

Cucurmosin induces apoptosis of BxPC-3 human pancreatic cancer cells via inactivation of the EGFR signaling pathway

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Abstract. Pancreatic cancer remains the fourth most common cause of cancer-related death in the United States. Potent therapeutic strategies are urgently needed for pancreatic cancer. Cucurmosin is a novel type 1 ribosome-inactivating protein (RIP) isolated from the sarcocarp of *Cucurbita moschata* (pumpkin). Due to its cytotoxicity, cucurmosin can inhibit tumor cell proliferation through induction of apoptosis on tumor cells, but the specific mechanism is still unclear. We explored the function of cucurmosin in BxPC-3 pancreatic cancer cells using multiple cellular and molecular approaches such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, flow cytometry, reverse transcription polymerase chain reaction (RT-PCR), Western blotting and transmission electron microscopy for observing typical changes and formation of apoptotic bodies. We found that cucurmosin inhibited the proliferation of BxPC-3 cells in a time- and dose-dependent manner, and increased the cell population in the G0-G1 phase. With increasing concentration of cucurmosin, the expression of EGFR, p-PI3K, Akt, p-Akt, mTOR, p-mTOR, P70S6K- α , p-P70S6K- α , 4E-BP1 and p-4E-BP1 at the protein level was decreased, whereas the expression of p-Bad and caspase-9 was elevated. However, the mRNA expression of EGFR did not change. These findings suggest that cucurmosin can down-regulate the expression of EGFR by targeting. Cucurmosin induces the apoptosis of BxPC-3 pancreatic cancer cells via the PI3K/Akt/mTOR signaling pathway.

Introduction

Pancreatic cancer is the fourth leading cause of cancer related deaths among men and women with an approximately 43,140 new cases and 36,800 in 2010 in United States (1). Patients diagnosed with pancreatic cancer typically have a poor prognosis. For patients at all stages, the 1- and 5-year survival rates are only 23% and 6%, respectively (2), because a majority of pancreatic cancer at the time of diagnosis is advanced and relative lack of early disease-specific symptoms (3). Since 1997, gemcitabine established its unshakable status as first-line therapy for advanced pancreatic cancer (4). In the past 10 years, Gemcitabine, with or without erlotinib, has been the standard chemotherapy in this setting but the benefit is only modest. A significant amount of studies on gemcitabine-based combinations chemotherapy, such as gemcitabine with fluorouracil, capecitabine, cisplatin, e.g., have not produced clear survival benefit (5). Therefore, the novel strategies are clearly needed.

The ribosome-inactivating proteins (RIPs) are RNA N-glycosidases (6,7) which inactivate ribosomes by site-specifically cleaving the single N-C glycosidic bond between adenine and ribose at A4324 in the 28 S rRNA, thus irreversibly inactivating ribosomes for protein synthesis. Based on the structure of the genes and mature proteins, RIPs can be classified into 3 types (8). Type 1; RIPs are single-chained proteins with a molecular weight of about 30 Kda. They are potent inhibitors of protein synthesis in the cell-free system, but are relatively non-toxic to intact cell. Type 2; RIPs are double -chained proteins which consist of an A-chain (catalytic subunit) corresponding to type 1 RIPs and a B-chain corresponding to lectin, linked by disulfide bond. The B-chain binds to galactosyl-terminated receptors on the target cell surface, facilitating the entry of the A-chain into the cytoplasm of the cell. Thus, some, but not all, type 2 RIPs are more potent toxin than type 1 RIPs because the latter can only enter into cells with difficulty. Type 3; RIPs is a kind of jasmonate-induced protein comprised of an N-terminal domain similar to other type 1 RIPs and an unrelated C-terminal domain of unknown function (9). Most RIPs are glycoproteins, with varying amount and type of sugars (10).

We isolate and purify cucurmosin from the sarcocarp of *Cucurbita moschata* (pumpkin). The weight of cucurmosin

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is about 28 KDa. Cucurmosin belongs to type 1 RIPs and possesses an rRNA N-glycosidase activity (11). Significantly, it can inhibit the proliferation of cancer cells, through the induction of apoptosis (12,13,14). In this study, we firstly investigated that cucurmosin can inhibit the proliferation and induce the apoptosis of human pancreatic cancer cell BxPC-3 by down-regulating the expression of EGFR at the protein level, but not at the gene level, and inactivate the downstream of EGFR, namely PI3K/Akt/mTOR signaling pathway.

Materials and methods

Antibodies and reagents. The primary rabbit polyclonal antibodies for EGFR (1:1000), Akt (1:1000), p-Akt (1:500), mTOR (1:1000), p-mTOR (1:1000), P70S6K- α (1:500), p-P70S6K- α (1:500), 4E-BP1 (1:500), p-4E-BP1 (1:750), and caspase-9 (1:1000) were obtained from BioWorld Technology, Inc. The primary rabbit monoclonal antibodies for p-PI3K (1:1000), Ras (1:500) and p-Bad (1:500) were obtained from Cell Signaling Technology. Cucurmosin that its purity was 97% was offered by Minghuang Chen Pro. (State Structural Chemistry Key Laboratory of Fujian Institute of Research on Structure of Matter, Chinese Academy of Sciences). All secondary antibodies were purchased from Beyotime Institute of Biotechnology (Beyotime, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma St. Louis, MO.

Cell line and cell culture. Human pancreatic cancer cell line BxPC-3 was purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China), and cultured in RPMI-1640 media (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin and streptomycin in a humidified cell culture incubator with 5% CO₂ at 37°C, and passed every 2-3 days to maintain logarithmic growth.

Cell growth inhibition studies by MTT assay. Human pancreatic cancer BxPC-3 cells (6x10³/well/100 μ l) were seeded in 96-well culture plates and incubated for 24 h, and then were treated with 0.03125, 0.0625, 0.125, 0.25, 0.5, 1 or 2 μ M cucurmosin for 24, 48, 72 h. Control cells were supplemented with 100 μ l RPMI-1640 culture medium. After treatment, cells were incubated with MTT 20 μ l (0.5 mg/ml) at 37°C for 4 h, and subsequently moved the culture medium and added dimethyl sulfoxide (DMSO) 200 μ l. The spectrophotometric absorbance of the samples at 570 nm wavelength was measured by Multiskan MK3 microplate reader (Thermo Scientific). Results were blotted as the mean \pm standard deviation. Each experiment was repeated 3 times.

Cellular shape changes by transmission electron microscope. BxPC-3 cells (3x10⁵/well) were seeded in 6-well plates. After incubation for 24 h, cells were treated with 1 μ M cucurmosin for 72 h. Control cells were supplemented with RPMI-1640 culture medium. Cells (1x10⁶) were harvested and washed 3 times with PBS, then centrifuged at 15,000 x g for 15 min, and subsequently fixed in 4% glutaraldehyde for 2 h. After fixation, samples were fixed in 1% osmic acid for 2 h, then gradually dehydrated by acetone and embedded with epoxy

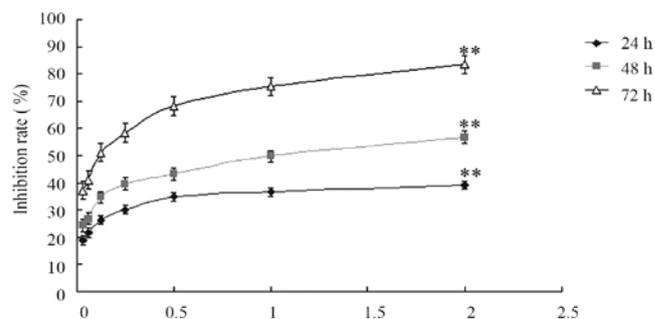


Figure 1. Inhibitory effect of cucurmosin on human pancreatic cancer cells. BxPC-3 cells were treated with various concentrations (0.03125, 0.0625, 0.125, 0.25, 0.5, 1 and 2 μ M) of cucurmosin for 24, 48 and 72 h. MTT assay was performed. The data shown are the means \pm SEM of 3 separate experiments. ** P<0.05 vs. control.

resin. Cells shape were observed under transmission electron microscope.

Flow cytometry cell cycle and apoptosis analysis. BxPC-3 cells (2x10⁶/well) were seeded in 90 mm plates. After attached for 24 h, cells were treated without control or with 0.0625, 0.25 and 1 μ M cucurmosin for 72 h. Cells (1x10⁶) were harvested and washed 3 times with PBS, and then respectively analyzed for their DNA content and apoptosis ratio by FACSCalibur (Becton-Dickinson, Mountain View, CA) using Cell Cycle Detection kit and Annexin V-FITC Apoptosis Detection kit (KeyGEN, Nanjing, China) according to the protocol of the manufacturer.

Reverse transcription polymerase chain reaction (RT-PCR) analysis for the expression of EGFR mRNA studies. BxPC-3 cells (2x10⁶/well) were seeded in 90 mm plates. After attached for 24 h, cells were treated without control or with 0.0625, 0.25 and 1 μ M cucurmosin for 72 h. Cells (1x10⁶) were harvested and washed 3 times with PBS. The total-RNA from each sample was isolated by TRIzol (Invitrogen) and purified by RNeasy mini kit and RNase-free DNase Set (Qiagen, Valencia, CA) according to the protocol of the manufacturer. One microgram of total-RNA from each sample was subjected to first strand combinational DNA (cDNA) synthesis using the TaqMan reverse transcriptase (RT) reagent kit (Fermentas) in a total volume of 20 μ l, including 6.25 units of MultiScribe RT and 25 pmol of random hexamers. RT reaction was done at 25°C for 5 min, followed by 42°C for 60 min, and 70°C for 5 min. After the RT, the polymerase chain reaction was undertaken. The primers used in this study are as follows: EGFR forward, 5'-TGCAGAATCCTGTCTATCACAATCA-3' and reverse, 5'-AGGGCTGTGCAATGTGCTGTT-3'; β -actin forward, 5'-TTCCTGGGCATGGAGTCCTGTGG-3' and reverse, 5'-CG CCTAGAAGCATTGCGGTGG-3'. The PCR reaction was used for a total volume of 20 μ l, including 2 μ l EGFR cDNA or 1 μ l β -actin cDNA. The thermal profile for PCR was 94°C for 5 min, 94°C for 45 sec, 60°C for 45 sec, 72°C for 90 sec and 72°C for 5 min, by 35 cycles. Each sample was tested in duplicated.

Western blot analysis. BxPC-3 cells (2x10⁶/well) were seeded 90-mm plates. After attached for 24 h, cells were treated

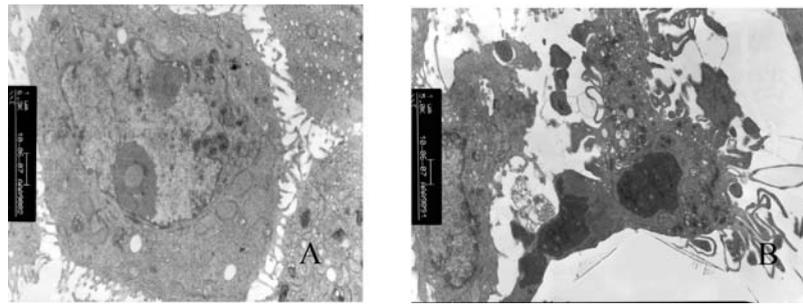


Figure 2. Morphologic change of pancreatic cancer BxPC-3 cells at 72 h after culture in 2 groups under transmission electron microscope. (A) BxPC-3 cells were treated without curcumin and supplemented with RPMI-1640 culture medium for 72 h (Control). (B) BxPC-3 cells were treated with 1 μ M curcumin for 72 h.

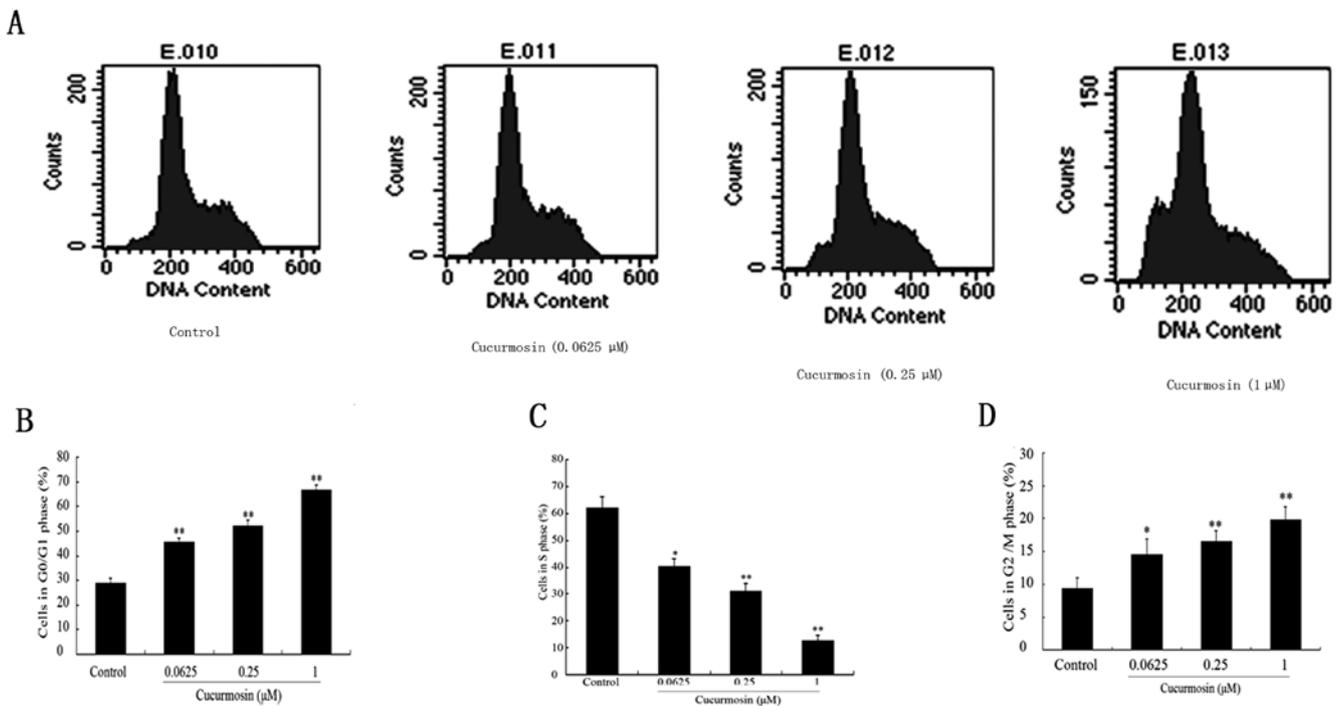


Figure 3. Flow cytometric analysis of curcumin-induced cell cycle arrest. After treatment without (control) or with 0.0625, 0.25 and 1 μ M curcumin for 72 h, BxPC-3 cells were harvested (1×10^6) and fixed with 1 ml of 70% ice-cold ethanol at 4°C. And then cells were stained with propidium iodide and analyzed by flow cytometric. (A) The diploid peak of apoptosis was detected in response to curcumin. The percentages of total cells in the G0/G1 phase (B), S phase (C) and G2/M phase (D) are shown as the means \pm SEM of 3 separate experiments. *P<0.01 vs. control, **P<0.05 vs. control.

with 0, 0.0625, 0.25 and 1 μ M curcumin for 72 h. Cells were harvested and washed 3 times with PBS. Each sample was lysed in lysis buffer [50 mmol/l Tris-HCl, PH 7.5, 100 mmol/l NaCl, 1 mmol/l ethylenediamine tetraacetic acid (EDTA), 0.5% Nonidet P-40, 0.5% Triton X-100, 2.5 mmol/l sodium orthovanadate, 10 μ l/ml protease inhibitor cocktail and 1 mmol/l phenylmethylsulfonyl fluoride (PMSF)] by incubating for 15 min at 4°C, and centrifuged at 12,000 g for 15 min. Protein concentrations were measured by BCA assay. Total proteins were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the gel was transferred onto nitrocellulose membrane. The membranes were blocked using 5% BSA, and then incubated with specific primary antibodies. The immunocomplexes were incubated with the appropriate horseradish peroxidase-conjugated anti-rabbit secondary antibodies, and then detected using the Luminata Forte Western HRP substrate (Millipore).

Statistical analysis. Statistical analysis was performed with the statistical analysis software SPSS 13.0. Comparisons were performed using the Student's t-test between 2 groups. Results are presented as means \pm SEM. P<0.05 was considered to indicate significant difference.

Results

Curcumin inhibits the proliferation and induces the apoptosis on human pancreatic cancer BxPC-3 cells. Besides rRNA N-glycosidase activity, curcumin exhibits strong cytotoxicity to cancer cells. To test these properties, we performed the MTT assay. After pancreatic cancer BxPC-3 cells were treated with 0.03125, 0.0625, 0.125, 0.25, 0.5, 1 or 2 μ M curcumin for 24, 48 or 72 h, the proliferation was inhibited in a time and dose-dependent manner in BxPC-3 cells (Fig. 1). The IC₅₀ values for 24, 48, and 72 h were 1.45 \pm 2.14, 0.54 \pm 1.78, 0.22 \pm 0.69 μ M, respectively (P<0.05).

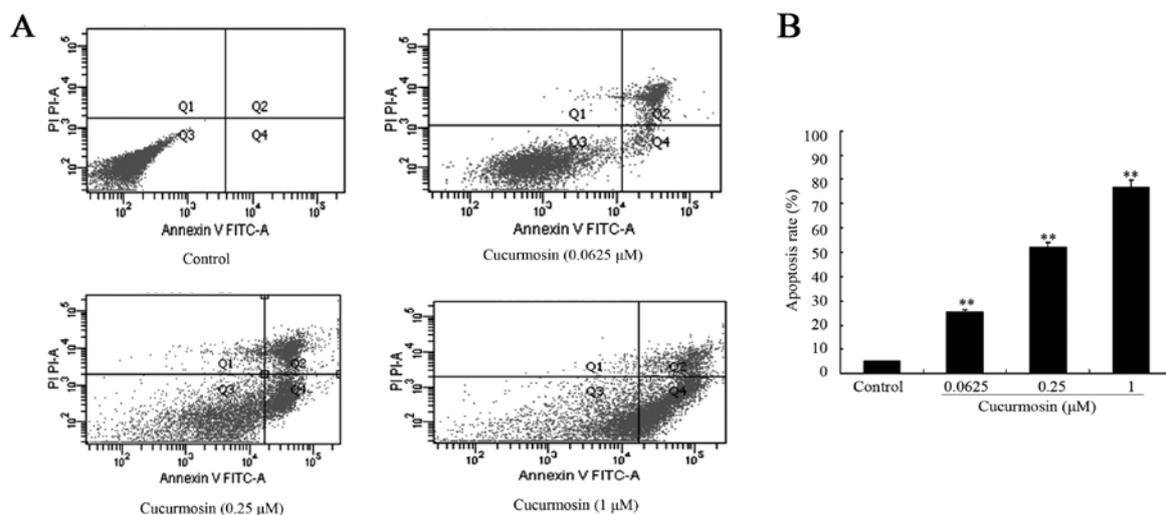


Figure 4. Flow cytometric analysis of cucurmosin-induced cell apoptosis rate. After treatment without control or with 0.0625, 0.25 or 1 μM cucurmosin for 72 h, BxPC-3 cells were harvested (1×10^6) and fixed with 1 ml of 70% ice-cold ethanol at 4°C. And then samples were analyzed by flow cytometry according to the protocol of the manufacturer. (A) The apoptosis was detected in response to various concentrations of cucurmosin. (B) The percentages of apoptotic cells are shown as the means \pm SEM of 3 separate experiments. ** $P < 0.05$ vs. control.

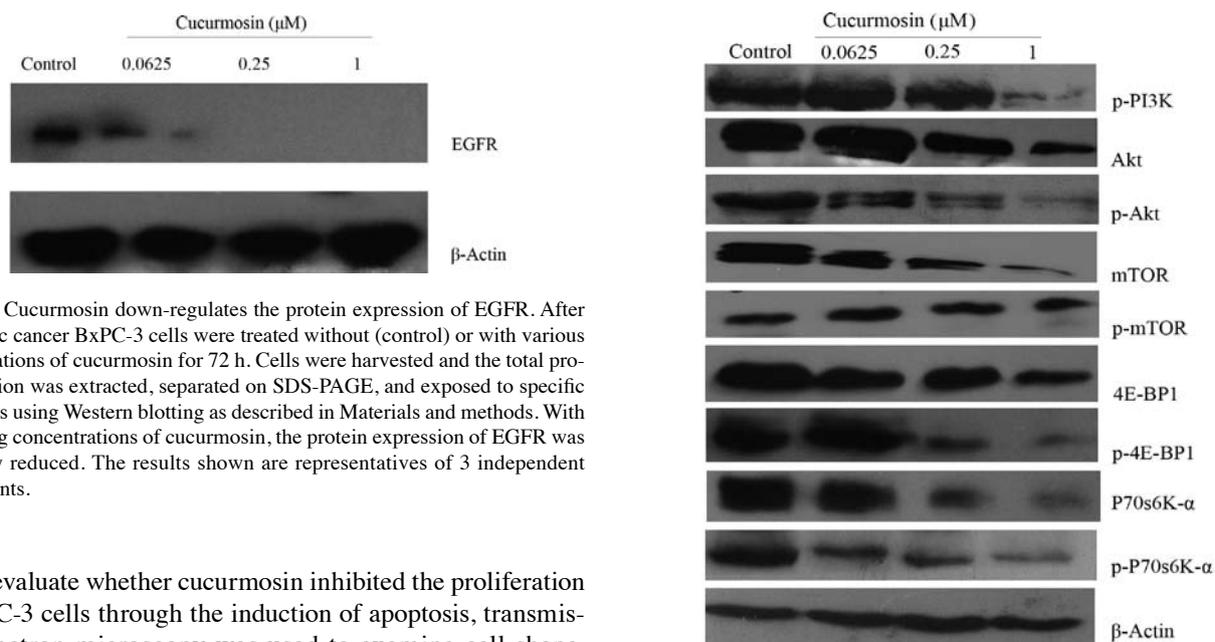


Figure 5. Cucurmosin down-regulates the protein expression of EGFR. After pancreatic cancer BxPC-3 cells were treated without (control) or with various concentrations of cucurmosin for 72 h. Cells were harvested and the total protein fraction was extracted, separated on SDS-PAGE, and exposed to specific antibodies using Western blotting as described in Materials and methods. With increasing concentrations of cucurmosin, the protein expression of EGFR was gradually reduced. The results shown are representatives of 3 independent experiments.

To evaluate whether cucurmosin inhibited the proliferation in BxPC-3 cells through the induction of apoptosis, transmission electron microscopy was used to examine cell shape. Consistently, after BxPC-3 cells were treated with 1 μM cucurmosin for 72 h, cells showed typical features of apoptosis, such as destroyed cellular structures, collapsed cells, and the appearance of a significant number of apoptotic bodies in BxPC-3 cells (Fig. 2). To further evaluate the apoptosis in BxPC-3 cells, DNA content and apoptosis ratios were analyzed by flow cytometry using the Cell Cycle Detection kit and the Annexin V-FITC Apoptosis Detection kit. We found that the cell cycle of pancreatic cancer BxPC-3 cell was arrested in G0/G1 phase (Fig. 3), and the apoptosis rates without (control) or with 0.0625, 0.25, 1 μM cucurmosin for 72 h were $5.31 \pm 0.21\%$, $25.17 \pm 1.32\%$, $51.79 \pm 2.32\%$ and $76.57 \pm 3.25\%$, respectively (Fig. 4) ($P < 0.05$).

Cucurmosin down-regulates EGFR protein expression and inactivates the PI3K/Akt/mTOR signaling pathway. EGFR mutations are rare in pancreatic tumors and have not been

Figure 6. Cucurmosin blocks the signal transduction of the PI3K/Akt/mTOR signaling pathway via down-regulating EGFR. Cells were treated without (control) or with various concentrations of cucurmosin for 72 h, and harvested. Total protein fractions were extracted, separated on SDS-PAGE, and exposed to specific antibodies using Western blotting as described in Materials and methods. With increasing concentrations of cucurmosin, the protein expressions of activated (phosphorylated) PI3K, total and activated (phosphorylated) Akt, total and activated (phosphorylated) mTOR, total and activated (phosphorylated) 4EBP-1 and total and activated (phosphorylated) P70s6K- α were gradually reduced. The results shown are representatives of 3 independent experiments.

associated with clinical prognosis, and treatment response (15), but increasing evidence suggests the dysregulation of EGFR pathways by overexpression or constitutive activation could promote tumor growth and metastasis, and that this is associated with poor prognosis and tumor aggressiveness in

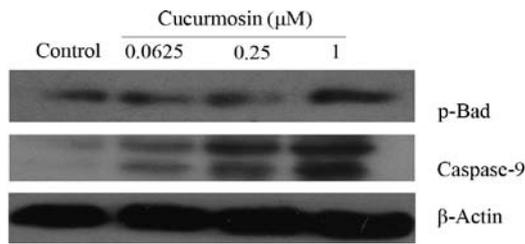


Figure 7. Curcumin increases activated (phosphorylated) Bad and pro-caspase-9, pro-apoptotic proteins of the Akt downstream targets. Cells were treated without (control) or with various concentrations of curcumin for 72 h, and harvested. Total protein fractions were extracted, separated on SDS-PAGE, and exposed to specific antibodies using Western blotting as described in Materials and methods. With increasing concentrations of curcumin, the protein expressions of activated (phosphorylated) Bad and pro-caspase-9 were elevated. The results shown are representatives of 3 independent experiments.

pancreatic cancer (16-20). In this study, we detected EGFR expression by Western blotting. The results show that EGFR expression was reduced after BxPC-3 cells were treated with various curcumin concentrations for 72 h (Fig. 5). This demonstrates that EGFR is an effective target for curcumin. To explore how curcumin induces the apoptosis in BxPC-3 cells via the EGFR pathway, the key components of the PI3K/Akt/mTOR signaling pathway were detected using Western blotting. Expressions of EGFR, p-PI3K, Akt, p-Akt, mTOR, p-mTOR, P70S6K- α , p-P70S6K- α , 4E-BP1 and p-4E-BP1 were decreased (Fig. 6), but expressions of p-Bad and caspase-9 were increased by curcumin in a dose-dependent manner (Fig. 7). This finding shows that curcumin might induce the apoptosis of pancreatic cancer cells through inactivating the PI3K/Akt/mTOR signaling pathway.

Curcumin does not alter EGFR gene transcription in BxPC-3 cells. In a recent study, Fujita *et al* illustrated that the high-level expression of EGFR mRNA was correlated with shorter disease-free-survival and overall-survival after gemcitabine-based-adjuvant chemotherapy (21). To investigate whether curcumin can down-regulate the EGFR mRNA expression, RT-PCR was performed. Conversely, we found that EGFR mRNA expression was not changed after BxPC-3 cells were treated with various curcumin concentrations for 72 h (Fig. 8). This is explained by the function of curcumin, in inhibiting only protein synthesis.

Discussion

We found that curcumin can inhibit the proliferation of human pancreatic cancer BxPC-3 in a time and dose-dependent manner. IC₅₀ for 24, 48 and 72 h were 1.45±2.14, 0.54±1.78, 0.22±0.69 μ M, respectively (P<0.05). The apoptosis rates without treatment or with 0.0625, 0.25, 1 μ M curcumin for 72 h were 5.31±0.21, 25.17±1.32, 51.79±2.32 and 76.57±3.25%, respectively (P<0.05). These findings show that curcumin inhibits the proliferation of pancreatic cancer BxPC-3 cell through the induction of apoptosis.

A comprehensive genetic analysis of 24 pancreatic ductal adenocarcinoma (PDAC) defined alterations in 12 cell-signaling pathways. The dysregulated pathways include Wnt/

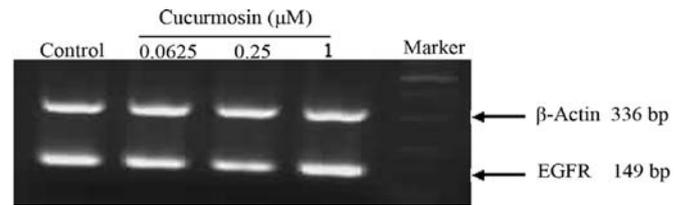


Figure 8. Curcumin does not affect EGFR gene transcription in pancreatic cancer BxPC-3 cells. Cells were treated without (control) or with various concentrations of curcumin for 72 h, and harvested. Total-RNA was extracted. RT-PCR detected the mRNA expression of EGFR. With increasing concentrations of curcumin, the mRNA expression of EGFR was not altered. The figures shown are representatives of 3 independent experiments.

Notch and Hedgehog signaling, each contributing to pancreatic tumorigenesis (22). Therefore, pancreatic cancer is a multi-target disease. Moore *et al* demonstrated improvement in survival to 6.24 vs. 5.91 months with the combination of gemcitabine and erlotinib, a small-molecule tyrosine kinase inhibitor that targets and blocks epidermal growth factor receptor (EGFR), compared to gemcitabine alone (23). This trial raised significant interest in targeting the EGFR signaling pathway in metastatic pancreatic cancer.

EGFR is 170 kDa protein belonging to 1 of 4 members of the ErbB family of transmembrane tyrosine kinase growth receptors (24). Activation of EGFR in tumors results in increased cell proliferation, reduced apoptosis, increased angiogenesis, increased motility, invasion and metastasis (25). It is believed to play an integral part in tumorigenesis of multiple epithelial cancers, including pancreatic cancer (26). The EGFR over-expression is observed in 30-89% of pancreatic cancers assayed by immunohistochemistry techniques (27,28). Its expression had been shown to correlate with worse outcome and more aggressive disease in few retrospective studies (16-20). Therefore, EGFR-targeted therapy is a significant strategy.

Curcumin belongs to type 1 RIP and is similar to trichosanthin in structure, but the cytotoxicity of curcumin is more potent than trichosanthin. Curcumin possesses an rRNA N-glycosidase activity and inhibits protein synthesis (29). In this study, we detected the EGFR expression at the protein and mRNA level using Western blotting and RT-PCR, respectively. With increasing concentrations of curcumin, the EGFR expression was gradually decreased (P<0.05), which shows that curcumin can down-regulate the EGFR expression in pancreatic cancer BxPC-3 cells, and EGFR is an effective target for curcumin. But the mRNA expression was not changed, which demonstrates that curcumin only inhibits the protein synthesis and does not disturb the transcription process.

The PI3K/Akt signaling pathway is an important regulator of cell growth and survival (30). In a various types of tumors, components of this signaling pathway are activated, permitting cancer cell growth and proliferation and evasion of apoptosis, contributing to tumorigenesis (31). Once EGFR binds to the corresponding ligand, the PI3K is directly activated or is activated via Ras (32). Activation of PI3K leads to the phosphorylation of Akt/PKB. Akt promotes cell survival through effects on numerous downstream targets, including

the inactivation of pro-apoptotic proteins such as Bad and caspase-9, or the activation of nuclear factor-kappa B (NF- κ B) resulting in transcription of anti-apoptotic genes (33-35). We found that expression levels of p-PI3K, Akt, p-Akt were down-regulated, but the expression levels of p-Bad and caspase-9 were elevated in response to cucurmosin treatment. This suggests that cucurmosin can inactivate the key components of the PI3K/Akt signaling pathway through inactivating EGFR. This is in accordance with a prior study, in which the EGFR inhibitor inhibited the PI3K/Akt pathway activation and induced apoptosis (36).

The mammalian target of rapamycin (mTOR) is an intracellular serine/threonine protein kinase positioned at a central point in a variety of cellular signaling cascades. EGFR and associated ligands transmit the signal to mTOR through the PI3K/Akt and Ras-Raf signaling, in particular, the PI3K/Akt/mTOR signaling pathway (37). mTOR signals through several downstream effectors, including the 4E-BP1 (eukaryotic initiation factor 4E-binding protein) family of translational repressors and the S6Ks, to initiate ribosome translation of mRNA into protein (38). Studies showed that mTOR controls cell cycle progression which was blocked in the G0/G1 phase through its cell growth effectors S6K1 and 4E-BP1 (39,40). We found the expression of mTOR, p-mTOR, P70S6K- α , p- P70S6K- α , 4E-BP1 and p-4E-BP1 at the protein level were decreased in a dose-dependent manner ($P < 0.05$). Meanwhile, the cell cycle of pancreatic cancer BxPC-3 cell was arrested in the G0/G1 phase using a Cell Cycle Detection kit by FACSCalibur. Cucurmosin can inactivate the activity of mTOR signaling through the PI3K/Akt signaling pathway. The blockade of the cell cycle might be due to down-regulation of P70S6K- α and 4E-BP1.

In summary, cucurmosin can down-regulate the EGFR expression in pancreatic cancer cell. But the effect is selective and not specific because cucurmosin might down-regulate many targets. Cucurmosin induced the apoptosis of pancreatic cancer cell via inactivating the PI3K/Akt/mTOR signaling pathway. The PI3K/Akt/mTOR signaling pathway can be activated or inhibited by a lot of molecules, present on the cell membrane or in the cytoplasm, and apoptosis is a complex process controlled by a number of signaling pathways. Here, we preliminarily investigated the mechanism of the induction of apoptosis for cucurmosin and future studies will further explore this mechanism.

Acknowledgements

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