Curcumin combined with glycyrrhetinic acid inhibits the development of hepatocellular carcinoma cells by down-regulating the PTEN/PI3K/AKT signalling pathway

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Abstract: Curcumin is an active component of turmeric, which is derived from the rhizomes of Curcuma longa. Glycyrrhetinic acid (GA) is a natural compound extracted from liquorice. Both curcumin and GA are widely used as anticancer agents for treating many human cancers. In this study, curcumin and GA were used separately and in combination to treat human hepatocellular carcinoma (HCC) HepG2 cells. MTT assays were used to evaluate cell proliferation. Flow cytometry was carried out to measure cell apoptosis and determine cell cycle progression. Western blot analyses were applied to determine the expression levels of B-cell lymphoma-2 (Bcl-2), B-cell associated X protein (Bax), phosphatase and tensin homolog (PTEN), phosphorylated phosphoinositide 3-kinase (PI3K) and AKT serine/threonine kinase 1 (Akt). The results showed that combined treatment with curcumin and GA resulted in a significant reduction in proliferation and an increase in apoptosis and G1 cell cycle arrest in HepG2 cells. A xenograft tumour model showed that curcumin and GA suppressed HCC development in vivo. Moreover, by knocking down the expression of PTEN, we confirmed that curcumin and GA exert their anticancer effects by inhibiting the PTEN/PI3K/Akt signalling pathway. Collectively, these results indicate that the combination of curcumin and GA could effectively inhibit the development of HepG2 cells by inhibiting PTEN/PI3K/Akt signalling and could be a promising treatment strategy for patients with HCC.

Keywords: Curcumin, GA, HCC, development, PTEN/PI3K/Akt

Introduction

Hepatocellular carcinoma (HCC) is the sixth most prevalent type of malignant tumour and the third leading cause of cancer-related mortality worldwide [1]. Currently, liver transplantation and surgical resection remain the most effective therapeutic approaches for treating HCC [2-4]. Due to uncontrolled tumour metastasis and frequent intrahepatic spread, the prognosis of HCC patients is still unsatisfactory [5]. Therefore, the identification of effective and safe drug treatments for HCC is urgently needed.

Traditional Chinese medicine (TCM) has been used to treat malignant tumours for a long time in China. TCM has attracted attention in recent years because greater than two-thirds of novel anti-cancer drugs are natural [6, 7]. Curcumin is also called diferuloylmethane and is obtained from Curcuma longa. Previous studies have demonstrated that curcumin has anti-inflammatory, antioxidant and anti-infective properties [8]. Recently, studies have found that curcumin serves as a potent anticancer drug for treating various types of human cancers [9-11].

Glycyrrhetinic acid (GA) can be extracted from Glycyrrhiza glabra and is a well-known TCM that has been frequently used for treating various diseases. GA is extensively reported to have many pharmacological activities, including anti-ulcer, anti-inflammatory, immunomodulatory and anti-tumourigenic properties [12]. GA also has antiviral activity against HIV, hepatitis B
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Table 1. Primers used for RT-qPCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5’-3’)</th>
<th>Reverse (5’-3’)</th>
</tr>
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<tbody>
<tr>
<td>PTEN</td>
<td>GTGCAGATAATGACAAG</td>
<td>GATTTGACGGCTCCTCT</td>
</tr>
<tr>
<td>PI3K</td>
<td>GAATCCATGGGAACCTGT</td>
<td>GGGAGGTTAATAAAGGT</td>
</tr>
<tr>
<td>AKT</td>
<td>TGCAGCATTGCTTCTTTG</td>
<td>TCTGGGCGTGAAACTCCT</td>
</tr>
<tr>
<td>GAPDH</td>
<td>CAAGGTCATCCATGACAA</td>
<td>GTCCACCCACCTGTGCTG</td>
</tr>
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viruses, and SARS (severe acute respiratory syndrome)-associated coronavirus [13]. Due to these properties, combining GA with other drugs could increase their pharmacological activities. In particular, when delivered with curcumin, GA was found to increase the anticancer effects of curcumin on prostate carcinoma and lung cancer. However, the effects of curcumin and GA treatment on HCC have not been elucidated.

Here, we investigated the effects of curcumin and GA on cell proliferation, cell cycle progression, and apoptosis in HCC HepG2 cells in vitro and in vivo. We presented evidence that curcumin combined with GA inhibited the development of hepatocellular carcinoma cells by blocking the PTEN/PI3K/Akt signalling pathway. Our study may provide a basis for using curcumin and GA for the clinical treatment of HCC.

Materials and methods

Chemicals and antibodies

Curcumin and GA were purchased from Sigma (St. Louis, MO, USA). Curcumin and GA were dissolved in dimethylsulfoxide (DMSO) and maintained as a stock solution at -20°C. The final concentration of DMSO was kept below 0.2% (v/v) throughout the study. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma (St. Louis, MO, USA). Primary antibodies against PTEN, PI3K, Akt, Bcl-2, Bax and GAPDH were obtained from Cell Signaling Technology (Beverly, MA, USA).

Cell culture and transfection

HCC cells (HepG2) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% foetal bovine serum (Invitrogen, Carlsbad, CA, USA). All cells were maintained at 37°C in a humidified chamber with 95% air and 5% CO₂. PTEN siRNA (siPTEN) and control siRNA were obtained from GenePharma (Shanghai, China). HepG2 cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions.

RNA isolation and quantitative real-time RT-PCR (qRT-PCR)

Total RNA from cells and tissues was extracted using TRIzol reagent (Invitrogen, CA, USA). cDNA was synthesized by using Oligo-dT or specific microRNA stem loop RT primers with 1 μg of total RNA according to the manufacturer’s instructions. qRT-PCR was performed by using the SYBR® Premix EX TaqTMiPCR Kit (Takara, Dalian, China) according to the manufacturer’s instructions and a Roche LightCycler® 480 II Real-Time PCR System. Human GAPDH was used as an internal control. The primers are shown in Table 1. The PCR reactions were performed under the following conditions: 98°C for 2 min, 98°C for 10 s, 55°C for 30 s, and 72°C for 30 s for 40 cycles, followed by 72°C for 5 min. The results were analysed using the Applied Biosystems Comparative CT Method [14].

Protein extraction and Western blot analysis

Total protein from cells and tissues was extracted using RIPA reagents (Beyotime Biotech, Shanghai, China) according to the manufacturer’s instructions. Cell lysates were washed with PBS three times and incubated in cold RIPA buffer for 30 min. Then, the cell lysates were centrifuged at 13,000 g for 15 min at 4°C. After that, 10% SDS-PAGE (Beyotime Biotech, Shanghai, China) was used to separate equal amounts of total protein, and the proteins were transferred to PVDF membranes (Millipore, MA, USA). After blocking with 5% non-fat milk for 1 h, the membranes were incubated with anti-Bcl-2 (1:1,500), Bax (1:1,000), PTEN (1:500), PI3K (1:1,000), Akt (1:1,000), p-Akt (1:2,000) and GAPDH (1:1,000) antibodies overnight at 4°C. After that, the membranes were incubated with the corresponding HRP-linked secondary antibodies (Santa Cruz Biotechnology, USA) for 2 h at room temperature. Positive bands were detected using an ECL kit (Thermo Scientific, Waltham, MA, USA).
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Cell proliferation assay

Cell growth was determined using MTT assays performed according to the manufacturer’s instructions. Cells (approximately 1×10⁴ cells/well) were seeded into 96-well culture plates 24 h prior to treatment. After 0, 24, 48 or 72 h of treatment, 20 µl of MTT (5 mg/ml) was added to each plate and incubated for 4 h at 37°C. Then, 150 µl of dimethyl sulfoxide (DMSO) was added to each plate and agitated for 10 min at 37°C to solubilize the crystals. The number of cells per plate was measured by using the absorbance (480 nm) at the indicated time points.

Cell apoptosis assay

HepG2 cells were seeded in 6-well plates (approximately 2×10⁶ cells/well) after treatment. Cell apoptosis was detected by using a annexin V-fluorescein isothiocyanate (FITC)/PI detection kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. In brief, cells were collected and stained with 5 µl of FITC-Annexin V and 3 µl of propidium iodide (PI). Finally, flow cytometry (EPICS, Xi-4, Beckman, CA, USA) was used to evaluate the percentage of apoptotic cells after incubation for 15 min at 37°C.

Cell cycle assay

Treated HepG2 cells in the log phase of growth were collected and fixed in 75% ethanol at -20°C for 16 h. For cell cycle analysis, treated cells were stained with propidium iodide and examined with a fluorescence-activated cell sorting (FACS) flow cytometer (BD Biosciences, CA, USA); DNA histograms were analysed with modified software. Each test was repeated in triplicate.

Nude mouse tumour xenograft model

All experimental procedures involving the use of animals were in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the Ethics Committee of the Affiliated Hospital of Hubei University of Chinese Medicine (Wuhan, China). Nude mice (6 weeks old, n = 12) received 200-µl subcutaneous injections of 4×10⁶ HepG2 cells in their left flank area. Once palpable tumours developed, the volumes of the tumours were measured with a calliper every 4 days, and the following formula was used: volume = (length × width²)/2. When the tumour volume reached an average of 75 to 100 mm³, the mice were divided randomly into two groups (six mice per group). The mice were then intratumourally injected with 100 µl of siPTEN or control siRNA three times per week for 2 weeks.

Statistical analysis

All statistical analyses were conducted using SPSS 17.0 software (SPSS Statistics Inc., IL, USA). The data are presented as the mean ± standard deviation (mean ± SD). The differences between groups were analysed by using Student’s t-test or one-way ANOVA. P<0.05 was considered statistically significant. All of the experiments were conducted at least three times.
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**Results**

*Curcumin combined with GA significantly inhibited the growth of HepG2 cells*

The effects of curcumin (5 µM) and/or GA on HepG2 cells were assessed by MTT assays. HepG2 cells treated with DMSO were used as the negative control (NC) group. As shown in **Figure 1**, treating the HepG2 cells for 24, 48 or 72 h with curcumin or GA obviously inhibited cell viability compared with that of the NC group (p<0.05). Cell growth was decreased significantly when HepG2 cells were treated with GA and curcumin compared with that in the groups treated with only curcumin or GA (p<0.05). These findings suggest that curcumin combined with GA markedly inhibits cell growth in HepG2 cells.

*Curcumin combined with GA induced G1-phase cell cycle arrest in HepG2 cells*

To investigate the role of curcumin and GA in cell cycle progression, we treated HepG2 cells with curcumin and/or GA. Cell cycle assays showed that curcumin- or GA-treated HepG2 cells had a significantly increased proportion of cells in the G1 phase compared with those in the NC treatment group (Figure 2, p<0.05). Additionally, we examined the effects of curcumin combined with GA on cell cycle progression and found that the proportion of HepG2 cells in the G1 phase was significantly increased in the curcumin-and GA-treated groups compared with that in the curcumin or GA treated group (Figure 2, p<0.05). These findings indicate that curcumin combined with GA significantly induces G1-phase cell cycle arrest in HepG2 cells.

*Curcumin combined with GA markedly induced apoptosis in HepG2 cells*

Prior to treatment, the apoptosis rate in curcumin- or GA-treated (5 µM) HepG2 cells was also measured. Following treatment with curcumin or GA for 48 h, the apoptosis rate markedly increased compared with that in the NC group (Figure 3A, p<0.05). Cell apoptosis was
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increased significantly when HepG2 cells were treated with the combination of GA and curcumin compared with that of cells treated with only curcumin or GA (Figure 3A, p<0.05).

Furthermore, the expression of B-cell lymphoma-2 (Bcl-2) and B-cell associated X protein (Bax) was also measured. As shown in Figure 3B, we found that Bcl-2 expression was downregulated after treatment with curcumin and/or GA for 48 h, whereas Bax expression was upregulated. These data indicate that curcumin combined with GA markedly induced apoptosis in HepG2 cells.

Curcumin combined with GA inhibited the PTEN/PI3K/AKT signalling pathway in HepG2 cells

To determine whether curcumin and/or GA influenced PTEN/PI3K/AKT signalling in HCC cells, we treated HepG2 cells with curcumin and/or GA. Figure 4A shows that treatment with curcumin or GA decreased PI3K and AKT mRNA expression and upregulated PTEN mRNA expression in HepG2 cells compared with that in the NC cells (p<0.05). The mRNA and protein levels of PI3K, AKT and p-AKT were decreased significantly when HepG2 cells were treated with the combination of GA and curcumin compared with those in the groups treated with curcumin or GA only, and the protein expression of PTEN was upregulated markedly in HepG2 cells (Figure 4B).

Curcumin combined with GA remarkably suppressed HCC development via the PTEN/PI3K/AKT signalling pathway in vivo

To investigate whether curcumin and/or GA inhibited HCC development in vivo, we used a xenograft tumour model to examine the biological effects. As shown in Figure 5A and 5B, the tumours formed by curcumin- and/or GA-trea-
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HepG2 cells were smaller in size and weight than the tumors formed by NC-treated HepG2 cells. Conversely, the tumors formed by siPTEN-transfected HepG2 cells were larger than those of the curcumin and GA treated group (p<0.05). Furthermore, Western blot assays revealed that curcumin and GA suppressed PI3K, AKT and p-AKT protein expression in HCC tissues and promoted PTEN levels, while the knockdown of PTEN partly reversed this effect.

Collectively, these results strongly demonstrate that curcumin combined with GA remarkably inhibits the development of HepG2 cells by down-regulating the PTEN/PI3K/AKT signalling pathway.

Discussion

With the improvement of modern medical technology and cancer prevention, the incidence...
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of HCC has decreased remarkably in the past few years. However, HCC remains the sixth leading cause of mortality in malignant diseases. Alternative therapies, including chemotherapy, radiotherapy, and radiochemotherapy, though effective, are not curative [15, 16]. In recent decades, several natural products originating from medicinal herbs have broadened our understanding of cancer treatment because of their extensive biological activities. Drugs such as emodin, curcumin, and GA have been demonstrated to have anti-cancer effects by inhibiting the proliferation, invasion, and metastasis of multiple malignant cancers [17-19]. Although many studies have revealed their pharmacological mechanisms, much research is still needed.

In the present study, the effects of curcumin combined with GA on proliferation, cell cycle progression and apoptosis in HCC cells and the possible mechanisms were investigated. The results indicated that curcumin combined with GA significantly inhibited cell growth in HepG2 cells and induced G2-phase cell cycle arrest. Cell apoptosis was increased significantly when HepG2 cells were treated with the combination of GA and curcumin. Furthermore, we demonstrated that curcumin combined with GA inhibited the development of HCC in vivo.

Numerous studies have shown that activation of the PI3K/Akt signalling pathway is essential for the development and progression of HCC and can modulate the malignant behaviour of HCC, such as cell proliferation, invasiveness, angiogenesis and metastasis [20-23]. Of note, PTEN, which acts as an inhibitor of the PI3K/Akt pathway, has been found to be inhibited in HCC due to gene deletions or mutation during oncogenesis [24, 25]. PTEN is an essential regulator of cell proliferation, differentiation, growth and apoptosis, and its deficiency is closely associated with HCC development and progression [26]. In addition, PTEN can inhibit tumour cell growth and invasion by suppressing the PI3K/Akt pathway [27].

Xu et al have demonstrated that curcumin inhibits the invasion and migration of FTC133 cells via downregulating the PI3K/Akt signalling pathway in thyroid cancer cells [28]. Xu et al suggested that curcumin inhibits tumour proliferation in lung cancer through the PI3K/Akt pathway [29]. Schee et al revealed that miR-22-3p, miR-143-3p and miR-192-5p regulated and were involved in the APC, TGFβ and PI3K pathways in colorectal cancer cells [30]. Herein, we discovered that GA combined with curcumin inhibited PI3K/Akt signalling through activating PTEN, suggesting that GA and curcumin may represent potential therapeutic drugs for HCC treatment.

In conclusion, to the best of our knowledge, this study is the first to focus on the effects of GA combined with curcumin against HepG2 cells. The data demonstrate that GA combined with curcumin significantly inhibits the development of HepG2 cells through suppressing the PTEN/PI3K/AKT signalling pathway. The conclusions of the present study indicate that GA and curcumin are potential drugs for treating HCC. However, the present study reveals some limitations as we have not examined the detailed relationship between GA and curcumin and PTEN/PI3K/AKT signalling in HCC cells. Additional clinical studies and large-scale statistical analyses should be performed to verify the results of the present study.

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Disclosure of conflict of interest

None.

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