Curcumin inhibits hypoxia-induced epithelial-mesenchymal transition in pancreatic cancer cells via suppression of the hedgehog signaling pathway

LEI CAO[1*], XUE XIAO[1*], JIANJUN LEI[2], WANXING DUAN[2], QINGYONG MA[2] and WEI LI[2]

[1]Department of Pharmacology, School of Basic Medical Sciences, Xi'an Jiaotong University
Health Science Center, Xi'an, Shaanxi; [2]Department of Hepatobiliary Surgery, The First
Affiliated Hospital of Xi'an Jiaotong University, Xi'an, Shaanxi 710061, P.R. China

Abstract. Hypoxic microenvironment, a common feature of pancreatic cancer, is associated with tumor proliferation, metastasis and epithelial-mesenchymal transition (EMT) changes. In recent years, many natural agents, including curcumin, have been proven to possess the ability to inhibit the progression of pancreatic cancer. However, whether curcumin is able to suppress hypoxia-induced pancreatic cancer progression and the underlying mechanisms are still not fully elucidated. The aim of the present study was to evaluate whether curcumin affects hypoxia-induced EMT and the activation of Hh signaling pathway in pancreatic cancer. The human pancreatic cancer cell line Panc-1, was treated with hypoxic condition and curcumin. Cell proliferation was assessed by the MTT assay. Wound healing assay and Transwell invasion assay were used to detect the migratory and invasive activity of cancer cells. The EMT-related factors, E-cadherin, N-cadherin, vimentin were detected by QT-PCR, western blot analysis and immunofluorescence staining. The Hh signaling-related factors, SHH, SMO and GLI1 were detected by western blot analysis. The results of present study showed that curcumin could not only inhibit the hypoxia-induced cell proliferation, migration and invasion in pancreatic cancer, but also mediate the expression of EMT-related factors. In addition, curcumin remarkably inhibited hypoxia-mediated activation of Hh signaling pathway. Taken together, these data indicate that curcumin plays an important role in suppressing hypoxia-induced pancreatic cancer metastasis by inhibiting the Hh signaling pathway. Curcumin might be a potential candidate for chemoprevention of this severe disease.

Correspondence to: Dr Wei Li, Department of Hepatobiliary Surgery, The First Affiliated Hospital of Xi'an Jiaotong University, 277 West Yanta Road, Xi'an, Shaanxi 710061, P.R. China
E-mail: 13572512207@163.com; 289421999@qq.com

*Contributed equally

Introduction

Pancreatic cancer is one of the most aggressive malignant diseases with a median survival time of less than 6 months and a 5-year survival rate of <5% (1). In 2015, it was estimated that 48,960 subjects will be newly diagnosed with pancreatic cancer and will account for 40,560 cancer-related deaths in the United States (1). Surgical resection remains the only way to cure this severe disease, however, due to the high metastatic rate, the majority of patients are diagnosed at an advanced inoperable stage, and less than 20% of patients are amenable for surgery (2). Even those patients with seemingly resectable pancreatic tumor are not always cured by surgery due to the microscopic systemic spread of cancer cells before the operation intervention (3). Currently, there are very limited therapeutic options for pancreatic cancer, therefore, more constructive and effective interventions for targeting cancer metastasis are urgently needed.

Hypoxia (low oxygen tension) is commonly found in solid tumors more than a few millimeters in size and is associated with a poor prognosis (4,5). Tumor hypoxia is strongly associated with enhanced tumor invasiveness, angiogenesis and distant metastasis (4). Hypoxia-inducible factor-1 (HIF-1), which belongs to the basic helix-loop-helix-periodic acid-Schiff domain transcription factor family, is a key mediator of the cellular response to hypoxia and is overexpressed in a wide variety of solid tumors, including pancreatic cancer (5). Our previous study identified that the Hedgehog (Hh) signaling modulated hypoxia induced pancreatic cancer epithelial to mesenchymal transition (EMT) and invasion (5).

EMT has been recognized both as a physiological mechanism for development and tissue remodeling, and as a pathological mechanism in cancer progression, during which cells lose their polarized epithelial traits and acquire mesenchymal characteristics (6,7). The initiation of cancer metastasis requires migration and invasion of cells, which is enabled by EMT (8). EMT cells are a resource for cancer stem-like cells which are more resistant to therapies, because of the survival advantage with increased anti-apoptotic activities (9). A typical characteristic of EMT is the loss of the cell-cell adhesion molecule E-cadherin expression and

gain of mesenchymal markers, such as vimentin, N-cadherin and others (7). Our recent study showed that hypoxic condition was able to induce EMT in HepG2 hepatocellular carcinoma cells (6).

Curcumin is a natural polyphenol present in turmeric that possess many biological activities, including anti-infectious, anti-inflammatory, anti-oxidant and chemopreventive effects (10). Recent studies have shown that curcumin is able to inhibit the proliferation, invasion and metastasis of a variety of tumor cells (10,11). Multiple cellular signaling pathways have been proven to be regulated by curcumin in cancer treatment including mitogen-activated protein kinase (MAPK), nuclear factor-κB (NF-κB), Akt, Wnt/β-catenin and others (10). Sun *et al* (12) recently indicated that curcumin could reverse pancreatic cancer progression by inhibiting the Hh signaling pathway. The Hh signaling pathway, which is considered to play an important role in tumorigenesis, is normally quiescent in adult pancreas and has been shown to be very active in pancreatic cancer (5). Our previous study showed that hypoxia-induced invasion and EMT process is intimate related with the Hh signaling pathway (5).

In the present study, we tested the hypothesis that curcumin is able to inhibit hypoxia-induced proliferation, invasion and migration as well as EMT progression of pancreatic cancer cells. We also investigated the effect of curcumin on hypoxia-induced activation of Hh pathway. Results from this study suggest that curcumin treatment may be a novel option for therapy of pancreatic cancer via the inhibition of the Hh signaling pathway.

Materials and methods

Cell culture and reagents. The human pancreatic cancer cell line, Panc-1, was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured in DMEM medium containing 10% dialyzed heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37°C. In experiments designed to assess the role of hypoxia, cells were first cultured in normoxic conditions to obtain the desired subconfluence level (65-70%) and then were incubated in strictly controlled hypoxic conditions (1% O_2). Exponentially growing cells in complete medium were pretreated for 1 h with 20 μM curcumin, followed by continual incubation in normal culturing conditions or hypoxic conditions for indicated time intervals according to the purpose of the experiment. Dulbecco's modified Eagle's medium (DMEM) and FBS were from Gibco (Grand Island, NY, USA). Curcumin was purchased from Sigma-Aldrich (St. Louis, MO, USA). Millicell Transwells for the invasion assays were obtained from Millipore (Billerica, MA, USA). Matrigel was from BD Biosciences (Bedford, MA, USA). Primary antibodies against E-cadherin, N-cadherin, vimentin, SHH, SMO and GLI1 were procured from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Nitrocellulose membranes were from Millipore. The BCA assay kit and the chemiluminescence kit were from Pierce (Rockford, IL, USA). Other reagents were purchased from common commercial sources. All drug solutions were freshly prepared on the day of testing.

MTT proliferation assays. Panc-1 cells were seeded in 96-well plates at the density of $1x10^4$ cells/well and incubated overnight in 10% FBS medium. The cells were then treated with curcumin in normoxic or hypoxic condition. After incubation for 24, 48 and 72 h at 37°C, 15 μl of MTT solution (5 mg/ml in phosphate-buffered saline (PBS) was added to each well, and then the cells were incubated for 4 h at 37°C. DMSO (100 μl) was then added to each well. The optical density (OD) value at 490 nm was determined using a spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA).

Wound healing assay. Cell migratory ability was detected by a wound-healing assay. Panc-1 cells were seeded in 24-well plates ($1.0x10^5$ cells/500 μl). After the cells grew to 90-100% confluence, a sterile pipette tip was used to produce a wound line between the cells. Cellular debris was removed by washing with PBS and then allowed to migrate for 24 h. Images were taken at time 0 and 24 h post-wounding under a Nikon Diaphot TMD inverted microscope (x10). The relative distance traveled by the leading edge from 0 to 24 h was assessed using Photoshop software (n=5).

Transwell Matrigel invasion assay. The invasion of pancreatic cancer cells was performed in Transwell chambers. The 8.0 μm pore inserts were coated with 25 μl Matrigel. The cell suspensions ($5x10^4$) were added to the upper chambers in DMEM containing 1% FBS. Simultaneously, 500 ml of DMEM containing 20% FBS was placed in the lower chambers. The cells were allowed to migrate for 48 h at 37°C. The non-invading cells were removed from the upper surface by scraping with a wet cotton swab. After rinsing with PBS, the filter was fixed and stained with crystal violet. The invasion ability was determined by counting the stained cells on the bottom surface. Three random fields were captured at x20 magnification (n=3).

Real-time quantitative PCR (QT-PCR). Total RNA was extracted from the pancreatic cancer cells using the Fastgen200 RNA isolation system (Fastgen, Shanghai, China) according to the manufacturer's protocol. Total RNA was reverse-transcribed into cDNA using the Fermentas RevertAid™ kit (MBI Fermentas, Burlington, Canada). The primer sequences were as follows: E-cadherin: forward, 5'-ATTCTGATTCTGCTG CTCTTG-3' and reverse, 5'-AGTCCTGGTCCTCTTCTCC-3'; N-cadherin: forward, 5'-TGTTTGACTATGAAGGCAGT GG-3' and reverse, 5'-TCAGTCATCACCTCCACCAT-3'; vimentin: forward, 5'-AATGACCGCTTCGCCAAC-3' and reverse, 5'-CCGCATCTCCTCCTCGTAG-3'; β-actin: forward, 5'-GACTTAGTTGCGTTACACCCTTTCT-3' and reverse, 5'-GAACGGTGAAGGTGACAGCAGT-3'.

The PCR reactions consisted of 30 sec at 95°C, followed by 40 cycles of 95°C for 5 sec, 60°C for 30 sec and 72°C for 30 sec. After each QT-PCR experiment, a dissociation curve analysis was conducted. The relative gene expression was calculated using the previously described $2^{-\Delta\Delta Ct}$ method (13).

Western blotting. Proteins were electrophoretically resolved on a denaturing SDS-polyacrylamide gel and electrotransferred onto nitrocellulose membranes. The membranes were initially blocked with 5% non-fat dry milk in Tris-buffered saline (TBS)
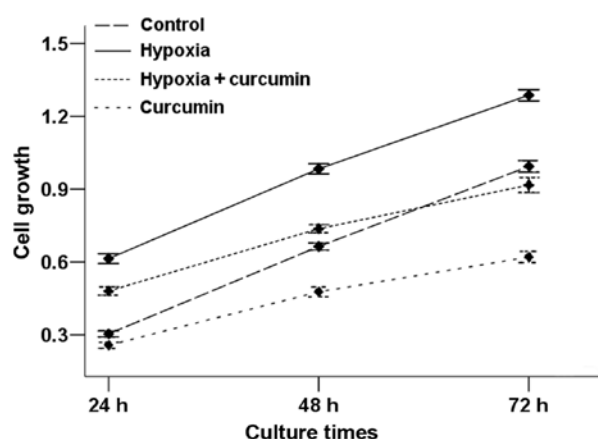
Figure 1. The effects of curcumin on hypoxia-induced pancreatic cancer cell proliferation. Pancreatic cancer cells were treated with hypoxia with or without 20 μM curcumin for 24, 48 or 72 h to analyze the cell proliferation rates.

for 2 h and then probed with antibodies against E-cadherin, N-cadherin, vimentin, SHH, SMO, GLI1 or β-actin (loading control). After co-incubation with the primary antibodies at 4°C overnight, membranes were blotted with the secondary antibody for 2 h at 37°C. The results were visualized using the ECL Western blotting substrate and photographed by GeneBox (SynGene).

Immunofluorescence microscopy. Panc-1 cells were fixed with 4% paraformaldehyde for 10 min at room temperature, permeabilized in 0.5% Triton X-100 for 10 min, and blocked in 1% BSA for 1 h. Fixed cells were then incubated with primary antibodies against E-cadherin (1:100), N-cadherin (1:100) and vimentin (1:200) at 4°C overnight. Cells were washed and incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG for 1 h in the dark. The cells were visualized by a fluorescent microscope (Nikon, Tokyo, Japan) using appropriate excitation and emission spectra at x400 magnification.

Statistical analysis. Statistical analysis was performed using SPSS software (version 17.0; SPSS, Inc., Chicago, IL, USA). Data are presented as the means ± SEM of three replicate assays. Differences between the groups were analyzed by analysis of variance (ANOVA). Statistical significance was set at $P<0.05$. All experiments were repeated independently at least three times.

Results

Curcumin inhibits the proliferation of Panc-1 cells. Our previous study showed that curcumin is able to inhibit the proliferation of HepG2 cells (6). In order to explore the role of curcumin on the proliferative ability of pancreatic cancer cell, Panc-1 cells were treated with hypoxic condition and curcumin alone or in combination. At the time-points indicated in Fig. 1, the proliferative rate of Panc-1 cells was determined by the MTT assay. The results demonstrated that the proliferation of Panc-1 cells increased in hypoxic condition compared with the control group and the increased rate of cell proliferation induced by hypoxic condition was reduced in the presence of curcumin. Curcumin alone was also able to inhibit the proliferative ability of Panc-1 cells.

Curcumin inhibits hypoxia-induced invasive ability of pancreatic cancer cells. The initiation of cancer metastasis requires migration and invasion of cells, which is enabled by EMT (14). In order to confirm whether curcumin could influence hypoxia-induced cancer cell invasive ability, we used a Transwell invasion assay. As shown in Fig. 2, hypoxia exposure significantly increased pancreatic cancer invasive ability, while curcumin decreased the average cell number that invaded into the lower chamber.

Curcumin inhibits hypoxia-induced wound closure of pancreatic cancer cells. Migration is an important aspect that leads to the ability of cancer cells to form metastasis. A wound-healing assay was then used to test the effect of curcumin on hypoxia-induced pancreatic cancer cell motility.
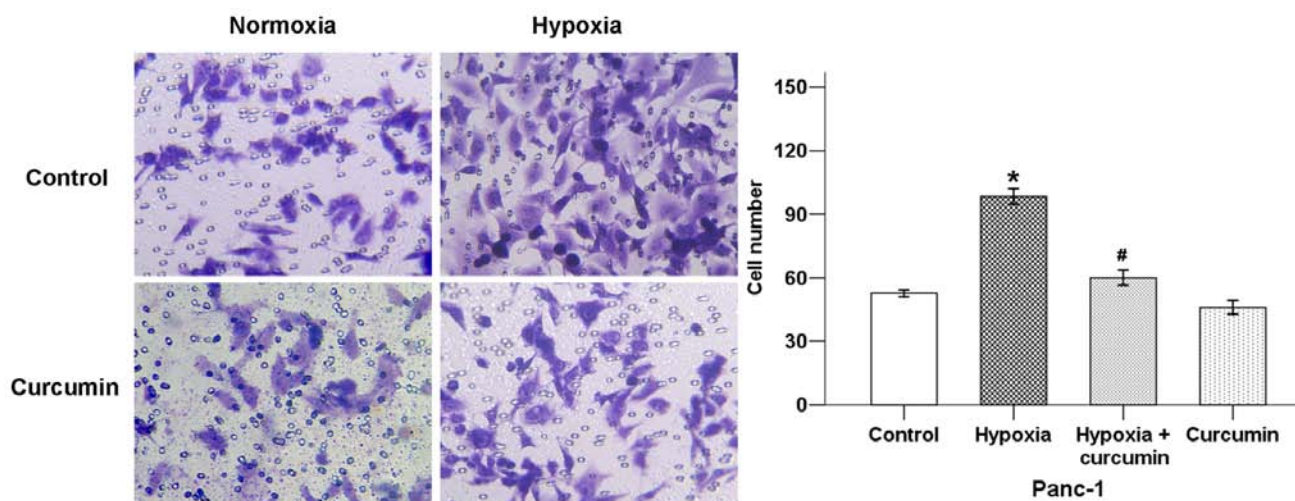

Figure 2. The effects of curcumin on hypoxia-induced invasive ability of pancreatic cancer cells. The images show the bottom side of the filter inserts with stained cells that have migrated through the filter pores after 48 h. The invasive ability of Panc-1 cells was promoted under hypoxic condition, whereas curcumin addition reduced the invasion of pancreatic cancer cells. *$P<0.05$ as compared with control group (normoxia); #$P<0.05$ as compared with hypoxia group.
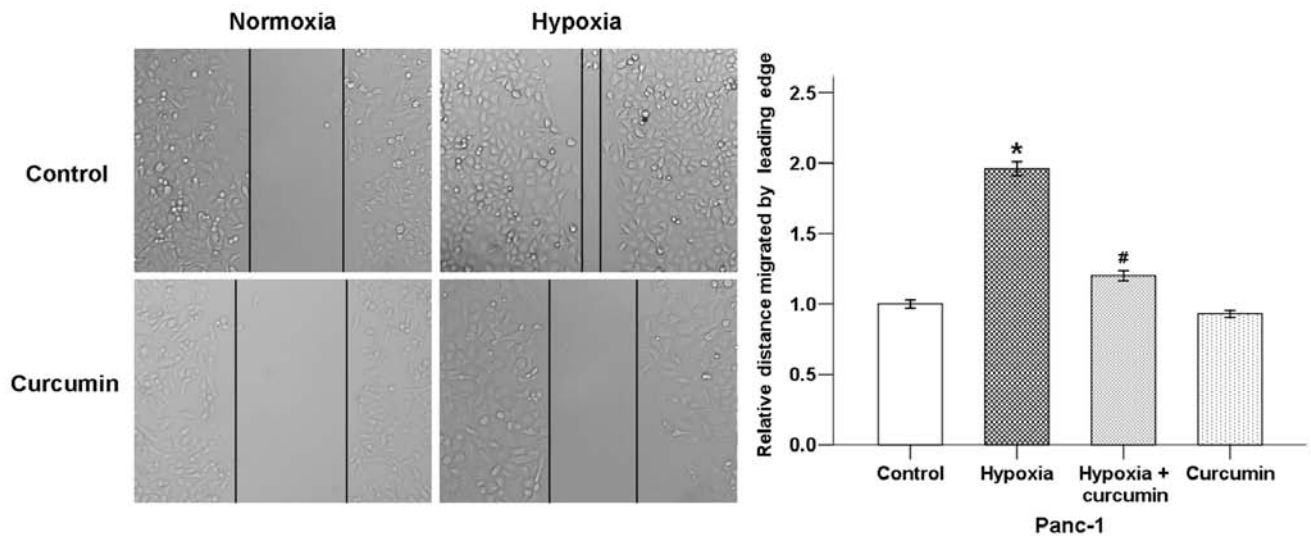
Figure 3. The effects of curcumin on hypoxia-induced migratory ability of pancreatic cancer cells. The confluent monolayer was wounded with sterile pipette tip and cells were allowed to migrate for 24 h. The migratory ability of Panc-1 cells was promoted under hypoxic condition, whereas curcumin addition reduced the migration of pancreatic cancer cells. *$P<0.05$ as compared with control group (normoxia); #$P<0.05$ as compared with hypoxia group.
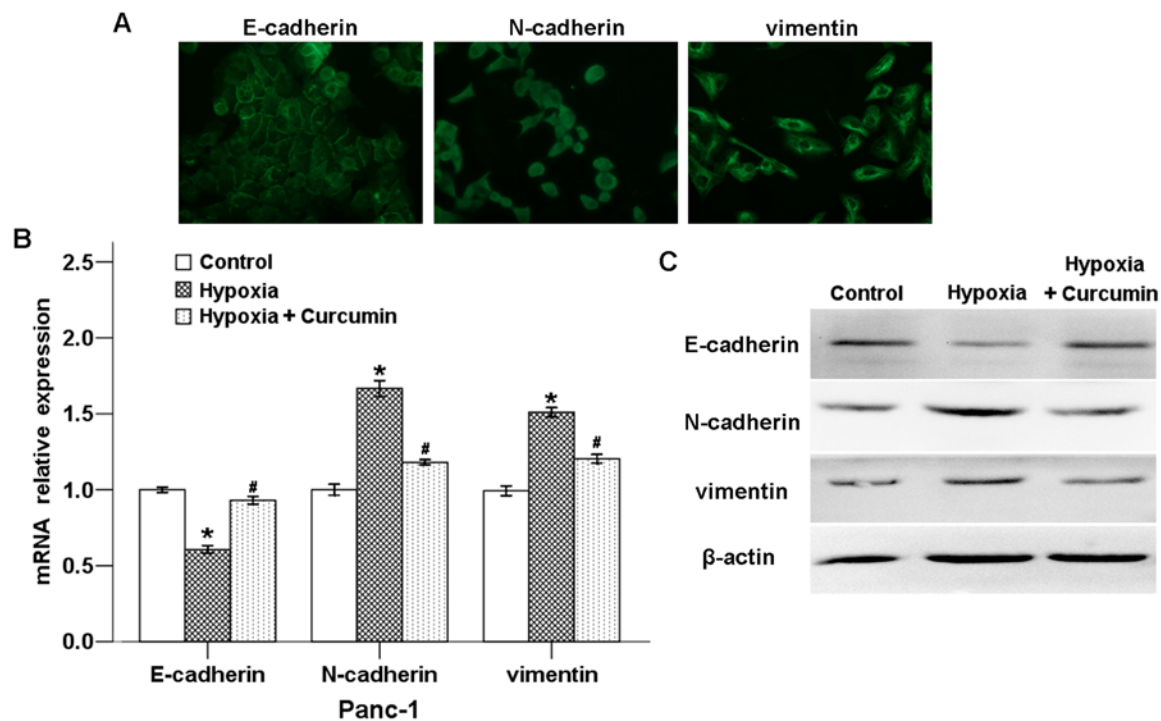

Figure 4. The effects of curcumin on expression of hypoxia-induced EMT-related factors of pancreatic cancer cells. (A) Panc-1 cells were labeled with fluorescence-conjugated antibodies against E-cadherin, N-cadherin and vimentin (green) (magnification, x400). (B) Treatment with cucumin diminished the effects of hypoxia-modulated expression of E-cadherin, N-cadherin and vimentin at the mRNA level in Panc-1 cells, as determined by QT-PCR. (C) Treatment with cucumin also counter-balanced the effects of hypoxia-modulated expression of E-cadherin, N-cadherin and vimentin at the protein level in Panc-1 cells, as determined by western blotting. *$P<0.05$ as compared with control group (normoxia); #$P<0.05$ as compared with hypoxia group.

Results showed that hypoxic condition significantly increased the migratory ability of Panc-1 cells after incubation for 24 h. Curcumin counter-balanced this effect of hypoxia (Fig. 3). These results indicate that curcumin inhibits migration and invasion of pancreatic cancer cells under hypoxic conditions.

Effects of curcumin on the expression of hypoxia-modulated EMT-related factors in pancreatic cancer cells. EMT contains

four important steps: loss of epithelial cell adhesion, gain of mesenchymal proteins and acquisition of a mesenchymal-like state, degradation of basement membranes and enhanced cell invasive ability that facilitate tumor cell invasion into stroma and in turn entrance to the circulation (7). To confirm the effect of curcumin on hypoxia-induced EMT, we determined the expression levels of EMT-related genes after the cells were treated in hypoxic condition with or without curcumin. As
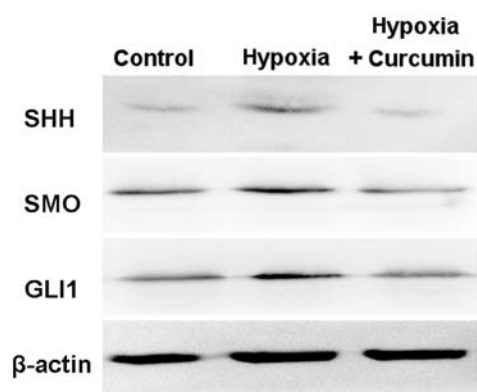
Figure 5. The effects of curcumin on hypoxia-induced Hh signaling activation of pancreatic cancer cells. Treatment with curcumin diminished the effects of hypoxia-induced expression of SHH, SMO and GLI1 at the protein level in Panc-1 cells, as determined by western blotting.

illustrated in Fig. 4A, the expression of E-cadherin was located in cell membrane, whereas N-cadherin and vimentin were localized in both cell membrane and cytoplasm. As shown in Fig. 4B, hypoxic condition downregulated the mRNA level of the epithelial marker E-cadherin, while the expression of mesenchymal markers N-cadherin and vimentin were strongly increased. Curcumin could significantly reverse all of these hypoxia-induced effects.

To evaluate the effects of hypoxia and curcumin on the expression of E-cadherin, N-cadherin and vimentin at protein level, we determined these proteins in Panc-1 cells using western blotting. As shown in Fig. 4C, curcumin counterbalanced the hypoxia-induced EMT-related factors at the protein level, and the trend was consistent with the mRNA results. These results indicate that hypoxic condition-induced EMT progression could be inhibited by curcumin.

Curcumin downregulates hypoxia-activated Hh signaling pathway. Hh signaling plays an important role in the initiation and progression of pancreatic cancer (15). As shown in Fig. 5, the protein levels of SHH, SMO and GLI1 were increased in Panc-1 cells under hypoxic condition, which indicated that Hh signaling was activated by hypoxia. Curcumin significantly decreased hypoxia-induced expression levels of SHH, SMO and GLI1. Taken together, our results demonstrate that curcumin inhibits hypoxia-induced EMT in Panc-1 cells via suppression of the Hh signaling pathway.

Discussion

Due to both the inherently aggressive biology of the disease and its late diagnosis in most cases, pancreatic cancer is one of the most aggressive malignant digestive carcinoma with an extremely high mortality rate (16). A number of studies have indicated that hypoxia and hypoxia-induced signaling pathways are highly associated with poor clinical outcome of patients diagnosed with pancreatic cancer, because of the enhanced cancer cell progression (17,18). Hypoxia-mediated target gene expression has been shown to stimulate proliferation, angiogenesis, metastasis, chemo-resistance and radio-resistance of tumor cells (19-21). In recent years, many

active compounds with anti-invasive and anti-metastatic properties, such as curcumin, α-mangostin and resveratrol, have been defined as new chemotherapeutic agents (6,22-23). Our previous study showed that curcumin inhibited hypoxia-induced HIF-1α accumulation in a hypoxia model induced by $CoCl_2$ and suppressed proliferation, migration, invasion and EMT of HepG2 cells in this environment (6). In the present study, we focused on the underlying mechanisms through which curcumin inhibits hypoxia-induced EMT in pancreatic cancer cell line Panc-1.

Our data showed that hypoxic condition could significantly modulate the expression of EMT-related factors, E-cadherin, vimentin and N-cadherin in Panc-1 cells, which further enhanced the capacity of the pancreatic cancer cells to migrate and invade the extracellular matrix. Curcumin was able to terminate these effects of hypoxic condition. In addition, we also tested the effects of hypoxic condition and curcumin on the activation of SHH, SMO and GLI1. Data showed that hypoxic condition significantly increased the expression levels of SHH, SMO and GLI1 in pancreatic cancer cells, whereas the addition of curcumin to the cell culture resulted in a decrease of these Hh pathway related factors.

Curcumin (diferuloylmethane) is a bioactive natural compound and a large number of experimental studies have shown that curcumin is able to suppress initiation, progression and metastasis of a variety of tumors, including pancreatic cancer (10,24). Youns et al (24) reported that curcumin could inhibit pancreatic cancer cell proliferation and upregulate the extrinsic apoptotic pathway through activation of caspase-3, caspase-8, Bid, Bax and downregulation of NF-κB and Bcl-2 genes. Curcumin and its analogues (UBS109 and EF31) could inhibit multiple angiogenic pathways and suppress tumor angiogenesis (25). In animal models of pancreatic cancer, the combination of curcumin and gemcitabine is much more effective than gemcitabine alone in the inhibition of tumor growth and anti-angiogenesis (26). A recent study showed that a novel synthetic analog of curcumin referred to as difluorinated-curcumin could inhibit cell survival, clonogenicity, migration, invasion, angiogenesis and the cancer stem cell (CSC) self-renewal capacity in human pancreatic cancer cells *in vitro* under hypoxic conditions, consistent with the inhibition of miR-21, miR-210, HIF-1α and CSC signature gene markers (27).

Accumulating evidence indicates that curcumin could inhibit tumor progression via multiple cellular signaling pathways, including MAPK, NF-κB, Akt, Wnt/β-catenin and Hh signaling pathway (10,12). The Hh signaling pathway, initiated through the binding of secreted Hh ligands to the membrane receptor patched1 (PTCH1), results in smoothened (SMO) dissociating, nuclear translocation and activation of the transcription factors of the GLI family. The expression of SMO and GLI1 is presumed to be the markers of the Hh pathway activation (5). Elamin et al (28) observed that curcumin caused inhibition of medulloblastoma cell growth and induction of apoptotic cell death by downregulating Hh pathway proteins, including SHH, PTCH1 and GLI1. In addition, curcumin also enhanced the anti-tumor effects of cisplatin and γ-rays by targeting pathways that are crucial for tumor survival. Slusarz et al (29) reported that curcumin caused major reductions in GLI1 mRNA concentrations in transgenic prostate

carcinoma mice. A recent study also revealed that resveratrol and curcumin synergistically caused apoptosis in cigarette smoke induced breast cancer cells through p21 (Waf/Cip1) mediated inhibition of Hedgehog-GLI cascade (30). In the present study, we showed that curcumin remarkably inhibited hypoxia-mediated activation of Hh signaling pathway.

Cancer metastasis is a process of dissemination of tumor cells from a primary tumor mass to a different site through blood vessels and lymphatic vessels. EMT is a characteristic feature of most metastatic cells and has been regarded as the possible first step in the complex process of metastasis (31). In this process, epithelial cells are transformed from highly differentiated, polarized and organized cells into undifferentiated, isolated mesenchymal-like cells with migratory and invasive properties (7). A typical characteristic of EMT is the loss of the cell-cell adhesion molecule E-cadherin expression and gain of mesenchymal markers, such as vimentin, N-cadherin, which in turn lead to reorganization of the cytoskeleton to acquire a more spindle-like morphology, and increased motility that involves dynamic actin microfilament networks (7,32). Our previous study showed that superoxide dismutase-induced hydrogen peroxide production can promote EMT in pancreatic cancer, leading to increased motility and invasion via activation of ERK signaling pathway (33). We have also verified that resveratrol plays an important role in suppressing the proliferation, migration and invasion of pancreatic cancer cells *in vitro* by modulating EMT-related factors via the PI-3K/Akt/NF-κB signaling pathway. Resveratrol was also able to suppress the migration and invasion of pancreatic cancer cells by inhibiting TGF-β-mediated EMT (23). In this study, we showed that curcumin is able to suppress hypoxia-induced activation of Hh pathway and, thus, inhibits pancreatic cancer cell invasive and migratory ability.

In conclusion, the present study demonstrates that curcumin plays an important role in suppressing hypoxia-induced proliferation, migration, invasion and EMT of pancreatic cancer cells *in vitro* by inhibiting the Hh signaling pathway. These results suggest that curcumin might be a potential anticancer agent for the treatment of pancreatic cancer.

Acknowledgements

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