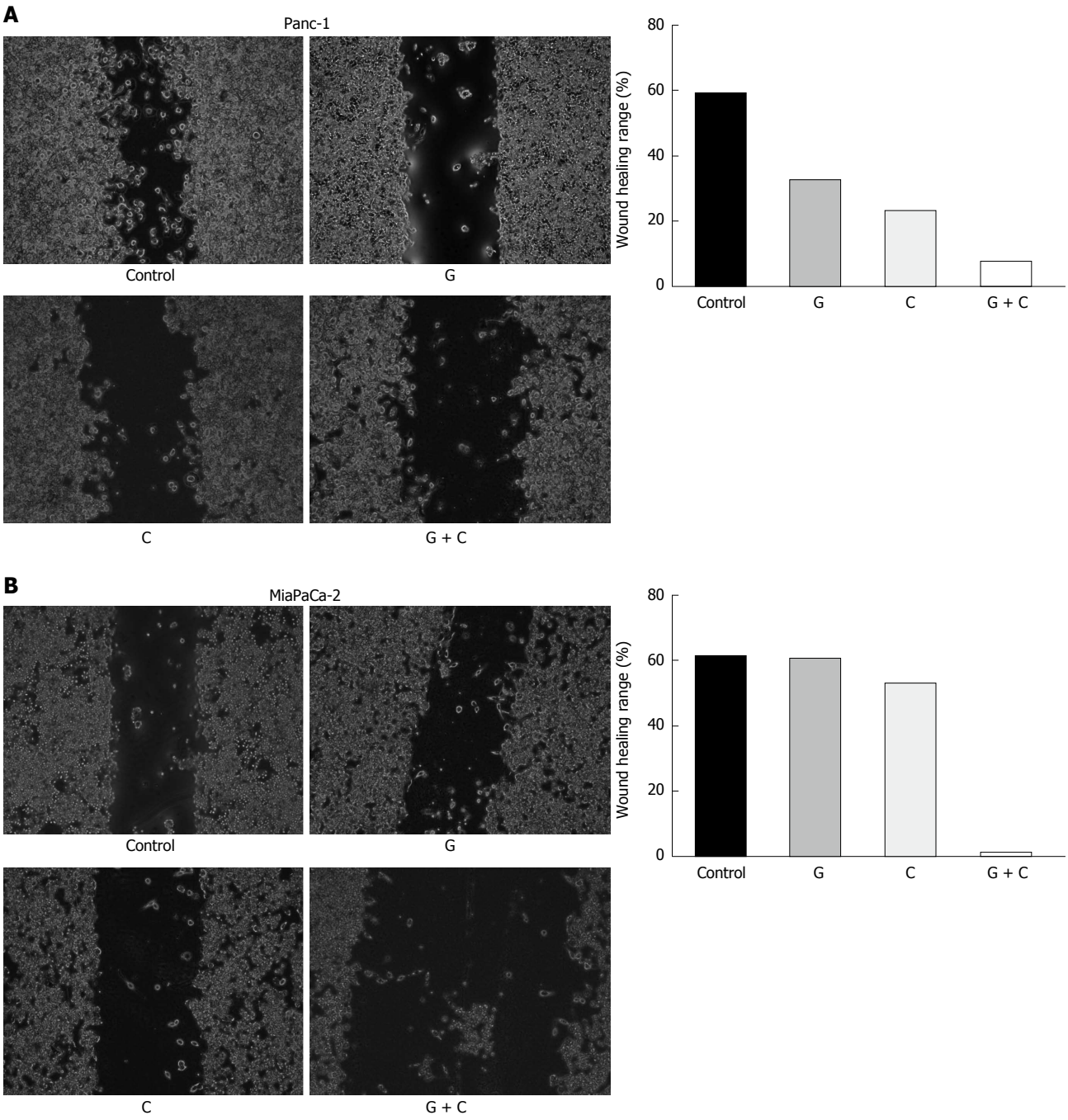


Figure 4 Migration of pancreatic cancer cells was inhibited after gemcitabine and clobenpropit combination in wound healing assay. The concentrations of gemcitabine in Panc-1 (A) and MiaPaCa-2 (B) were 5 and 15 μmol/L, respectively. The concentration of clobenpropit was 50 μmol/L. G: Gemcitabine; C: Clobenpro-

man pancreatic cancer cells *in vitro* in cell culture as well as *in vivo* in tumor xenograft mice. There was no adverse health effect due to clobenpropit in mice as monitored by body weight. The enhanced cytotoxicity of gemcitabine and clobenpropit combination might result from disruption of EMT through H_4 receptor.

Three kinds of pancreatic cancer cells (Panc-1, MiaPaCa-2 and AsPC-1) were used in this study, and all the cells had H_4 receptors. The H_4 specific antibody showed immunoreactivity mainly as a band at 73000 and 40000 Da, which is consistent with a previous report[32].

Increasing evidence indicates that cancer cells are subjected to the EMT, a process by which epithelial cells undergo remarkable morphologic changes characterized by a transition from an epithelial to a mesenchymal phenotype leading to increased motility and invasion[33]. EMT is a developmental process which plays an important role in tumor progression and metastasis in diverse solid tumors, including pancreatic cancer[25]. E-cadherin, a calcium-dependent transmembrane glycoprotein, is one of the most important molecules in cell-cell adhesion in epithelial tissue[34], which localized on the surface of epithelial cells in regions of adherent junctions[35]. It plays a major role in malignant cell transformation, and especially in tumor development and progression. Loss of E-cadherin is associated with invasion and metastasis of

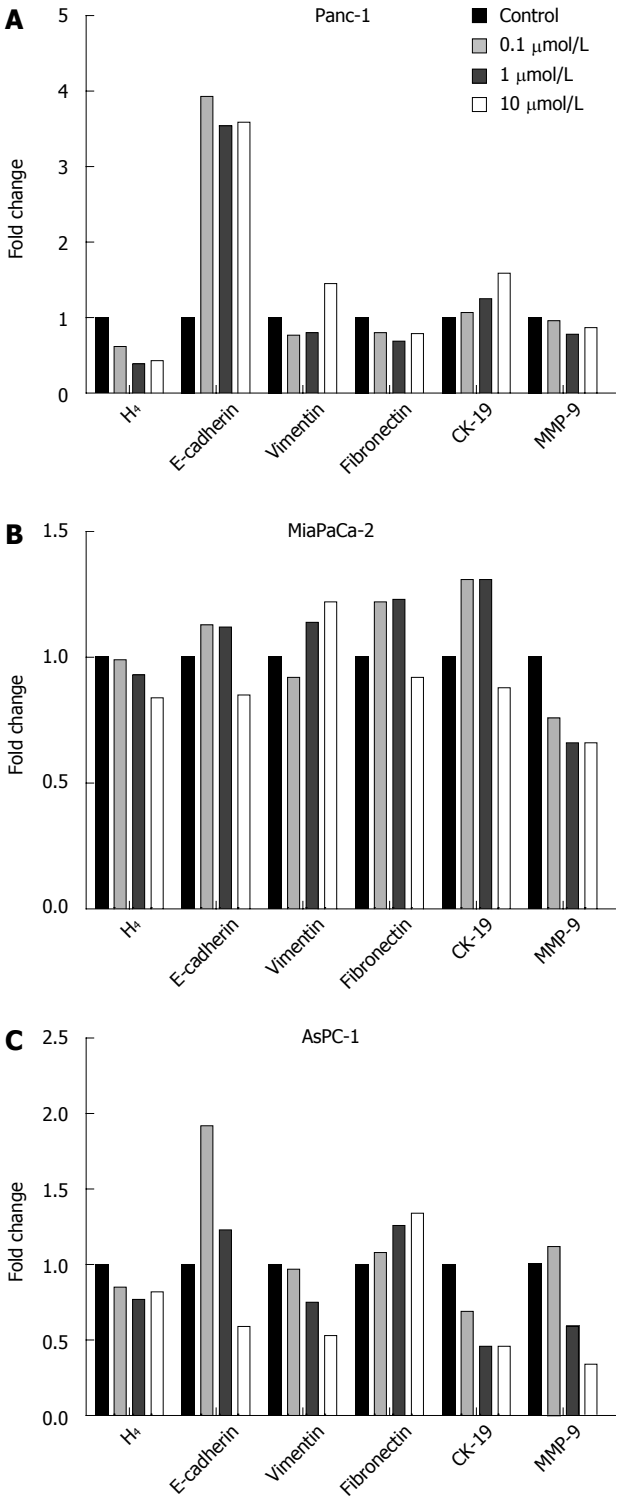


Figure 5 Changes of epithelial and mesenchymal markers of pancreatic cancer cells by clobenpropit. Real-time polymerase chain reaction shows that E-cadherin was up-regulated (A), whereas MMP-9 and vimentin were down-regulated (B, C) in pancreatic cancer cells after treatment with different concentrations of clobenpropit.

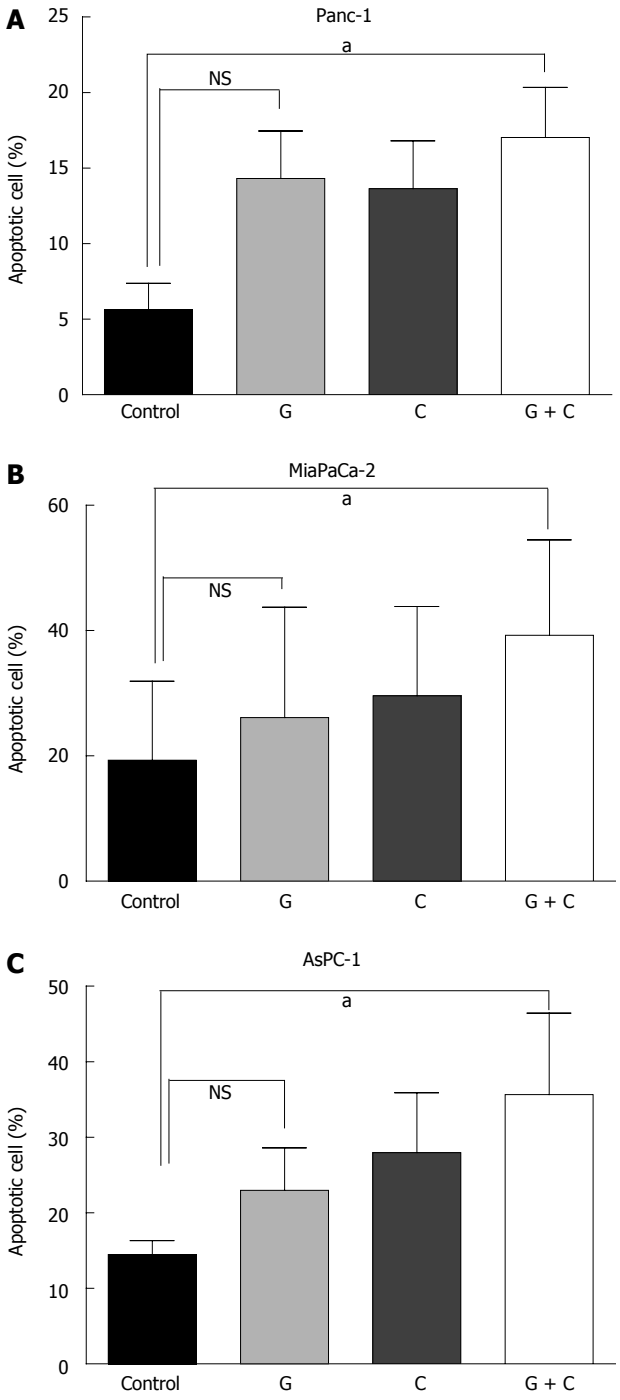


Figure 6 Enhanced apoptosis of pancreatic cancer cells by gemcitabine and clobenpropit combination treatment. Clobenpropit enhanced apoptotic cell death in combination of gemcitabine in human pancreatic cancer cells. The percentage of apoptotic cells was determined by fluorescein isothiocyanate-labeled annexin V assay followed by flow cytometry. Statistically significant differences ($^{a}P < 0.05$ *vs* control group) of the combination treatment of gemcitabine (5 μmol/L) and clobenpropit (50 μmol/L) compared with control in Panc-1 (A), MiaPaCa-2 (B) and AsPC-1 (C). G: Gemcitabine; C: Clobenpropit.

tumors[36]. Furthermore, the loss of E-cadherin expression has been associated with a poor clinical outcome in several cancers[25,37,38]. The H_4 receptor agonist increased the expression of E-cadherin in this study, both *in vitro* in cell culture and *in vivo* in xenograft mouse. Clobenpropit could play an important role with interfering cell migration and increasing chemosensitivity of gemcitabine in pancreatic cancer cells through inhibition of EMT process and up-regulation of E-cadherin.

Transcriptional repressors of E-cadherin such as Zeb1, Zeb2, Twist, Snail and Slug are associated with

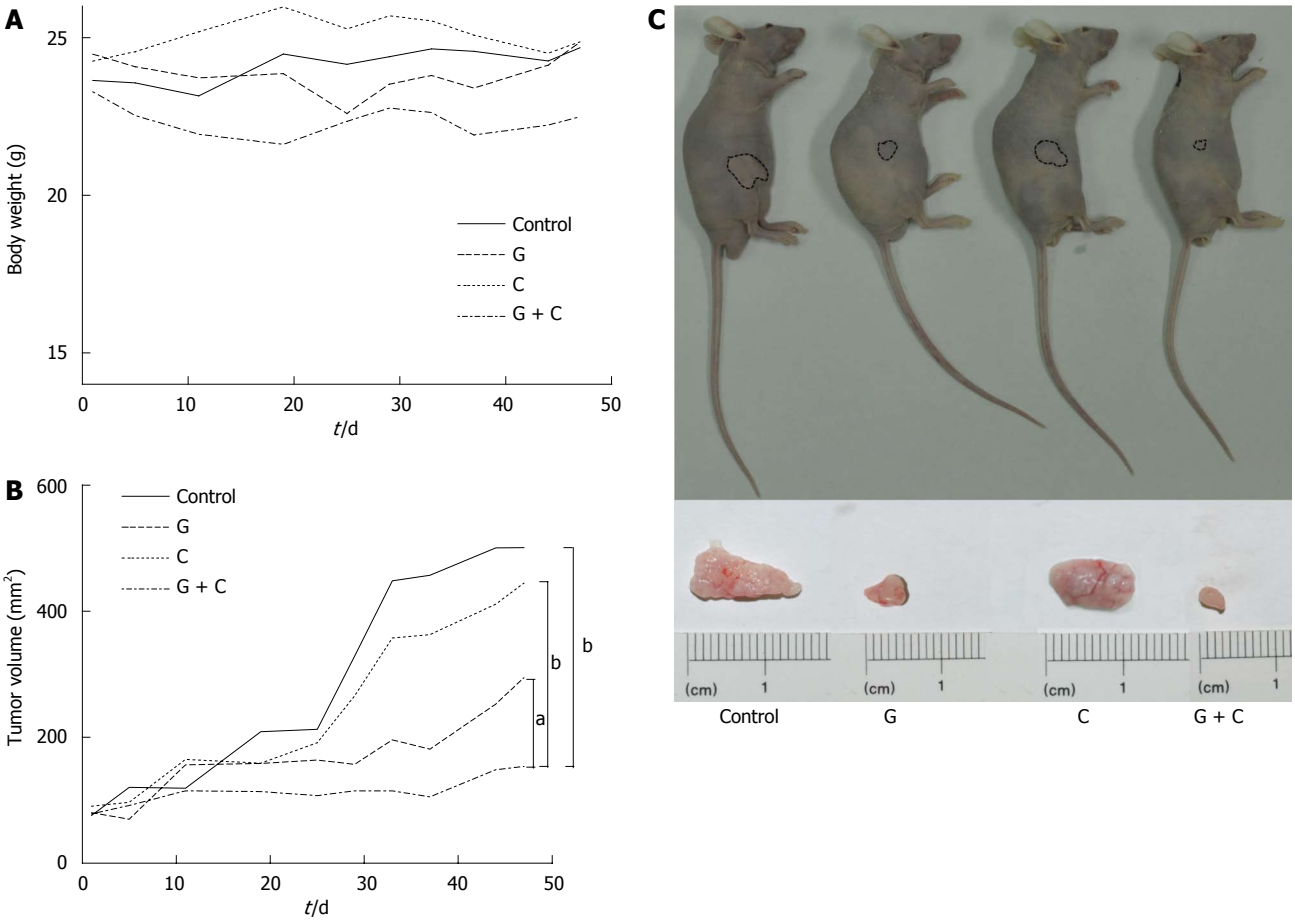


Figure 7 Inhibition of tumor growth in Panc-1 xenograft mouse by gemcitabine and clobenpropit combination treatment. Body weight (A) and tumor volume (B) curves for Panc-1 xenograft mouse model with administration of vehicle (control), gemcitabine, clobenpropit or their combination. There was no significant difference of body weight between the groups. Tumor volume of gemcitabine and clobenpropit combination therapy group was significant lower than control and other treatment groups. Tumor bearing mice and excised tumor of each treatment group (C). G: Gemcitabine; C: Clobenpropit. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control.

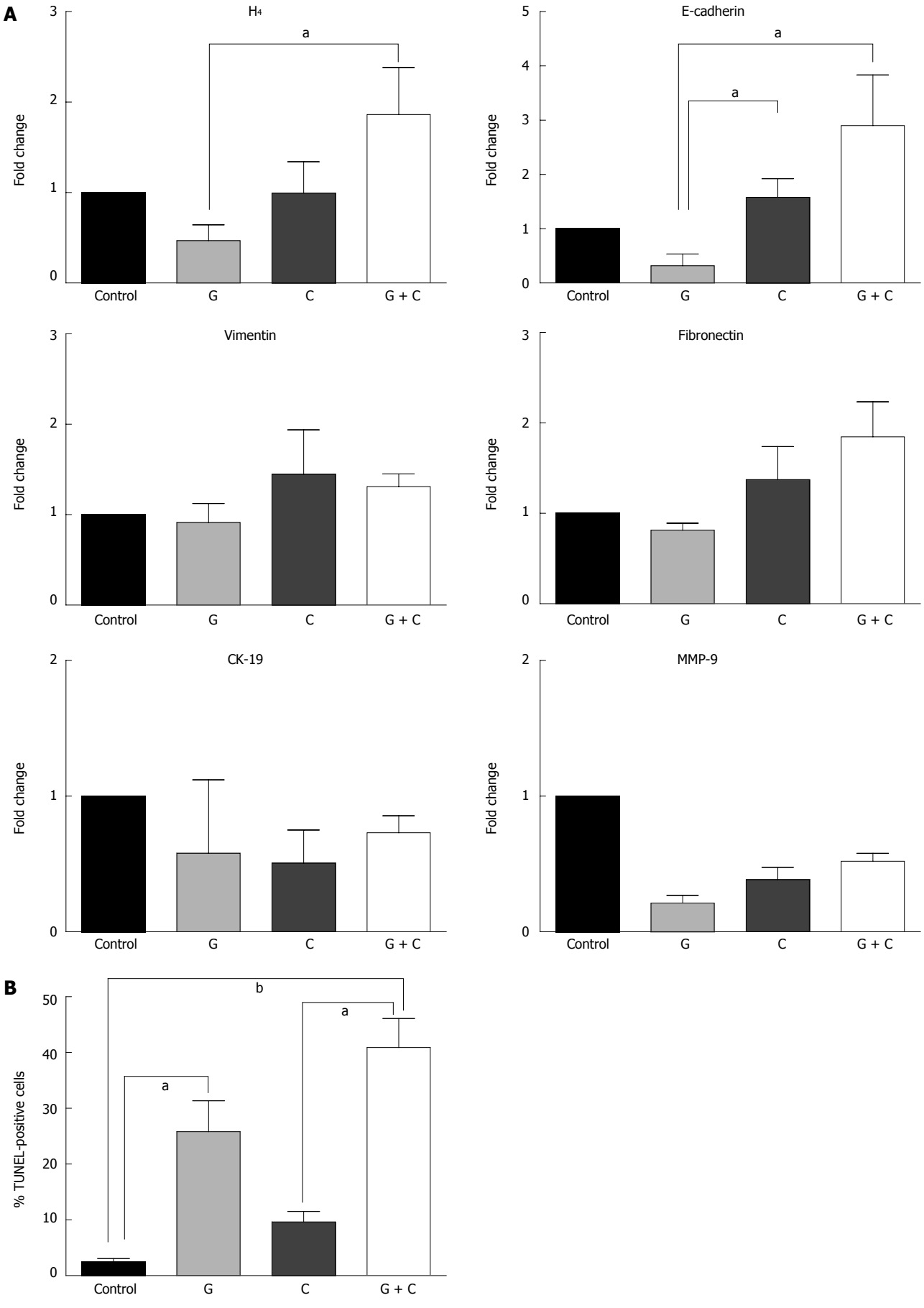
EMT[39-42]. Diverse signal pathways such as Wnt cascade, TGF-β and PI3K/Akt pathway are connected with these transcriptional repressors of E-cadherin[43,44]. Zeb1 expression was decreased in the clobenpropit treated mice group compared with the control and gemcitabine alone groups. Zeb1 could act as the main transcriptional repressor of E-cadherin in this study although the relationship between Zeb1 and H_4 receptor remains unsolved.

Vimentin is a mesenchymal marker which is up-regulated with EMT[45]. Down-regulation of vimentin after clobenpropit administration suggests that H_4 receptor agonist disrupts the EMT process. For the invasion and metastasis of tumors, breakdown of the ECM should be present[46]. Clobenpropit may protect the ECM from breakdown by down-regulation of MMP-9, preventing invasion or metastasis of pancreatic cancer.

The change of epithelial markers or mesenchymal markers after administration with clobenpropit was different according to the cells. E-cadherin was increased in Panc-1 only, while vimentin was decreased in AsPC-1 only. MMP-9 was decreased in MiaPaCa-2 and AsPC-1. This can be explained by the different expressions of EMT markers according to the cancer cells[25]. However, it was coherent that the change of EMT markers indicated the disruption of the EMT process by clobenpropit in this study.

The chemosensitivity of pancreatic cancer cells varies, and Panc-1, MiaPaCa-2 and AsPC-1 are known to be resistant to gemcitabine[25]. In order to clarify the effect of clobenpropit, we used the most chemoresistant cells (Panc-1) in the xenograft model. It is known that pancreatic cancer cells undergoing EMT with increased expression of Snail and Twist become invasive and develop chemoresistance[47]. In addition, EMT reversion by silencing Zeb1 increases cellular sensitivity to gemcitabine[25]. The apoptosis of pancreatic cancer cells was significantly increased after gemcitabine and clobenpropit combination treatment in present study. Moreover, the tumor volume of the xenograft mouse was significantly decreased in the combination group compared with control and clobenpropit or gemcitabine alone groups, and TUNEL stain also showed increased apoptosis in the combination group. These results support the idea that therapeutic targeting to reverse EMT could increase chemosensitivity in pancreatic cancer. However, further studies are needed to clarify the molecular alterations which reverse EMT through H_4 receptor.

In conclusion, clobenpropit enhanced gemcitabine-induced apoptosis in human pancreatic cancer cells by inhibition of EMT process. The novel role of the H_4 re-


A
H4
E-cadherin
Vimentin
Fibronectin
CK-19
MMP-9
Fold change
Control
G
C
G + C
a
B
% TUNEL-positive cells
b

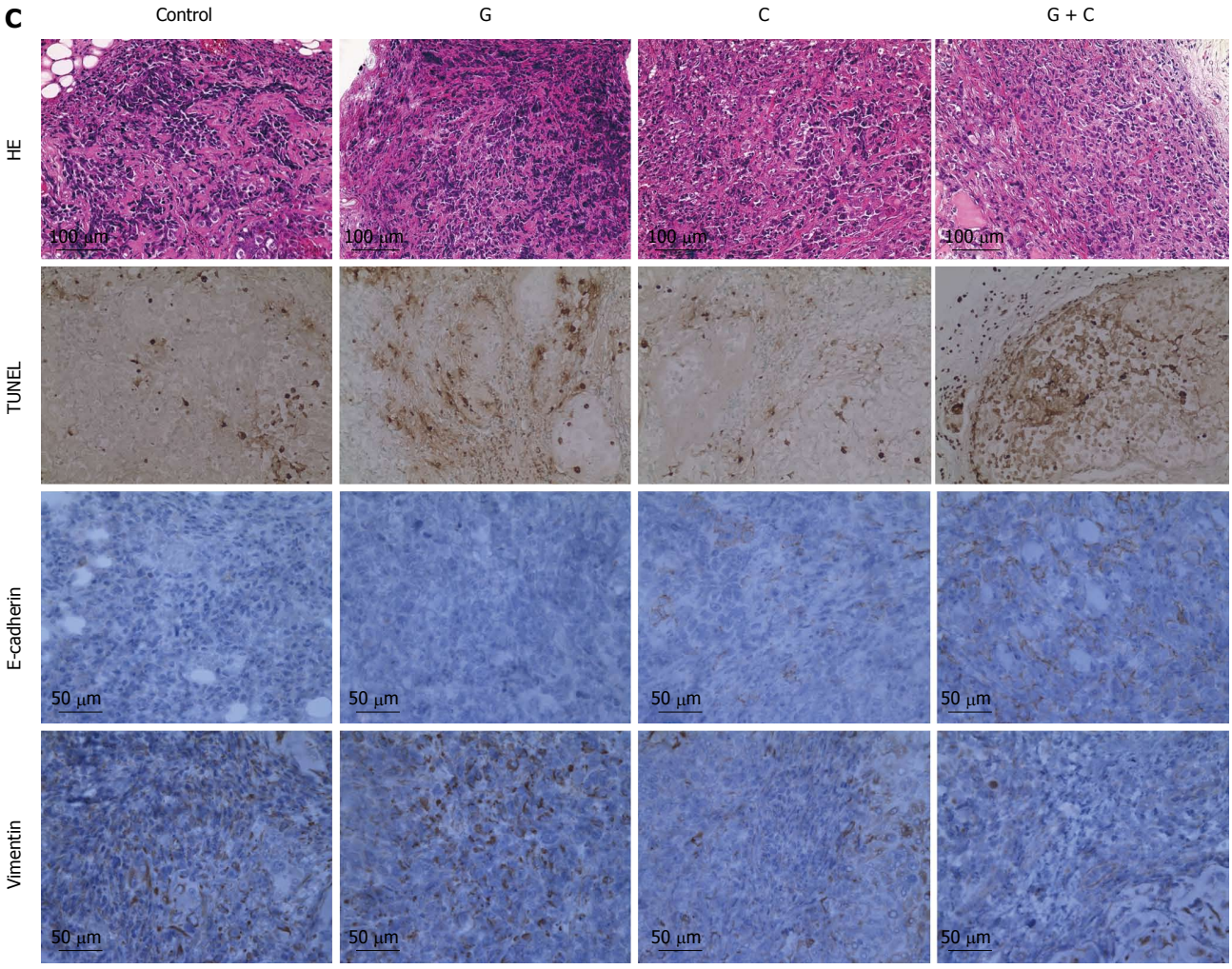


Figure 8 Effect of gemcitabine and clobenpropit combination treatment in Panc-1 xenograft mouse. A: Real-time polymerase chain reaction shows increased E-cadherin expression after clobenpropit treatment compared with gemcitabine alone; B: Immunohistochemical staining shows up-regulation of E-cadherin in gemcitabine and clobenpropit combination group. Apoptotic index calculated with TUNEL staining shows increased apoptosis in gemcitabine and clobenpropit combination group; C: Pathological evaluation of tumor tissue determined by hematoxylin and eosin staining, TUNEL staining and immunohistochemistry of E-cadherin and vimentin; D: E-cadherin was also increased in clobenpropit alone and combination group by Western blotting, whereas Zeb1, the repressor of E-cadherin, was decreased in combination group. G: Gemcitabine; C: Clobenpropit. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control.

ceptor in carcinogenesis of pancreatic cancer represents a new therapeutic molecular target and clobenpropit could be a promising drug. Further studies are required to reveal the mechanism of EMT inhibition *via* H_4 receptor.

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COMMENTS

Background

Histamine is associated with carcinogenesis through activation of its 4 membrane-specific receptors. Pancreatic cancer, which is a very aggressive human cancer, is highly resistant to conventional chemotherapy. Therefore, new treatment options including targeting histamine receptors in pancreatic cancer are required.

Research frontiers

Recently, the modulation of the histamine receptor by clobenpropit disrupts

the epithelial-mesenchymal transition (EMT) process, and results in inhibition of tumor growth in cholangiocarcinoma. Similarly, EMT plays a critical role in tumor progression and metastasis of pancreatic cancer. The research hotspot is to evaluate the anti-tumor effect and mechanism of clobenpropit in pancreatic cancer.

Innovations and breakthroughs

Clobenpropit emphasized gemcitabine-induced apoptosis of pancreatic cancer. The enhanced cytotoxicity of gemcitabine and clobenpropit may result from disruption of EMT through H_4 receptor.

Applications

The study results suggest that the combination of clobenpropit and gemcitabine may be a promising treatment in pancreatic cancer.

Peer review

The present study reported that clobenpropit enhanced the anti-tumor effect of gemcitabine in pancreatic cancer cells through inhibition of the EMT process. The authors' experimental methods, including *in vitro* and *in vivo* experiments, are not only appropriate but also clear for getting results. Accordingly, these results about anti-tumor effects *via* inhibition of EMT are helpful for a new paradigm of pancreatic cancer therapy.

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