Curcumin reverses the epithelial-mesenchymal transition of pancreatic cancer cells by inhibiting the Hedgehog signaling pathway

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Abstract. Curcumin, a phenolic compound extracted from Zingiberaceae turmeric, has strong anti-inflammatory, antioxidant and antitumor properties. However, the anti-carcinogenic mechanism of curcumin has yet to be fully elucidated. Epithelial-mesenchymal transition (EMT) induced by transforming growth factor-β1 (TGF-β1) is involved in the promotion of tumor invasion and metastasis, and is closely related to the drug resistance of tumor cells. The abnormal activation of Hedgehog signaling also plays an important role in tumorigenesis and metastasis. In order to investigate whether curcumin can reverse the TGF-β1-stimulated EMT of pancreatic cancer PANC-1 cells, and its possible mechanism, the pancreatic cancer cell line PANC-1 was stimulated with TGF-β1 (5 ng/ml) for 7 days to induce formation of EMT, and the TGF-β1-stimulated PANC-1 cells were treated with different concentrations of curcumin (10, 20 and 30 µmol/ml) for 48 h. The growth inhibition rate of the cells was measured by MTT assay, apoptosis was detected by flow cytometry, the expression levels of Shh, GLI1, E-cadherin and vimentin were detected by western blot analysis, and cell invasion and migration ability were examined by Transwell cell invasion assay and wound healing assay. Following stimulation with TGF-β1, the expression levels of Shh, GLI1 and vimentin in the TGF-β1-stimulated group were significantly increased, compared with those in the control group (P<0.01, respectively). The expression levels of E-cadherin in the TGF-β1-stimulated group were significantly decreased, compared with those in the control group (P<0.01). The scratch wounds in the curcumin-treated group healed slower compared with those in the control group (P<0.01). Curcumin significantly inhibited the invasion and migration of TGF-β1-stimulated PANC-1 cells. These results indicate that curcumin can inhibit the proliferation of TGF-β1-stimulated PANC-1 cells, it can induce apoptosis, and reverse the EMT. The possible underlying molecular mechanisms are through inhibition of the Shh-GLI1 signaling pathway.

Introduction

Curcumin is a phenolic compound that is extracted from Zingiberaceae turmeric and has strong anti-inflammatory, antioxidant and antimutation properties. Recent studies have shown that curcumin can inhibit the growth, invasion and metastasis of a variety of tumor cells, it can induce apoptosis through a variety of mechanisms, and can improve the sensitivity of tumor cells to chemotherapeutic drugs and radiotherapy (1,2). Transforming growth factor-β1 (TGF-β1) can induce tumor cell epithelial-mesenchymal transition (EMT) (3,4). EMT is involved in tumorigenesis and development and plays an important role in the promotion of tumor invasion and metastasis and it is also closely related to drug resistance of tumor cells (5,6). In some particular physiological or pathological conditions, the epithelial cells lose their polarity, convert into interstitial cells and are able to move freely in the cell matrix. The most important characteristic of EMT cells is the reduction or loss of the epithelial marker E-cadherin (7,8) and the induction of the mesenchymal markers such as fibronectin protein, vimentin.

Studies have shown that the abnormal activation of Hedgehog (Hh) signaling plays a significant role in tumorigenesis and metastasis (9,10). The Hh signaling pathway is a highly conserved signaling pathway, composed of the signal molecule Hh, two membrane receptors patched (Ptd) and smoothened...
(Smo), and the downstream nuclear transcription factor Gli. Gli proteins play a key role in the Hh signaling pathway. If having no ligands, Ptc binds with Smo and inhibits the function of Smo. When Hh presented, Hh ligand binds to its receptor Ptc, Smo is released and activated and, ultimately, the entire length of Gli transported to the nucleus and activated the transcription of target genes. Gli target genes are involved in tumor cell growth, apoptosis, metastasis, EMT and neovascularization (11).

In the present study, we stimulated the pancreatic cancer PANC-1 cells with TGF-β1, we treated the cells with different concentrations of curcumin and then detected the proliferation and apoptosis of each group of cells. We analyzed the expression levels of Shh, GLI1, E-cadherin and vimentin and evaluated the invasion and migration ability of each group of cells in vitro. The aim of the present study was to investigate whether curcumin can reverse the TGF-β1-induced EMT of pancreatic cancer PANC-1 cells and to explore its possible mechanism.

Materials and methods

Cell lines and reagents. Pancreatic cancer PANC-1 cells were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Antibodies of rabbit anti-human Shh, GLI1, vimentin and E-cadherin were purchased from Cell Signaling Technology (Beverly, MA, USA). Mouse anti-rabbit secondary antibody was obtained from Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd (Beijing, China). Annexin V/PI apoptosis detection kit was purchased from Multisciences Biotech Co., Ltd. (Hangzhou, China). DMEM and fetal bovine serum were purchased from Gibco (Grand Island, NY, USA). Curcumin and MTT were purchased from Sigma-Aldrich (Irvine, CA, USA). TGF-β1 was purchased from R&D Systems China Co., Ltd. (Shanghai, China). BD Matrigel™ Matrix basement membrane was purchased from BD Biosciences (Franklin Lakes, NJ, USA). Transwell insert (8 µm) was purchased from Corning Incorporated (Corning, NY, USA).

Cell treatment. The pancreatic cancer cell line PANC-1 was grown in complete Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and streptomycin in 5% CO₂ at 37°C. Cells were changed for fresh medium every 3 days. Cells were cocultured with TGF-β1 (5 ng/ml) for 7 days. Then, the TGF-β1-stimulated pancreatic cancer cells were treated with different concentrations of curcumin (10, 20 and 30 µmol/ml) for 48 h.

Cell proliferation assay (MTT). MTT was used to investigate the cellular growth inhibition rate. Following digestion with 0.25% trypsin, 1x10⁴ TGF-β1-stimulated pancreatic cancer cells were seeded in a 96-well plate and allowed to adhere to the plate for 24 h at 37°C. The cells were then divided into the control group and the curcumin-treated group (10, 20 and 30 µmol/ml) and were treated for 48 h. Thereafter, the cells in 96-well plates were centrifuged at 500 x g for 3 min, with 100 µl supernatant in each well removed. Then, 10 µl (5 mg/ml) MTT in PBS was added to each well and the microtiter plates were incubated for an additional 4 h at 37°C. DMSO (100 µl) was then added to each well and OD570 value was detected with enzyme-labeled immunoassay instrument after shaking for 10 min. The growth inhibition rate was calculated as follows: Inhibition rate = (1 - A_{treatment group}/A_{control group}) x 100%.

Cell apoptosis assay by flow cytometry. Cell apoptosis was investigated with the Annexin V-FITC apoptosis detection kit according to the corresponding protocols: 1x10⁵ TGF-β1-stimulated pancreatic cancer cells were seeded in 6-well plates for 24 h at 37°C. Then, the cells were divided into the control and the curcumin-treated group (10, 20 and 30 µmol/ml). Forty-eight hours later, cells in 6-well plates were collected and centrifuged at 800 x g for 5 min. After adding buffer solution (50 µl), V-FITC (5 µl) and PI (10 µl) for 5 min, cells were transferred for flow cytometry assay.

Western blot analysis of Shh, GLI1, vimentin and E-cadherin. After PANC-1 cells were cocultured with TGF-β1 (5 ng/ml) for 7 days, the expression levels of Shh, GLI1, vimentin and E-cadherin were detected by western blot analysis.

For western blot analysis, we prepared separation and spacer gels. Cells in the above groups were collected separately and total proteins in each group were extracted with the one-shot method. After mixing with 4X loading buffer, boiling for 5 min, and centrifuging at 12,000 rpm for 3 min, 20 µl of the resulting sample was used for SDS-PAGE analysis at 100 V. When the separation step was completed, the gel was washed once with Millipore H₂O, electroblotted onto transfer membranes at 100 V for 2 h, and then blocked with blocking reagent for 2 h. The blots were incubated with corresponding primary antibodies for 24 h. After washing four times (10 min each time) with Tris-buffered saline with Tween-20 (TBST), the membranes were incubated 1 h with secondary antibodies conjugated with horseradish peroxidase. The membranes were again washed four times (10 min each time) with TBST and incubated with ECL. Band Leader software was used for densitometry analysis of protein bands with GAPDH as internal control.

Subsequently, the TGF-β1-stimulated pancreatic cancer PANC-1 cells were treated with curcumin (30 µmol/ml) for 48 h. The expression levels of Shh, GLI1, vimentin and E-cadherin were also detected by western blot analysis, as described above.

Transwell cell invasion assay. Transwell chambers were placed in a 24-well plate. Matrigel was mixed with serum-free DMEM (1:3). Each small chamber was supplemented with 30 µl of the Matrigel mixture, and then placed at 37°C for 30 min. Then, 30 µl serum-free DMEM containing 0.1% BSA was added to each chamber. Cells were then divided into the TGF-β1-stimulated PANC-1 control group and the curcumin-treated group (30 µmol/ml). Cells of each group (3x10⁴) were resuspended in 300 µl serum-free DMEM with 0.1% BSA and seeded in the transwell upper chamber. DMEM (600 µl) containing 10% FBS was added to the lower chamber of the transwell. The cells were then incubated at 37°C, 5% CO₂ for 48 h, the culture medium was absorbed and the cells of the microporous membranes were gently wiped off with a cotton swab; the invaded cells of the lower surface of the microporous membrane were retained in ice methanol for 1 min and H&E stained. The invading cells were counted in five randomly selected high magnification fields (x200). The experiment was repeated 5 times.
Wound healing assay. TGF-β1-stimulated pancreatic cancer cells (1x10^6) were seeded in 6-well plates. Cells were divided into the TGF-β1-stimulated PANC-1 control group and the curcumin-treated group (30 µmol/ml). Upon 80% confluence, cells were scraped across the cell monolayer using a plastic 200 µl tip. Photomicrographs were captured at 24 h. The measured ratio of the remaining wound area relative to the initial wound area was quantified and reported. The data are described as closed width/scratch width (%). The experiment was repeated 5 times, independently.

Statistical analysis. SPSS 13.0 software was used for statistical analysis and data are shown as the means ± SD. Statistical differences were determined by t-test analysis and P<0.05 was considered to indicate a statistically significant result.

Results

Expression level of Shh, GLI1, E-cadherin and vimentin in TGF-β1-stimulated PANC-1 cells. The Hh signaling pathway is abnormally activated in a variety of tumors and is associated with development and invasion, and plays an important role in the transfer process. Hh signaling target genes are related to tumor cell growth, apoptosis, metastasis, EMT. To explore whether TGF-β1 induces the EMT of PANC-1 cells through activation of the Hh signaling, the expression levels of Shh, GLI1, E-cadherin and vimentin in TGF-β1-stimulated PANC-1 cells were detected by western blot analysis.

Taking GAPDH as internal control, the results demonstrated that the expression levels of Shh and GLI1 in the TGF-β1-stimulated group were significantly increased, compared with those in the control group (t=7.30, P<0.01; t=9.12, P<0.01, respectively). The expression level of E-cadherin in the TGF-β1-stimulated group was significantly decreased, compared with that in the control group (t=7.89, P<0.01), and the expression level of vimentin in the TGF-β1-stimulated group was also significantly elevated, compared with that in the control group (t=8.15, P<0.01). These results suggest that the TGF-β1-stimulated PANC-1 cells are able to induce activation of the Hh signaling pathway and the EMT of PANC-1 cells, as shown in Fig. 1.

Curcumin inhibits proliferation and induces apoptosis of TGF-β1-stimulated PANC-1 cells. TGF-β1 can induce pancreatic cancer PANC-1 cell EMT. Our experimental results also suggest that following stimulation with TGF-β1, the proliferation, invasiveness and migration ability of PANC-1 cells increased and the cell morphology changed, suggesting the occurrence of EMT.

Curcumin can inhibit the growth of various tumor cells through a variety of mechanisms and it can induce apoptosis. In order to verify the role of curcumin in TGF-β1-stimulated PANC-1 cells, we treated the cells with different concentrations of curcumin (10, 20 and 30 µmol/ml) for 48 h. The results indicated that as the curcumin concentration increased, its proliferation inhibitory effect on TGF-β1-stimulated PANC-1 cells also increased. Using t-test analysis, the proliferation inhibiting effect of the 10 µmol/ml curcumin-treated group was significantly higher than that of the control group (P=0.01). The proliferation inhibiting effect of the 20 µmol/ml curcumin-treated group was significantly higher than that of the 10 µmol/ml curcumin-treated and the control group (P=0.01, respectively), and the proliferation inhibiting effect of the 30 µmol/ml curcumin-treated group was also significantly higher than that of the 10 and 20 µmol/ml curcumin-treated and the control group (P=0.01, respectively) as shown in Table I.

Table I. Growth inhibition rates of TGF-β1-stimulated PANC-1 cells treated with curcumin detected by MTT (%). The data are described as closed width/scratch width (%). The experiment was repeated 5 times, independently.

<table>
<thead>
<tr>
<th>Group</th>
<th>Growth inhibition rate</th>
</tr>
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<tbody>
<tr>
<td>Control group</td>
<td>0</td>
</tr>
<tr>
<td>Curcumin (10 µmol/ml)</td>
<td>13.60±3.39</td>
</tr>
<tr>
<td>Curcumin (20 µmol/ml)</td>
<td>17.43±3.70</td>
</tr>
<tr>
<td>Curcumin (30 µmol/ml)</td>
<td>62.18±6.87</td>
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*P<0.01 vs. control group; †P<0.01 vs. 10 µmol/ml curcumin group; ‡P<0.01 vs. 10 and 20 µmol/ml curcumin group.
The experimental results of the flow cytometry also showed that when the curcumin concentration increased, the apoptosis of TGF-β1-stimulated PANC-1 cells also increased. With t-test analysis, the apoptosis of the 10 µmol/ml curcumin-treated group was significantly different from that of the control group (P<0.01). The apoptosis of the 20 µmol/ml curcumin-treated group was significantly different from that of the 10 µmol/ml curcumin-treated and the control group (P<0.01, respectively), and the apoptosis of the 30 µmol/ml curcumin-treated group was also significantly different from that of the 10 and 20 µmol/ml curcumin-treated and the control group (P<0.01, respectively) as shown in Table II, Fig. 2.

**Table II. Apoptosis rate of TGF-β1-stimulated PANC-1 cells treated with curcumin detected by flow cytometry (%).**

<table>
<thead>
<tr>
<th>Group</th>
<th>Apoptosis rate</th>
</tr>
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<tbody>
<tr>
<td>Control group</td>
<td>7.62±0.32</td>
</tr>
<tr>
<td>Curcumin (10 µmol/ml)</td>
<td>9.52±0.56</td>
</tr>
<tr>
<td>Curcumin (20 µmol/ml)</td>
<td>12.92±0.67</td>
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<tr>
<td>Curcumin (30 µmol/ml)</td>
<td>35.57±1.26</td>
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*P<0.01 vs. control group; *P<0.01 vs. 10 µmol/ml curcumin group; *P<0.01 vs. 10 and 20 µmol/ml curcumin group.

Effects of curcumin on the expression levels of Shh, GLI1, E-cadherin and vimentin in TGF-β1-stimulated PANC-1 cells. The antitumor mechanism of curcumin is highly complicated and involves several signaling pathways, such as EGFR, HER-2, Wnt/β-catenin and its downstream signaling molecules ERK, AKT, NF-κB, STATs. In order to investigate whether curcumin is able to reverse the EMT of TGF-β1-stimulated PANC-1 cells by inhibiting the Hh signaling pathway, the TGF-β1-stimulated PANC-1 cells were treated with 30 µmol/ml curcumin for 48 h, and the expression levels of Shh, GLI1, vimentin and E-cadherin were then detected by western blot analysis.

Taking GAPDH as internal control, the results were obtained by semi-quantitative analysis of the gray scales of bands using Band Leader software. The results revealed that the expression levels of Shh and GLI1 in the curcumin-treated group were significantly decreased compared with those in the control group (t=14.66, P<0.01; t=7.46, P<0.01, respectively), the expression level of E-cadherin in the curcumin-treated group was significantly increased compared with that in the control group (t=4.37, P<0.01), and the expression level of vimentin in the curcumin-treated group was also significantly decreased compared with that in the control group (t=5.00, P<0.01). The results suggest that curcumin inhibits the activation of the Hh signaling pathway and reverses the EMT of TGF-β1-stimulated PANC-1 cells, as shown in Fig. 3.
ph physiological or pathological conditions in which the epithelial cells with polarity lose their polarity and convert into interstitial cells with the ability to move freely in the cell matrix. The tumor cells that acquire EMT phenotype have increased invasion ability and are easy to transfer. To verify whether curcumin can inhibit the metastatic ability of TGF-β1-stimulated PANC-1 cells, we performed Transwell cell invasion assay and wound healing assay. The results showed that cell invasion in the curcumin-treated group (30 µmol/ml) was significantly decreased compared with that in the control group ($t=7.68$, $P<0.01$), as shown in Fig. 4A. The scratch wound in the curcumin-treated group healed slower than that in the control group ($t=12.90$, $P<0.01$), as shown in Fig. 5A. Representative images of invasion and wound healing are shown in Figs. 4B and C, and 5B and C. The results suggest that curcumin inhibits cell invasion and migration of TGF-β1-stimulated PANC-1 cells.

Figure 4. Transwell cell invasion assay of curcumin on TGF-β1-stimulated PANC-1 cells. (A) Invasion assay of curcumin on TGF-β1-stimulated PANC-1 cells. (B) Images of invasion in the control group (magnification, x200). (C) Images of invasion in the curcumin-treated group (magnification, x200). $P<0.01$ vs. control group, mean ± SD, n=5. Control group, TGF-β1-stimulated PANC-1 cells. Curcumin-treated group, TGF-β1-stimulated PANC-1 cells treated with curcumin (30 µmol/ml).

Discussion

Abnormal and sustained activations of several signaling pathways are the main mechanisms of malignant tumor development, invasion, metastasis, recurrence and resistance. Curcumin has chemopreventive and therapeutic roles in a variety of tumor cells, including breast, gastric, colorectal, lung, pancreatic and prostate cancer (12-14). The antitumor mechanism of curcumin is to regulate multiple signaling molecules, such as downregulation of EGFR, HER-2, SHH/GLI1 Wnt/β-catenin and its downstream signaling molecules ERK, AKT, NF-κB, STATs, and upregulation of p21, p27, cell cycle kinase inhibitors (13,15-17).

Curcumin can inhibit pancreatic cancer cell proliferation, induce apoptosis, anti-angiogenesis, chemosensitizing, but its mechanism is not yet fully understood. Curcumin may inhibit EGFR, STAT-3, NF-κB and their downstream target genes,
multidrug resistance-associated protein 5 expression (18-21).
Curcumin inhibits Panc-28 and L3.6PL pancreatic cancer cell proliferation through downregulation of NF-xB-dependent genes and Sp1, Sp2, Sp3 transcription factors (18). Curcumin can suppress the transplantation tumor growth of L3.6PL pancreatic cells in nude mice through intraperitoneal injection (20). Curcumin can improve the antiproliferative effects of gemcitabine on BxPC-3, Panc-1, MiaPaCa-2 pancreatic cancer cells and can induce apoptosis (19,22). In animal models of pancreatic cancer, the combination of curcumin and gemcitabine is more effective than gemcitabine alone in the inhibition of tumor growth and anti-angiogenesis (18,21). The chemotherapy sensitizing effect of curcumin is partly due to the inhibition of the expression of STAT-3 and NF-xB-regulated genes such as cyclins, c-Myc, Bcl-2, Bxl-x1, MMPs and VEGF (18,21).

Studies have found that Hh signaling mediated tumor development. The cross-talk between the Hh signaling with other signaling pathways, such as EGFR, Wnt/β-catenin and transforming growth factor-β (TGF-β)/TGF-β receptors, also play important roles (23). Morton et al (23) also found that the Hh signaling pathway has a synergistic effect with activated K-ras signaling by reducing tumor cell dependence on sustained activation of MAPK and PI3-K/Akt/mTOR signals, thereby enhancing the K-ras-induced pancreatic cancer development. In pancreatic ductal adenocarcinoma the downstream nuclear transcription factor GLI1 of the Hh signaling pathway is not completely dependent on upstream Shh-Ptch-Smo signals and is also regulated by TGF-β and KRAS signals (24).

TGF-β1, a multifunctional polypeptide cytokine, has an impact on a variety of cell growth, differentiation, apoptosis and immune regulations. The signaling pathway of TGF-β1 is to inhibit epithelial cell growth and to induce apoptosis of epithelial cells. However, it has been found that the majority of tumor cells can secrete TGF-β1 by autocrine or paracrine modes. The high level of TGF-β1 in the tumor tissue can not only promote the growth of tumor cells, and inhibit apoptosis, but it can also promote the occurrence of EMT. Pancreatic cancer cells secrete TGF-β1; the tumor-derived TGF-β1 plays an important role in pancreatic cancer microenvironment. miR-23a regulates TGF-β-induced EMT by suppression of E-cadherin and contributes to EGFR-TKI resistance in lung cancer cells (25).

In our experiment, the proliferation, invasion and migration activities of pancreatic cancer Panc-1 cells increased significantly by stimulation with TGF-β1 (5 ng/ml) for 7 days. The expression levels of Shh and GLI1 increased, E-cadherin decreased, while vimentin expression increased as well. Compared with the control group, the difference was significant. These results indicate that TGF-β1 promotes EMT transition of Panc-1 cells.

Subsequently, we treated the TGF-β1-stimulated Panc-1 cells with different concentrations of curcumin. We found that with the gradual increase of the concentration of curcumin, the inhibition of cell proliferation also increased, and 30 μmol/ml of curcumin significantly inhibited the proliferation of Panc-1 cells. After the Panc-1 cells were treated with curcumin, the rate of apoptosis was significantly increased. Our experimental results suggest that curcumin inhibits Panc-1 cell proliferation and induces cell apoptosis in vitro.

Following treatment with curcumin, the expression levels of Shh and GLI1 were significantly decreased in TGF-β1-stimulated Panc-1 cells. GLI protein is the downstream transcription factor and plays a key role in the Hh pathway. GLI1 is also a target gene of the Hh signal. Our results suggest that curcumin inhibits Hh signaling in TGF-β1-stimulated Panc-1 cells.

The most important changes of EMT are reduced or lost expression of epithelial markers E-cadherin, and elevated expression of interstitial cell markers, such as fibronectin, vimentin (8,9). Our experimental study also found that the expression levels of Shh and GLI1 were significantly decreased, as was the expression of vimentin, while the expression of E-cadherin increased in TGF-β1-stimulated Panc-1 cells treated with curcumin. Furthermore, cell invasion significantly decreased, and the scratch wound healed slower in the curcumin-treated group. The results suggest that curcumin inhibits the cell invasion and migration of TGF-β1-stimulated Panc-1 cells, and reverses the EMT of TGF-β1-stimulated Panc-1 cells.

These findings suggest that TGF-β1 induces the EMT of the pancreatic cancer Panc-1 cells through activation of Hh signaling. Curcumin inhibits cell proliferation, induces apoptosis, decreases cell invasion and migration, and reverses the EMT of the TGF-β1-stimulated Panc-1 cells by inhibiting the Hh signaling pathway.

Acknowledgements

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References

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