

RESEARCH ARTICLE

Synergistic Anti-tumor Effect of KLF4 and Curcumin in Human Gastric Carcinoma Cell Line

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Abstract

Krüppel-like factor 4 is a transcription factor which plays an important role in development and progression of various carcinomas. Curcumin characterized by excellent anti-cancer properties is regarded as a serviceable natural compound used in carcinoma therapy. This study aimed at exploring the impact of KLF4 overexpression in cooperation with curcumin on the proliferation, apoptosis and invasion of human gastric carcinoma BGC-823 cells. Flow cytometry analysis, CCK-8 assays, transwell assays and Western blot results showed that KLF4 overexpression combined with curcumin had significant anti-proliferation, pro-apoptosis and anti-invasion effects on BGC-823 cells. We also found that KLF4 had synergistic effects with curcumin, better promoting apoptosis and inhibiting proliferation and invasion of gastric carcinoma cells. These results indicate that KLF4 could be used as a potential therapeutic target; curcumin could act as an auxiliary and provide a promising therapeutic strategy in stomach cancer.

Keywords: KLF4 - curcumin - stomach cancer - proliferation - migration - invasion

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Introduction

Gastric carcinoma is one of the most invasive and aggressive malignancy, with serious threatens to patients' health. Gastric cancer is considered as the fourth most common cancer worldwide which is caused by eating disorders, *Helicobacter pylori* (HP), carcinogenic chemicals and so on (Ekinci et al., 2014; Gryko et al., 2014; Huang et al., 2014). It is investigated that dietary improvements and prevention in HP infection due to the use of antibiotics can effectively cause a steady decrease in incidence and mortality rates of stomach cancer (Ekinci et al., 2014). While the present situation of gastric cancer therapy is still barely satisfactory, it is imperative to get a detailed understanding of the molecular biology of gastric carcinoma. It will be undoubtedly a general trend that molecular therapy combined with anti-tumor natural compound are employed in gastric carcinoma treatment.

The Krüppel-like factor (KLFs) family transcription factors play important role in the regulation of multiple biological functions, including proliferation, apoptosis, differentiation, inflammation, migration and tumor formation (Tiwari et al., 2013). KLF4 known as a zinc finger type of transcription factor has been investigated highly expressed in the skin, intestine, testis, lung, bone and various tumor tissues. KLF4 also called gut-enriched Krüppel-like factor (GKLF) has drawn much attention due to its tumor suppressive activity and growth arrest. It is reported that KLF4 is downregulated in a number of cancers (Flandez et al., 2008; Zhang et al., 2012).

Yoon et al. (2005) found that KLF4 was sufficient in cell proliferation of colon cancer. Wei et al. (2005) reported that alteration of KLF4 expression plays a critical role in gastric cancer cell growth and apoptosis. Zhang et al. (2012) demonstrated that KLF4 inhibited the proliferation, invasion and metastasis of gastric cancer. However, its role in cancer is not fully conclusive as it is also identified to act as an oncogene in some cancers.

Curcumin, a polyphenolic natural product, is the main active secondary metabolite of tumeric extracted from the rhizomes of *Curcuma longa*, which is used widely in cooking and traditional Chinese medicines (Mehta et al., 2014; Zang et al., 2014). It is reported that curcumin has been employed as an anticancer compound which effectively inhibits proliferation, invasion, migration, angiogenesis and metastasis in various cancer cells involving colon cancer, breast cancer, lung cancer, thyroid cancer and prostate cancer (Feng et al., 2014; Xu et al., 2014). Increasing evidence indicates that curcumin has a range of molecular targets and influences numerous biochemical and molecular process. Moreover, curcumin is safe for humans and there is no dose-limiting toxicity when curcumin is administered at doses up to 10 g/day (Lu et al., 2014; Xue et al., 2014).

In the present study, we attempt to estimate the effect of KLF4 overexpression cooperated with curcumin on proliferation, apoptosis and invasion of human gastric carcinoma cell line BGC-823 in vitro. In addition, we also identified whether curcumin had synergistic action with KLF4 for the purpose of providing insights toward gastric cancer treatment.

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Materials and Methods

Cell culture, transfection and treatment

The human gastric carcinoma BGC-823 cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI medium (Gibco Invitrogen Corporation, Carlsbad, CA) supplemented with 10% Fetal bovine serum (Gibco Invitrogen Corporation, Carlsbad, CA), 2 mM L-glutamine, 100 IU/ml penicillin and 100 mg/ml streptomycin at 37°C in a 5% CO₂ incubator. Cells of KLF4 low-expression were screened for transfection by means of western blot. The plasmid pCMV-KLF4; provided by JRDUN Biotechnology (Shanghai) Co., Ltd. Plasmid KLF4 (3 mg) or mock-vehicle plasmid were transfected into BGC-823 cell lines in 6-well plates (1 mg/ml) using the lentiviral vector, according to the manufacturer's instructions. Cells were treated with curcumin (15 µmol/l) for 48 h, and then were subjected to cell apoptosis and viability assay.

Quantitative reverse transcription (qRT)-PCR

Total RNA was isolated from transfected cells, mock cells and non-transfected cells using TRIzol (Invitrogen) according to the protocol. High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) was employed to synthesize cDNAs. Quantitative real-time PCR was preceded using SYBR Green PCR Master Mix (Applied Biosystems) in a 7900 Real-Time PCR System (Applied Biosystems). GAPDH was performed as the reference gene. The following primers were used: for KLF4, 5' AACCTGGCGGACATCAAC 3' (forward), 5' AGCACGAACCTGCCCCATC 3' (reverse); for GAPDH, 5' CACCCACTCCTCCACCTTTG 3' (forward), 5' CCACCACCCTGTTGCTGTAG 3' (reverse). The PCR cycles were 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Each reaction was performed in triplicate and analyzed individually. The results were analyzed by ABI Prism 7300 SDS Software.

Cell viability assay

For cell viability assay, cells (5×10⁵ cells/ml) were loaded in 96-well plates and treated with DMSO for the indicated times. CCK8 (10 µL) was added to each well with treated cells and incubated at 37°C for another 4h. Absorbance was read at 450 nm using a microplate reader.

Cell apoptosis assay

With or without curcumin treatment for 24 hours, cells were stained with annexin V–fluorescein isothiocyanate and apoptosis rates were analyzed using a flow cytometer (FACSCalibur, BD Biosciences).

Invasion assay

The invasion assay was carried out by using a modified two chamber plates with a pore size of 8 µm. Cells were harvested and resuspended in serum free medium in the upper chamber coated with 150 mg Matrigel (BD Biosciences, Bedford, MD). After 24 h incubation at 37°C, no invasive cells were gently removed from the top of the matrigel with a cotton-tipped swab. Invasive cells at the

bottom of the matrigel were fixed in methanol, stained with 1% crystal violet and counted under a microscope. Results were averaged from three independent experiments.

Western blotting

Proteins from cell samples were separated by SDS polyacrylamide gel electrophoresis, followed by electrotransfer to a nitrocellulose membrane by means of a transfer cell (Bio-Rad, Hercules, CA, USA). The proteins were then transferred to nitrocellulose membrane and incubated overnight at 4°C with the following antibodies: anti-cyclin D1, anti-E-cadherin, anti-Bax, anti-VEGF (Santa Cruz, CA, USA), anti-PI3K, anti-JNK, anti-ERK (Cell Signaling Technology, Beverly, MA, USA), anti-BCL-2. Immunoreactive bands were detected by reaction with the ECL detection system reagents (Amersham, Arlington Heights, IL, USA) and exposure to X-ray film, which was the developed and photographed.

Statistical analysis

Results were expressed as mean values± SEM of three independent experiments. For statistical tests, Prism 5.0 (GraphPad Software, San Diego, CA, USA) was used. *p* values less than 0.05 were considered significant.

Results

KLF4 Overexpression and curcumin inhibit the proliferation of BGC-823 cell promote apoptosis

To identify the roles of KLF4 expression in BGC-823 cells proliferation and apoptosis by real time quantitative PCR, the mammalian cell expression plasmids for KLF4 overexpression were constructed. As shown in Figure 1A, compared with the mock, the level of KLF4 mRNA was increased strongly in BGC-823 cells transfected with the KLF4 expression plasmid. The western blotting Figure 1B intuitively showed the high KLF4 expression of pCMV-KLF4.

We further evaluate the effects of KLF4 overexpression and cooperation with curcumin on the proliferation of BGC-823 cell. We detected the OD (450 nm) value of BGC-823 cell by CCK-8 to generate cell growth curves. As shown in Figure 1C, mock treated with curcumin, KLF4 overexpression and KLF4 overexpression treated with curcumin significantly inhibits BGC-823 proliferation at 72h, as compared to the control group. Figure 1D and 1E) showed that the cells of mock treated with curcumin, pCMV-KLF4 and pCMV-KLF4 treated with curcumin significantly decreased in S and G2 compared with the mock. Flow cytometric analysis displayed that G1 arrest before the apoptosis appeared in BGC-823 cell.

KLF4 and curcumin suppress BGC-823 cell proliferation by regulating PI3K/Akt and JNK/MAPK signal pathways

PI3K and cyclinD1 are crucial proteins in PI3K/Akt signal pathway regulating cell proliferation and cell cycle (Rusyn et al., 2000; Wang et al., 2001; Kim et al., 2009). JNK known as a stress-activated protein kinase involved in JNK/MAPK signal pathway play important role in regulating cell proliferation (Wang et al., 2001). As is shown in Figure 1F, the levels of *p*-PI3K, cyclinD1

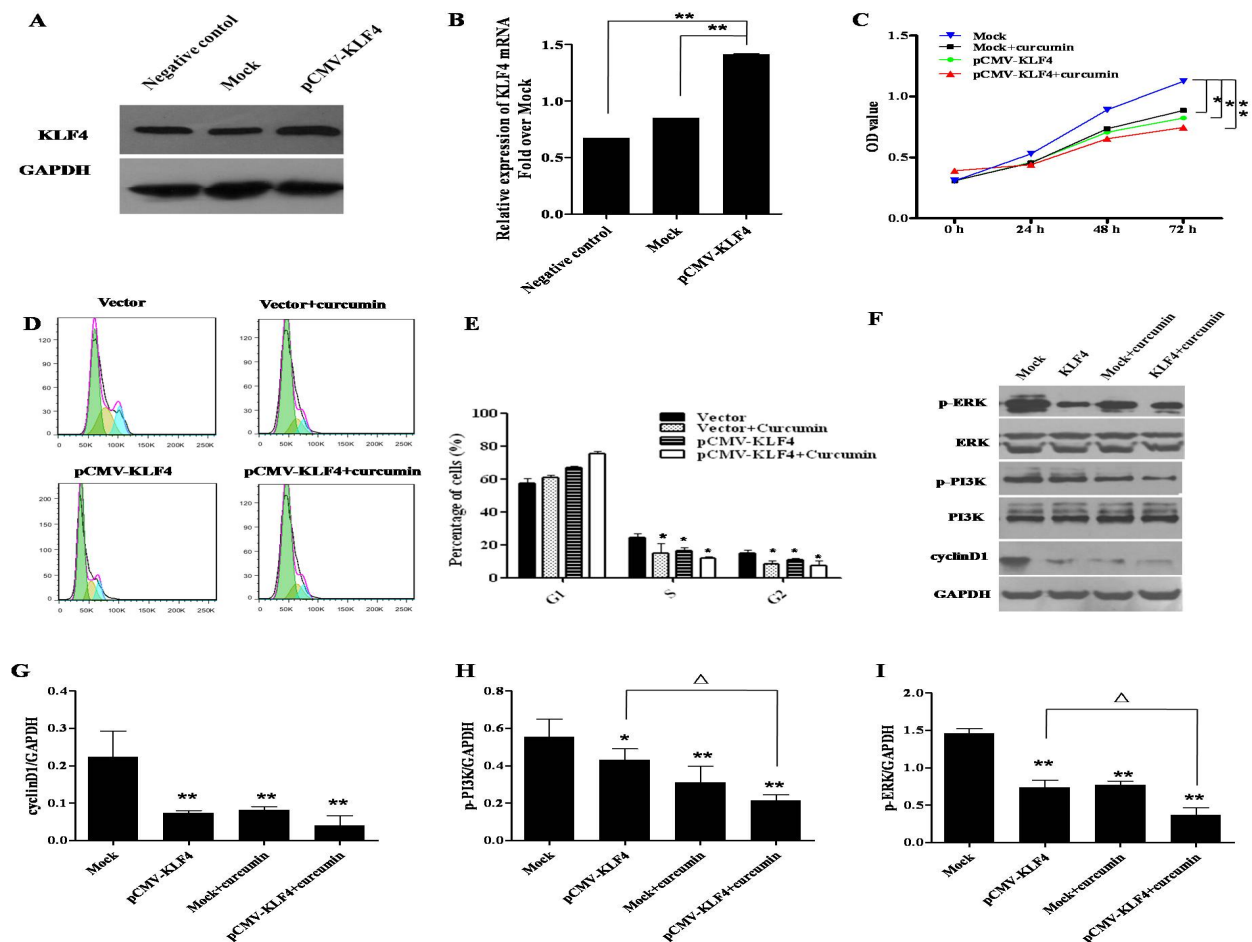


Figure 1. Effects of KLF4 Expression and Curcumin on Proliferation of BGC-823 Cells. A) BGC-823 cells were transfected with KLF4 plasmids. At 48 hr post-transfection, cells were lysed for WB analysis at 24 hr post-transfection using antibodies against the indicated proteins. GAPDH was detected as control for sample loading; B) Total RNA was isolated from cell samples and the mRNA level of KLF4 was analyzed by quantitative RT-PCR; C) Cell proliferation was measured at the indicated time post-transfection by CCK-8 test as described in the Methods and Materials; D, E) Effects of KLF4 expression and curcumin on cell-cycle distribution were detected by flow cytometry; F) Cells were transfected with the indicated plasmids or treated with curcumin and lysed for WB analysis at 24 h using antibodies against the indicated proteins. GAPDH was detected as control for sample loading; G, H, I) The cyclinD1, *p*-PI3K and *p*-ERK contents of mock cells, curcumin treated cells, KLF4 overexpression cells and curcumin treated transfected cells. Data were presented as mean $\pm$ SD, n=3, * P <0.05, ** P <0.01, Δ P <0.05, $\Delta\Delta$ P <0.01

and *p*-JNK are decreased in KLF4 expression plasmid transfected cells and curcumin treated transfected cells. In addition, the Figure 1H and 1I shows that *p*-PI3K and *p*-JNK levels of curcumin treated transfected cells are notable less than that of KLF4 overexpression cells. The results implied that KLF4 overexpression and cooperation with curcumin inhibit BGC-823 cell proliferation by regulating PI3K/Akt and JNK/MAPK signal pathways.

KLF4 Overexpression and curcumin encourage the apoptosis of BGC-823 cell

To estimate the effect of KLF4 overexpression, curcumin and combined action of both contribute to the inhibition of BGC-823 cell apoptosis, we examine the influence of them on cell apoptosis with Annexin-PI staining by flow cytometry. Figure 2A and 2B shows that mock treated with curcumin, KLF4 overexpression and KLF4 overexpression seeded with curcumin notably induce the apoptosis of human BGC-823 cells compared with the vector. In the Figure 2B, compared with the pCMV-KLF4, the pCMV-KLF4 treated with curcumin significantly promotes the apoptosis of BGC-823.

KLF4 and curcumin promote BGC-823 cell apoptosis by regulating apoptotic factors and ERK/MAPK signaling

As previous result showed that KLF4 overexpression and curcumin significantly accelerate the process of BGC-823 cell apoptosis, we further measure the expression quality of apoptotic factors in mock cells, curcumin treated mock cells, pCMV-KLF4 plasmid-transfected cells and curcumin treated transfected cells (Figure 2C). The activation of Bax and Bcl-2 was detected by means of western blotting. Bax playing a positive role on apoptosis was significantly up-regulated compare to mock cells. On the contrary, the Bcl-2 expression in curcumin treated cells, and transfected cells seeded with curcumin are lower than the mock cells. The relative value of Bax/Bcl-2 was shown in Figure 2D. The Bax/Bcl-2 value of transfected cells treated with curcumin is notably higher than the transfected cells. Since ERK/MAPK signal pathway regulates the process of cell apoptosis (McCain, 2013), we furthermore detected the expression level of *p*-ERK. The level of *p*-ERK was decreased notably in curcumin treated transfected cells compared with KLF4 overexpression cells (Figure 2F). These indicated that KLF4 and curcumin

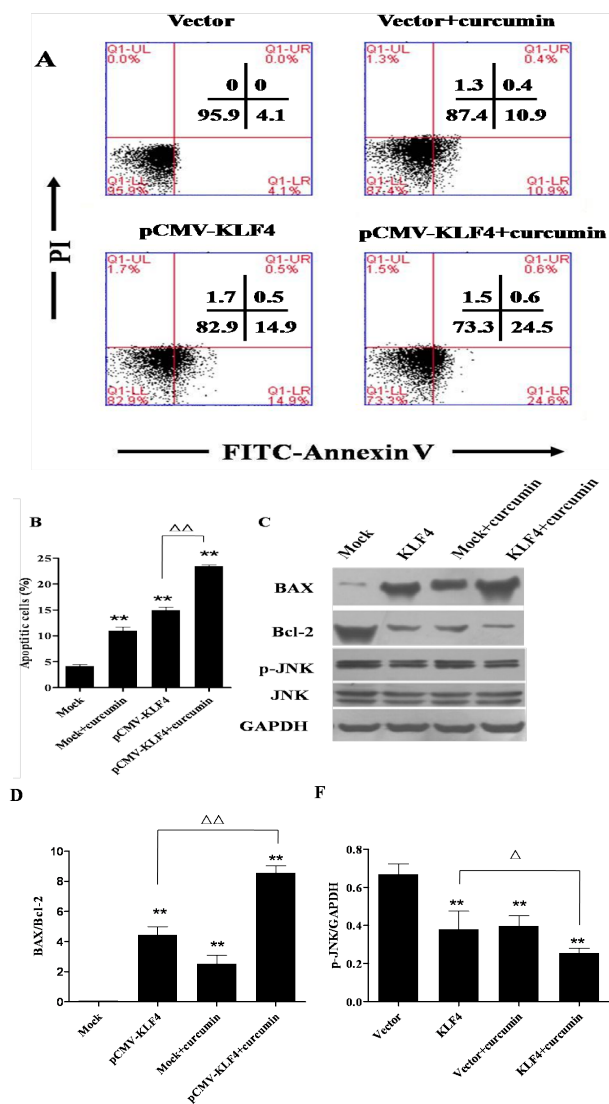


Figure 2. Effects of KLF4 Expression and Curcumin on Apoptosis of BGC-823 Cells. A, B) BGC-823 cells were transfected with KLF4 overexpression plasmids, control plasmids, or both treated with curcumin. After 48 hr, cell apoptosis was analyzed with Annexin-PI staining by flow cytometry; C, D, E) Cells were lysed for WB analysis at 24 h using antibodies against the indicated proteins including BAX, Bcl-2, p-JNK and p-JNK. GAPDH was detected as control for sample loading. Data were presented as mean±SD, n=3, **P*<0.05, ***P*<0.01, Δ *P*<0.05, $\Delta\Delta$ *P*<0.01

promoted BGC-823 cell apoptosis by regulating apoptotic factors and ERK/MAPK signal pathway

KLF4 and curcumin inhibit invasion of BGC-823 cells by down-regulating E-cadherin level

The ability of cells to cross matrigel revealed the invasiveness of the BGC-823 cell. The effects of KLF4 and curcumin on invasion of human gastric carcinoma BGC-823 cells were identified by transwell assay. Figure 3A implies that the vector treated with curcumin, pCMV-KLF4 and pCMV-KLF4 treated with curcumin significantly suppress the invasion of BGC-823 cell. Compared with the pCMV-KLF4, the effect of pCMV-KLF4 treated with curcumin on BGC-823 cell invasion was more dramatic. The amount of invading cells crossing

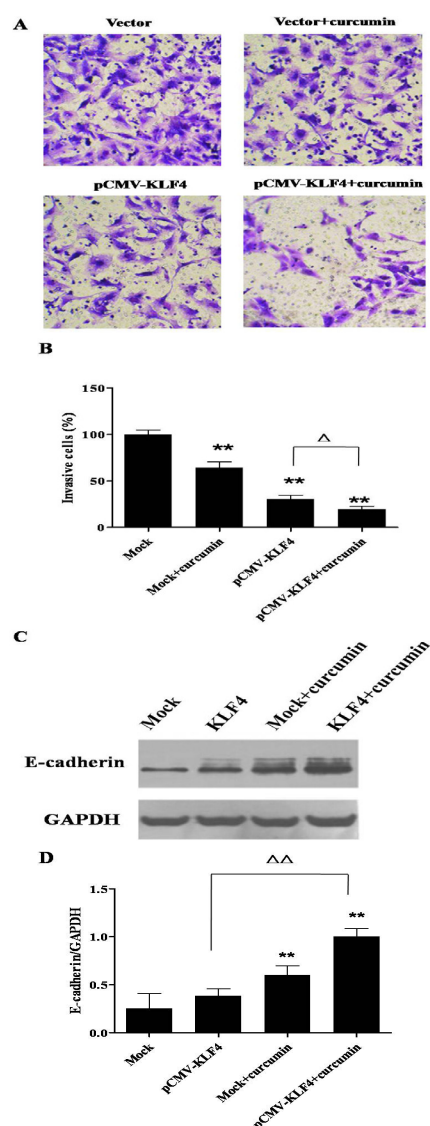


Figure 3. KLF4 Overexpression and Curcumin Inhibits Invasion of BGC-823 Cells. A, B) BGC-823 cell invasion assay was performed after re-expression of KLF4 BGC-823 cells, control group or KLF4 BGC-823 cells treated with curcumin; C, D) Cells were lysed for WB analysis at 24 h using antibodies against the indicated protein of E-cadherin. GAPDH was detected as control for sample loading. Data were presented as mean±SD, n=3, **P*<0.05, ***P*<0.01, Δ *P*<0.05, $\Delta\Delta$ *P*<0.01

through the matrigel basement membrane is shown in Figure 3B.

E-cadherin is reported as a kind of Ca^{2+} dependent glycoprotein, which performs essential part in cell invasion and migration (Lubkov and Bar-Sagi, 2014; Pamies, 2014). As showed in Figure 3A and 3B, the levels of E-cadherin expression are higher in curcumin treated cells, KLF4 plasmid transfected cells and curcumin treated transfected cells. In the Figure 3C and 3D, the levels of E-cadherin are notably higher in curcumin treated cells, KLF4 overexpression cells and both treated cell compared with the control group. The E-cadherin level of curcumin treated transfected cells is significantly higher than that of KLF4 expression plasmid transfected cells. The results implied that KLF4 and curcumin inhibit invasion of BGC-823 cells by down-regulating E-cadherin level.

Discussion

Stomach cancer is the second-greatest cause of cancer death globally and patients died from gastric carcinoma are more than those died from other malignancies in China. Both molecular therapy and natural anti-cancer product treatment toward stomach cancer are immediate areas of research focus. KLF4, a transcription factor expressed abundantly in the epithelium of the digestive tract, has been reported that KLF4 expression plays a critical role in gastric cancer development and progression (Wei et al., 2005). Curcumin as a common natural food pigment exhibit favorable anti-cancer activity which be used in many kinds of neoplasms treatment. Our study including identifying the anti-proliferation, pro-apoptotic and anti-invasive effect and mechanism of KLF4, curcumin and their combination may help to better understand how KLF4 and curcumin affect gastric carcinoma cell physiological activity and provide theoretical basis for clinical early diagnosis and intervention of gastric cancer.

The dominate findings of this study are the following:

i) KLF4, curcumin and their combination can effectively suppress the proliferation of human gastric carcinoma BGC-823 cells. The anti-proliferation effect of KLF4 overexpression and curcumin combination was more significantly than either of them. The impact of KLF4 and curcumin on cell cycle result showed that KLF4, curcumin and their combination arrested cell cycle at G1 period. PI3K/Akt and JNK/MAPK single pathways have been reported as import pathways that closely correlate with the proliferation, differentiation and migration of tumor cells (Hinoi et al., 2014; Li et al., 2014). Western blotting result implied that the levels of *p*-PI3K, cyclinD1 and *p*-ERK in KLF4, curcumin and their combination were plummeting compared with the control, and the combination of KLF4 overexpression and curcumin lead notable decrease of *p*-PI3K, cyclinD1 and *p*-ERK. These findings indicated that KLF4 and curcumin can effectively inhibit the proliferation of human gastric cancer BGC-823 cells by regulating PI3K/Akt and ERK/MAPK single pathways and arrested cell cycle at G1 period by down-regulating the expression of cyclinD1 (Meng et al., 2012). The combination of KLF4 overexpression and curcumin play a better role than either of them individually. ii) KLF4, curcumin and their co-work play a positive part in BGC-823 cell apoptosis. The cooperation of KLF4 overexpression and curcumin exhibited a more significant efficiency in inducing the process of apoptosis according to the flow cytometry analysis. Bax and Bcl-2 are the proteins which play important role in cell apoptosis (Theron et al., 2013; Pryczynicz et al., 2014). The further study on evaluating apoptotic proteins levels of BAX and Bcl-2 arrived at the consistent conclusion. JNK/MAPK signal pathway play crucial roles in tumor cell apoptosis (Meng et al., 2012; You et al., 2013; Bloch et al., 2014). *p*-JNK levels of KLF4 overexpression cells, curcumin treated cells and co-treated cells were notable less than the control. The above results explained that KLF4 promoted the apoptosis of BGC-823 cells by regulating JNK-MAPK signal pathway and combined with curcumin can achieve better result.

Previous study reported that KLF4 expression plays a critical role in gastric cancer development and progression (Wei et al., 2005; Zhang et al., 2012). Effect of KLF4 on gastric carcinoma BGC-823 cell invasion is discussed in our study. The result of whether KLF4 suppresses the invasion of BGC-823 cell showed that overexpression of KLF4 and combined with curcumin can effectively depress the invasion of gastric carcinoma cells. The western blotting result showed the expression level of E-cadherin was decreased more significantly in curcumin treated KLF4 overexpression cells.

In conclusion, this study identified the anti-apoptotic/pro-proliferative and anti-invasive roles of KLF4 and co-work with curcumin in human gastric carcinoma BGC-823 cells, and explained the corresponding mechanism. These findings may provide the insights toward the molecular mechanism of stomach cancer progression and pathogenesis and benefit the development of therapeutical approaches for the disease.

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