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ORIGINAL ARTICLE

Cryptocaryone induces apoptosis in human hepatocellular carcinoma cells by inhibiting aerobic glycolysis through Akt and c-Src signaling pathways

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Abstract

Hepatocellular carcinoma (HCC) is the most common form of liver cancer, with the second highest mortality rate in all cancer. Energy reprogramming is one of the hallmarks of cancer, and emerging evidence showed that targeting glycolysis is a promising strategy for HCC treatment. Cryptocaryone has been shown to display promising anti-cancer activity against numerous types of cancer. Previous study also indicated that cryptocaryone induces cytotoxicity by inhibiting glucose transport in cancer cells, but the detailed mechanism still needs to be elucidated. Therefore, this study aimed to investigate the relationship between the anti-cancer effect and glycolytic metabolism of cryptocaryone in human HCC cells. In this study, we found that cryptocaryone potently induced growth inhibition by apoptotic cell death in HCC cells. Cryptocaryone also suppressed the ATP synthesis, lactate production and glycolytic capacity of HCC cells. Mechanistic investigations showed that phosphorylation of Akt and c-Src, as well as the expression of HK1 were impeded by cryptocaryone. Moreover, cryptocaryone markedly increased the expression level of transcription factor FoxO1. Importantly, clinical database analysis confirmed the negative correlation between HK1 and FoxO1. High expression levels of HK-1 were positively correlated with poorer survival in patients with HCCs. These results suggest that cryptocaryone may promote cell apoptosis by inhibiting FoxO1-mediated aerobic glycolysis through Akt and c-Src signaling cascades in human HCC cells. This is the first study to indicate that cryptocaryone exerts anti-cancer property against human HCC cells. Cryptocaryone is a potential natural product worthy of further development into a promising candidate for HCC treatment.

Keywords: Apoptosis, Cryptocaryone, Glycolysis, Hepatocellular carcinoma

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1. Introduction

According to GLOBOCAN 2020, liver cancer is the sixth most common cancer and the third leading cause of cancer-related death, with an incidence rate of 4.7% and a mortality rate of 8.3%, respectively [1]. Hepatocellular carcinoma (HCC), the most common form of liver cancer (over 90% of liver cancer cases), suffered from an unfavorable 5-year survival rate (approximately 18%) due to the difficulty of early diagnosis, high recurrence rates and acquired resistance to the systemic therapies [2–4]. Currently, systemic target therapy (Sorafenib [5], Lenvatinib [6], and Regorafenib [7]), immune checkpoint inhibitors (Nivolumab [8], Pembrolizumab [6]) or a combination of both (Atezolizumab + Bevacizumab [9]) are the first- and second-line treatments against for advanced HCC treatments. However, many of these therapies succumb to limited anti-cancer activity, adverse treatment-related events and acquired resistance [10,11]. Therefore, novel strategies against HCC are urgently needed.

Recently, emerging studies are theorizing that metabolic reprogramming may be another hallmark of cancer [12]. As Otto Warburg discovered that tumor cells prefer utilizing glucose via glycolysis even in the oxygen-rich condition in the 1920s, the existence of the phenomenon termed the Warburg effect (aerobic glycolysis) in HCC was further proven by many studies [13–15]. Furthermore, this metabolic shift has been shown to play an essential role in the rapid proliferation [16], invasion [17], and sorafenib resistance [18] in HCC. Hexokinase (HK), phosphofructokinase (PFK), and pyruvate kinase (PK) are the rate-limiting enzymes of the glycolysis process. Overexpression of these enzymes has been observed in HCC and is associated with poor overall survival of HCC patients [19,20]. In recent years, emerging evidence suggests that the transcription factor family Forkhead box O proteins (FoxOs) are one of the principal regulators of cancer metabolism [21]. The hepatic knockout of FoxO1/3/4 have been shown to increase glycolysis compared with WT mice [22]. Furthermore, a recent study discovered that CircRPN2 could suppress HCC glycolysis and progression by targeting FoxO1 expression [23]. Hence, the effective agents targeting FoxO1-regulated glycolysis may be a promising novel strategy against HCC.

Over the past decades, natural products have been proven to be a vital source of novel liver cancer treatments [24]. Even though the mechanism of these compounds varies, many studies have suggested that many of them exert their anti-HCC activity by targeting glycolytic pathways [25,26]. Cryptocaryone is a dihydrochalcone derivative identified in many *Cryptocarya* plants [27–30]. Several studies have shown cryptocaryone possesses an anti-cancer effect against leukemia [29], prostate [31], ovarian [32], and oral [33] cancer cells. Compelling study proposes that cryptocaryone induces cytotoxicity and decreases the glucose uptake in cancer cells with an unknown mechanism [34]. However, the anti-cancer effect of cryptocaryone on human HCC cells is mostly unknown. Whether and how cryptocaryone impacts aerobic glycolysis in human HCC cells has not been well investigated. Therefore, we conducted this study to investigate the relationship of cryptocaryone with cytotoxic effect and glycolysis metabolism, and further elucidate its mechanism of action in human HCC cells.

2. Materials and methods

2.1. Materials

Cryptocaryone was isolated from the leaves of *Cryptocarya chinensis* with 98% purity as previously described [35]. Sulforhodamine B (SRB), Dimethyl sulfoxide (DMSO), and other chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA). Src activator peptides were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The following primary antibodies were used in Western blot analysis and purchased from Cell Signaling Technology (Danvers, MA, USA): PARP (Cat# 9542), LC3B (Cat# 3868), p-Akt (S473, Cat# 4060), p-ERK (T202/Y204, Cat# 4370), p-JNK (T183/Y185, Cat# 4668), p-p38 (T180/Y182, Cat# 45,110), p-Src (Y416, Cat# 2101), FoxO1 (Cat# 2880), p-FoxO1 (T256, Cat# 84,192), ATF6 (Cat# 65,880), PERK (Cat# 5683), eIF2α (Cat# 5324), p-eIF2α (S51, Cat# 3398), IRE1α (Cat# 3294), XBP1 (Cat# 27,901), PDI (Cat# 3501), Calnexin (Cat# 2679), Mcl-1 (Cat# 5453), Bcl-2 (Cat# 4223), Bcl-XL (Cat# 2764), HK1 (Cat# 2024), HK-2 (Cat# 2867), PFKP (Cat# 8164), PKM1/2 (Cat# 3190), and GAPDH (Cat# 5174). Primary antibodies against Caspase 3 (Cat# 19677-1-AP) were purchased from Proteintech (Rosemont, IL, USA). The horseradish peroxidase-linked secondary antibodies Anti-rabbit IgG (Cat# 7074) were purchased from Cell Signaling Technology.

2.2. Cell culture

Three human HCC cell lines, SK-Hep-1, HuH-7, and HA22T/VGH were employed in this study.

SK-Hep-1 and HA22T/VGH were obtained from the American Type Culture Collection (Manassas, VA, USA) and the Bioresource Collection and Research Center (Hsinchu, Taiwan), respectively. HuH-7 cells were kindly provided by Dr. Yi-Hsien Hsieh (Chung Shang Medical University, Taichung, Taiwan). SK-Hep-1 and HA22T/VGH cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (Thermo). HuH-7 cells were cultured in low-glucose DMEM (Thermo) supplemented with 10% FBS. All cell lines were maintained in accordance with a previously described protocol [36].

2.3. Cell viability analysis

The SRB assay was used to measure the viability of human HCC cells. Cells were seeded into 96-well plates at a density of 5000 cells/well and treated with indicated agents for 24 or 48 h. Then 50 μL of 10% trichloroacetic acid were added directly into each well and incubated at room temperature for 15 min. After washing each well with PBS and air-drying in the air, 50 μL of 0.4% SRB solution were added into each well and incubated at room temperature for 15 min. The plates were washed with 1% acetic acid to remove excess dyes and air-dried. Finally, the remaining SRB were dissolved by adding 100 μL of 10 mM Tris-base solution into each well, and the absorbance of each well at 490 nm were measured with a microplate reader (Molecular Devices, San Jose, CA, USA).

2.4. Western blot analysis

After treatment, cells were harvested and subjected to RIPA lysis buffer containing protease and phosphatase inhibitors (Roche, Basel, Switzerland). Protein lysates were prepared by the method based on our previous work [37]. Equal amounts of proteins (20–40 μg) were separated by SDS-PAGE (10–15% of polyacrylamide gel) and transferred to 0.2 μm PVDF membranes (Bio-Rad, Hercules, CA, USA). Membranes were then blocked with 5% non-fat dry milk (in Tris-buffered saline with 0.05% Tween-20, TBST) for 1 h at room temperature. After blocking, the membranes were subjected to overnight incubation with primary antibodies (diluted with 5% BSA in TBST) at 4 °C. The membranes were washed three times with TBST and further incubated with secondary antibody for 1 h at room temperature. After another three-time washes with TBST, the membranes were incubated with ECL reagents (Millipore, Burlington, MA, USA). The signals were collected with a UVP imaging system (Analytik Jena, Jena, Germany). The collected data were further quantified using Image J software.

2.5. Quantification of apoptosis

Cells were seeded into 96-well plates at a density of 5000 cells/well and treated with the indicated concentration of cryptocaryone for 24 h. DNA fragmentation was measured with Cell Death ELISA^{PULS} kit (Roche) according to the manufacturer's protocol [38].

2.6. Analysis of ATP and lactate production

Cells were seeded into 96-well plates at a density of 5000 cells/well and treated with the indicated concentration of cryptocaryone for 24. The ATP and lactate levels were measured with luminescent ATP detection kit (Abcam, Cambridge, UK) and colorimetric Lactate assay (Cell Biolabs, San Diego, CA, USA) as previously reported [39].

2.7. Bioenergetics analysis

The basal extracellular acidification rate (ECAR) and glycolytic capacity were measured by the Seahorse XF24 analyzer (Agilent, Santa Clara, CA, USA) according to the manufacturer's instructions of Glycolysis Stress Test Kit (Agilent). Cells (2×10^4) were seeded into XF24 cell culture microplates. On the next day, these cells were treated with or without cryptocaryone for 24 h and the XF24 sensor cartridge was hydrated with XF Calibrant inside a non-CO_2 37 °C incubator. On the following day, for basal ECAR detection, each well was washed twice and incubated in non-buffered DMEM (Gibco, without the addition of sodium bicarbonate) for 1 h inside a non-CO_2 37 °C incubator. The basal ECAR of cryptocaryone-treated cells was then recorded for 1 h. For the Glycolysis Stress Test, each well was washed twice and incubated in XF base medium (supplemented with 2 mM Glutamine) for 1 h inside a non-CO_2 37 °C incubator. Glucose, oligomycin and 2-deoxyglucose (2-DG) were dissolved in the XF base medium (final concentration: 10 mM, 1 μM, and 100 mM, respectively) and loaded into the designated injection ports on the sensor cartridge. The ECAR of cryptocaryone-treated cells was then measured with the automatic sequential injection of glucose, oligomycin, and 2-DG. Finally, after ECAR measurement, cryptocaryone-treated cells were subjected to SRB assay to obtain the cell density index for data normalization.

2.8. Plasmid and siRNA transfection

The pUSEamp-myr-Akt1 plasmids and the empty vector (pUSEamp) were kindly provided by Prof. Chien-Huang Lin (Taipei Medical University, Taipei, Taiwan). Transfection of these plasmids with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was conducted as described previously [37]. The siRNA targeting FoxO1 and control siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Transfection of these siRNAs with Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) was conducted according to the manufacturer's protocol.

2.9. Quantitative PCR analysis

Total RNA was isolated from indicated cells using the Trizol reagent (Thermo Fisher Scientific, MA, USA) following the manufacturer's protocol. 1 μg of total RNA were subject to the GoScript™ Reverse Transcription system (Promega Corporation, WI, USA) for the synthesis of cDNA. qPCR was performed with the GoTaq™ qPCR Master Mix (Promega Corporation, WI, USA) on a 7900HT Fast Real-Time PCR System (Thermo Fisher Scientific, MA, USA). The sequence of the primers for qPCR were: HK1, (F) 5′-CTG CTG GTG AAA ATC CGT AGT GG-3′, (R) 5′- GTC CAA GAA GTC AGA GAT GCA GG-3′; GAPDH, (F) 5′-TGA TGA CAT CAA GAA GGT GAA G-3′, (R) 5′- TCC TTG GAG GCC ATG TGG GCC AT-3′. The CT values of HK1 were normalized to the expression level of GAPDH. Relative expression (Fold) was calculated using the $2^{-\Delta\Delta Ct}$ method.

2.10. In silico *analysis of publicly available HCC database*

The All RNA-seq and ChIP-seq sample and signature search (ARCHS^4) is a data repository that includes over 200,000 standardized gene counts from sequencing samples of the Gene Expression Omnibus (GEO) and Sequence Read Archive (SRA). Herein, we analyzed the correlation between the expression of FoxO1 and HK1 in HCC tissues from ARCHS^4 with Correlation AnalyzeR and Pearson's χ^2 test. The prognostic value of HK1 in HCC was determined by Kaplan–Meier analysis using the GSE20140 dataset of the GEO database.

2.11. Data and statistical analysis

All data are presented as mean ± standard deviation, and all statistical analyses were performed with GraphPad Prism software (San Diego, CA, USA). One-way ANOVA followed by Tukey's post hoc test were performed when multiple groups were analyzed. The non-parametric Kruskal–Wallis tests with Dunns' post hoc test were performed when comparing normalized data (to the control group). $p < 0.05$ was considered statistically significant.

3. Results

3.1. Cryptocaryone inhibits cell growth and induces apoptosis of human HCC cells

To evaluate the anti-cancer effect of cryptocaryone (chemical structure showed in Fig. 1A), three human HCC cell lines were treated with the indicated concentration of cryptocaryone and subjected to the cell viability analysis. As shown in Fig. 1B, cryptocaryone displayed a concentration-dependent growth inhibition in SK-Hep-1, HuH-7, and HA22T cells with IC_{50} values of 1.37 ± 0.03 μM, 1.60 ± 0.06 μM, and 1.93 ± 0.09 μM, respectively. Previous study has shown that the growth-inhibitory effect of cryptocaryone against cancer cells is related to apoptotic cell death [31]. Moreover, autophagy plays either pro-death or pro-survival roles in cancer [40]. The effect of cryptocaryone on these pathways in human HCC cells was examined by Western blot analysis. After cryptocaryone treatment, the cleavage form of Caspase 3 and Poly (ADP-ribose) polymerase (PARP) were significantly increased while the expression of autophagy marker LC3-II remained unaltered (Fig. 1C-D). Furthermore, cryptocaryone also significantly increased the apoptosis levels in a concentration-dependent manner in SK-Hep-1 cells (Fig. 1E). All these results demonstrate that cryptocaryone can induce apoptotic cell death in human HCC cells.

3.2. Cryptocaryone suppresses the activation of Akt and c-Src signals and up-regulates the expression of FoxO1

Previous studies have shown that mitogen-activated protein kinases (MAPKs), Akt, and c-Src signaling pathways regulate glucose metabolism and apoptosis in cancer [41,42]. Therefore, we suspect these signaling pathways may play an essential role in the growth-inhibitory effect of cryptocaryone. To verify this hypothesis, we investigated the signal transduction in cryptocaryone-treated SK-Hep-1 cells. As shown in Fig. 2A-B, the phosphorylation of these kinases was obviously increased by serum stimulation, and the treatment of cryptocaryone significantly attenuated the activation of Akt and c-

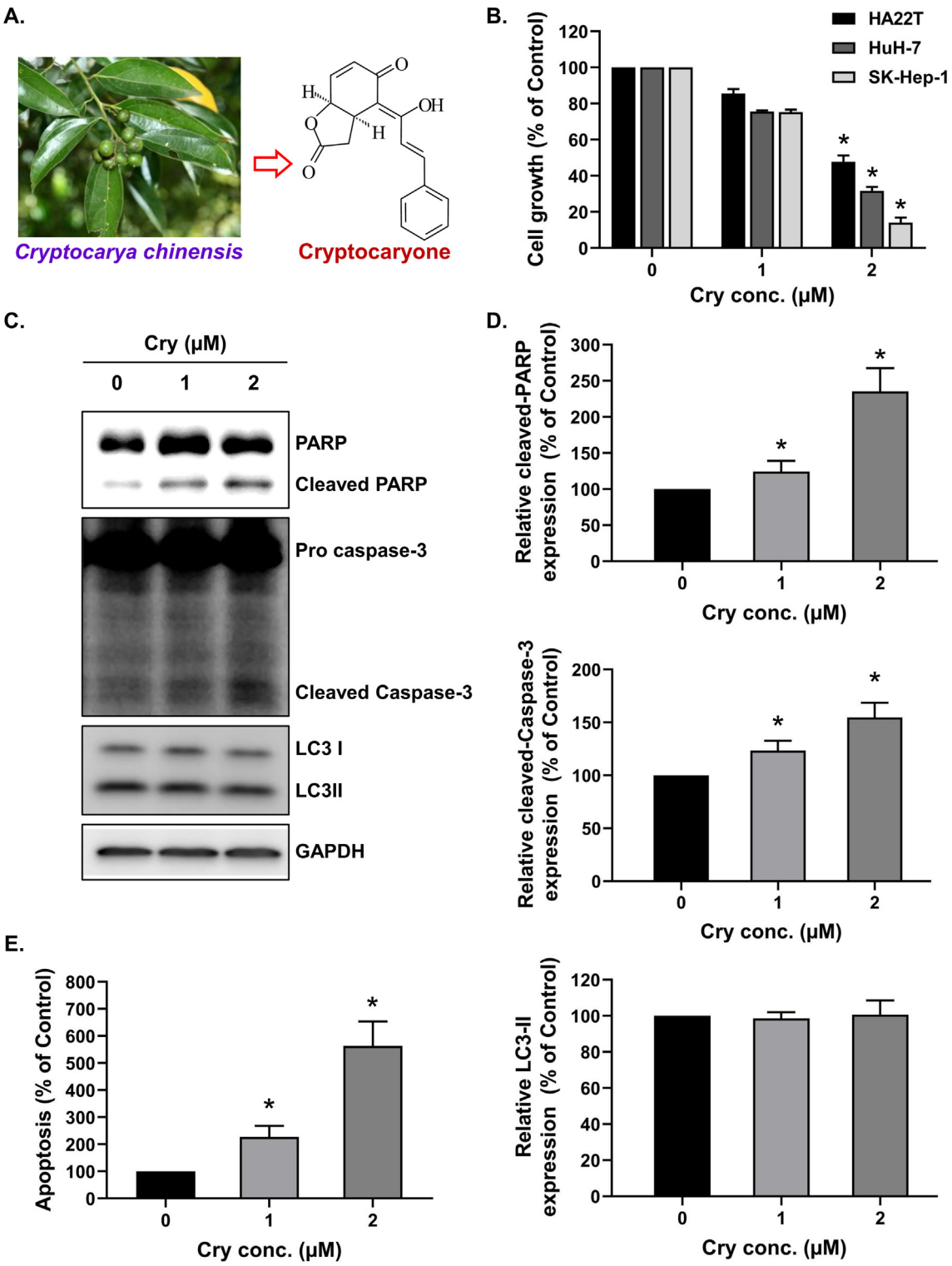


*Fig. 1. **Effect of cryptocaryone cell growth and apoptosis of human HCCs.** (A) Cryptocaryone (Cry) was isolated from the leaves of Cryptocarya chinensis. (B) HA22T, HuH-7, and SK-Hep-1 were treated with different concentrations of Cry (0, 1, and 2 μM) for 48 h, then cell growth was determined using SRB assay. (C) SK-Hep-1 were treated with the indicated concentrations of Cry for 24 h. Then, cell lysates were subjected to Western blot analysis for PARP, Caspase 3, and LC3B. GAPDH was employed as an internal control. (D) The quantitative densitometry analysis of the western bolts was performed with ImageJ software. (E) The quantitative assessment of oligonucleosomal DNA fragmentation was used to identify apoptotic cell death in Cry-treated SK-Hep-1 cells. Data are shown as means ± standard deviation of at least three independent experiments. *$p < 0.05$, significantly different from the control.*

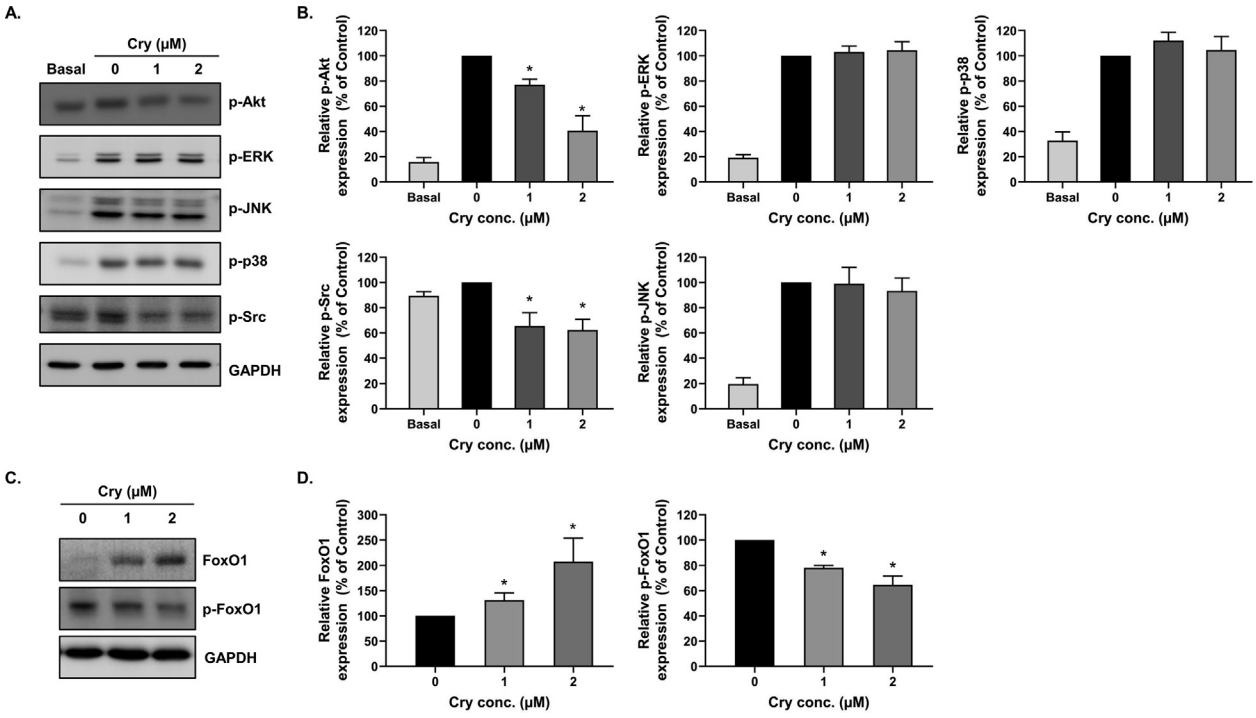


Fig. 2. ***Effect of cryptocaryone on apoptosis-related signaling pathways and transcription factor FoxO1.*** *(A) Quiescent SK-Hep-1 cells were treated with or without (Basal) 10% FBS containing medium in the absence or presence of cryptocaryone (Cry) for 15 min, and cell lysates were then subjected to Western blot analysis for of p-Akt (S473), p-ERK (T202/Y204), p-JNK (T183/Y185), p-p38 (T180/Y182) and p-c-Src (Y416). (C) SK-Hep-1 were treated with the indicated concentrations of Cry for 24 h. Cell lysates were subjected to Western blot analysis for FoxO1 and p-FoxO1 (T256). (B&D) Quantified levels of indicated phosphorylated and total proteins were determined using ImageJ software. GAPDH was employed as an internal control. Data are shown as means ± standard deviation of at least three independent experiments. *p < 0.05, significantly different from the control.*

Src kinases. Since Akt and c-Src regulate the transcription activity of FoxO1 via phosphorylation, resulting in cytosolic translocation and degradation [43], we further analyzed whether the cryptocaryone-induced suppression of Akt and c-Src signals affect the expression and the phosphorylation of FoxO1. We observed a significant increase in the expression of FoxO1 accompanied by a significant decrease in phosphorylation of FoxO1 in cryptocaryone-treated SK-Hep-1 cells (Fig. 2C-D). These findings suggest that cryptocaryone may induce apoptosis by increasing the expression of FoxO1 via Akt and c-Src signaling pathways in HCC cells.

3.3. Cryptocaryone induces apoptosis of human HCC cells through Akt signaling pathway

To explore the role of Akt pathway in cryptocaryone-mediated apoptosis, SK-Hep-1 cells were transfected with either a Myr-Akt (myristoylated, constitutively active form of Akt) plasmid or an empty plasmid. As shown in Fig. 3A-B, cryptocaryone-impeded Akt phosphorylation was dramatically prevented by Myr-Akt transfection. Additionally, the transfection of Myr-Akt plasmid significantly reduced cryptocaryone-induced cleavage of PARP (Fig. 3C-D) and the expression of FoxO1 (Fig. S1 https://www.jfda-online.com/journal/vol31/iss4/10/). Finally, the transient transfection of Myr-Akt obviously antagonized the growth-inhibitory effect of cryptocaryone (Fig. 3E). These results revealed that cryptocaryone promotes apoptosis via inhibiting Akt activation in human HCC cells.

3.4. Cryptocaryone induces apoptosis of human HCC cells through c-Src signaling pathway

We next investigated whether c-Src pathway is involved in cryptocaryone-induced apoptosis of human HCC cells. Pharmacological c-Src activator (SFA) was used for mechanistic investigation. Firstly, we observed that cryptocaryone-induced inhibition of c-Src phosphorylation was prevented by c-Src activator (Fig. 4A). c-Src activator also significantly reduced the cleavage of PARP caused by cryptocaryone (Fig. 4A-B) and the expression of FoxO1 (Fig. S1 https://www.jfda-online.com/journal/vol31/iss4/10/). Importantly, stimulation of cells with c-Src activators obviously antagonized the growth-inhibitory effect of cryptocaryone (Fig. 4C). Taken together, we

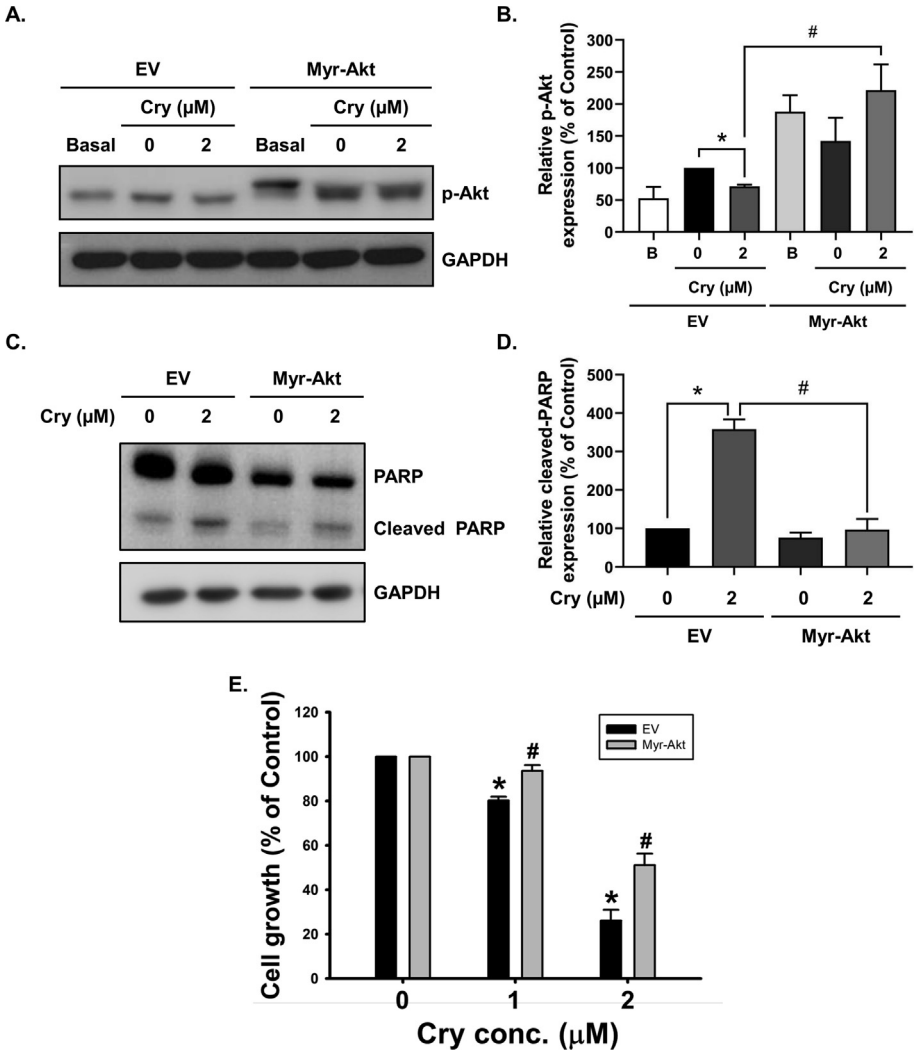


Fig. 3. ***Akt pathway is involved in cryptocaryone-induced apoptosis of HCC cells.*** *(A) SK-Hep-1 cells-transfected with empty vector (EV) or Myr-Akt were treated with vehicle (basal) or 10% FBS in the absence (control) or presence of cryptocaryone (Cry) for 15 min. Then, cells were harvested and lysed for the detection of p-Akt by Western blot analysis. (C) SK-Hep-1 cells-transfected with empty vector (EV) or Myr-Akt were treated with or without Cry for 24 h. Cell lysates were subjected to Western blot analysis for PARP. (B&D) Quantified levels of indicated phosphorylated and total proteins were determined using ImageJ software. GAPDH was employed as an internal control. (E) The cell growth was determined using SRB assay. Data are shown as means ± standard deviation of at least three independent experiments. *p < 0.05, significantly different from the control. #p < 0.05, significantly different from the EV group treated with Cry.*

suggest that c-Src pathway is also the important route for cryptocaryone-induced apoptosis in human HCC cells.

3.5. Cryptocaryone exerts apoptotic cell death by disrupting glycolysis metabolism

Based on a previous study which showed that glucose metabolism may be involved in the anti-cancer effect of cryptocaryone [34], we sought to investigate the bioenergetic effect and mechanism underlying cryptocaryone-induced apoptosis of human HCC cells. First, we examined whether cryptocaryone affects the intracellular ATP content and lactate production, two indicators of the Warburg effect, in cancer cells. Measurement of lactate production (indicated by extracellular acidification) allows confirmation of cells switching their energy production preference to glycolysis. As shown in Fig. 5A-B, cryptocaryone concentration-dependently inhibited the ATP synthesis and lactate production in SK-Hep-1 cells. The Seahorse XF Extracellular Flux Analyzer was further employed to investigate the effect of cryptocaryone on glucose

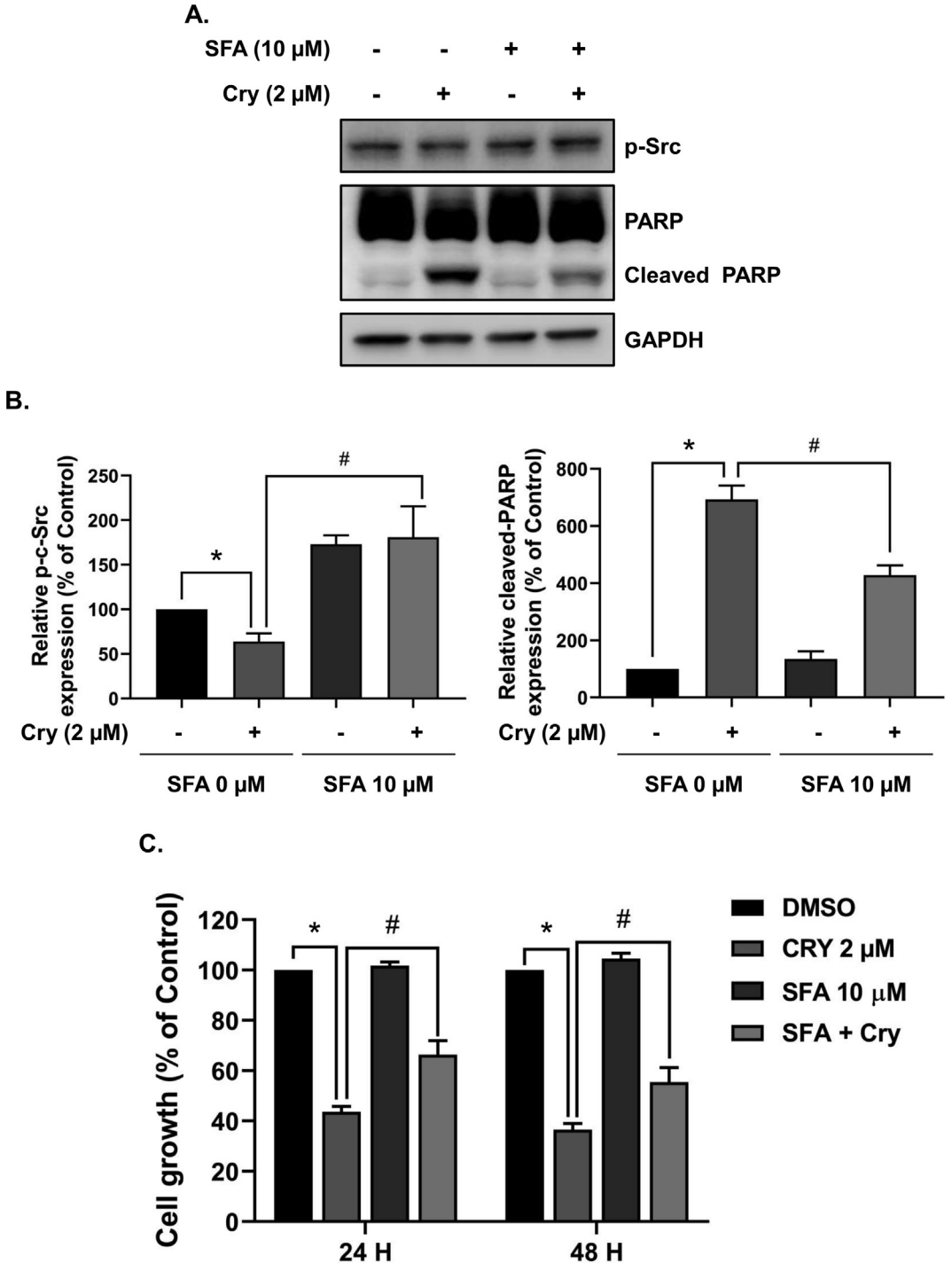


Fig. 4. ***c-Src pathway is involved in cryptocaryone-induced apoptosis of HCC cells.*** *(A) SK-Hep-1 cells were treated with or without c-Src activator (SFA) in the absence or presence of cryptocaryone (Cry) for 24 h. Then, cells were harvested and lysed for the detection of p-Src and PARP by Western blot analysis. GAPDH was employed as an internal control. (B) The quantitative densitometry analysis of the western bolts was performed with ImageJ software. (C) SK-Hep-1 cells were treated with or without Src activator in the absence or presence of 2 μM Cry for 24 h and 48 h. Then, cell growth was determined using SRB assay. Data are shown as means ± standard deviation of at least three independent experiments. *p < 0.05, significantly different from the control. #p < 0.05, significantly different from the Cry-treatment alone group.*

metabolism. We measured the baseline extracellular acidification rate (ECAR, an indicator of glycolytic flux) in cryptocaryone-treated SK-Hep-1 cells. As shown in Fig. 5C, cryptocaryone significantly suppressed the ECAR of cells at 0 min and 60 min. Furthermore, the glycolytic stress kit revealed that cryptocaryone inhibited the glycolysis, glycolytic capacity, and glycolytic reserve of SK-Hep-1 cells

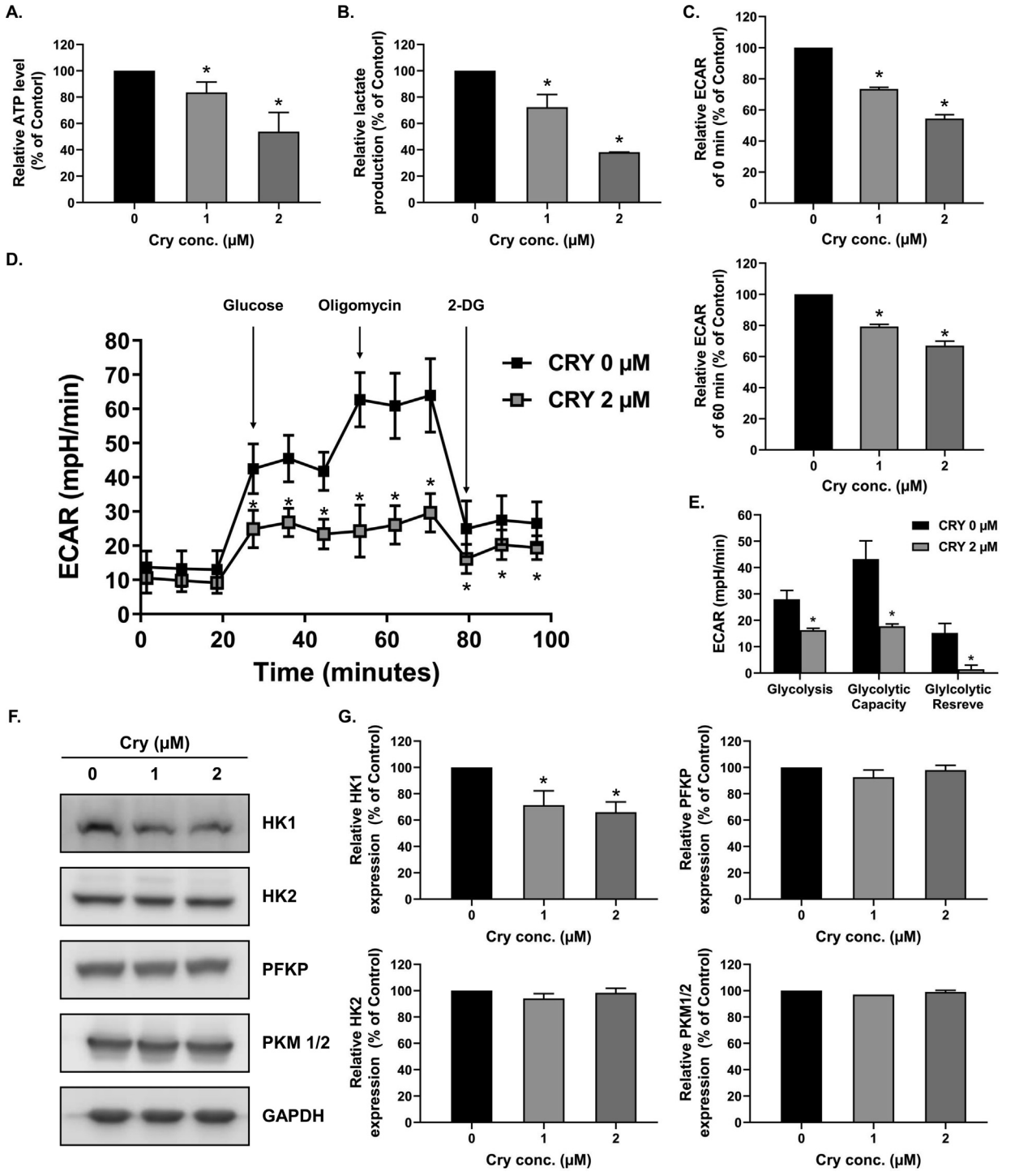


Fig. 5. ***Cryptocaryone inhibits glycolysis metabolism and HK1 expression in human HCC cells.*** *SK-Hep-1 were treated with the indicated concentrations of cryptocaryone (Cry) for 24 h. (A) The intracellular ATP content and (B) lactate production was measured by assay kit. (C) The basal glycolytic activity of immediately (left) and 60 min (right) after 24 h treatment of Cry was measured using the Agilent Seahorse XF24 analyzer. (D) The glycolytic capacity was measured using Glycolysis Stress Test. (E) Key parameters of glycolytic flux calculated from Glycolysis Stress Test. (F) Cell lysates were subjected to Western blot analysis for HK1, HK2, PFKP and PKM1/2. GAPDH was employed as an internal control. (G) The quantitative densitometry analysis of the western bolts was performed with ImageJ software. Data are shown as means ± standard deviation of at least three independent experiments. *$p < 0.05$, significantly different from the control.*

(Fig. 5D-E). Next, we assessed the impact of cryptocaryone on a series of key glycolytic proteins, including HK1, HK2, PFKP, and PKM 1/2. Interestingly, only the expression of HK1 was significantly repressed by cryptocaryone in SK-Hep-1 cells (Fig. 5F-G) and the mRNA expression of HK1 was also significantly inhibited by cryptocaryone (Fig. S2 https://www.jfda-online.com/journal/vol31/iss4/10/). Additionally, HuH-7 and HA22T cells were employed to further assess the effect of cryptocaryone on glycolysis metabolism. As shown in Fig. S3 https://www.jfda-online.com/journal/vol31/iss4/10/, cryptocaryone treatment increased the expression of FoxO1 and inhibited the expression of HK1 in a concentration dependent manner. Furthermore, glycolysis was also inhibited by cryptocaryone treatment. Collectively, these results indicate that HK1 may be the crucial target underlying cryptocaryone-mediated aerobic glycolysis-dependent apoptosis in human HCC cells.

3.6. FoxO1-mediated inhibition of HK1 expression is essential for cryptocaryone-induced apoptosis of human HCC cells

In order to verify the importance of the FoxO1/HK1 axis in the cryptocaryone-induced decrease in cell viability, siRNA targeting FoxO1 was employed. As shown in the Fig. 6A-C, siFoxO1 not only significantly increased the expression of HK1 but also increased the mRNA expression of HK1 in cryptocaryone-treated HCC cells. Additionally, the impairment of glycolysis caused by cryptocaryone treatment was also reversed by the transfection of siFoxO1 (Fig. 6D). Most importantly, the effect of cryptocaryone on cell viability was also significantly hindered by the transfection of siFoxO1 (Fig. 6E). These data suggest that cryptocaryone inhibits the growth of HCC cells by inhibiting the expression of HK1 and glycolysis, and FoxO1 plays a pivotal role in the regulation HK1 expression.

3.7. HK1 expression is negatively relative to FoxO1 and is associated with poorer survival in HCC patients

To verify the *in vitro* observations, we examined the relationship between HK1 and FoxO1 in HCC patients. Using Correlation AnalyzeR with Chi-square analysis [44], we discovered a significantly negative correlation between HK1 and FoxO1 mRNA expression (Fig. 7A). Furthermore, Kaplan–Meier survival analysis of the GEO database revealed that HCC patients with lower HK1

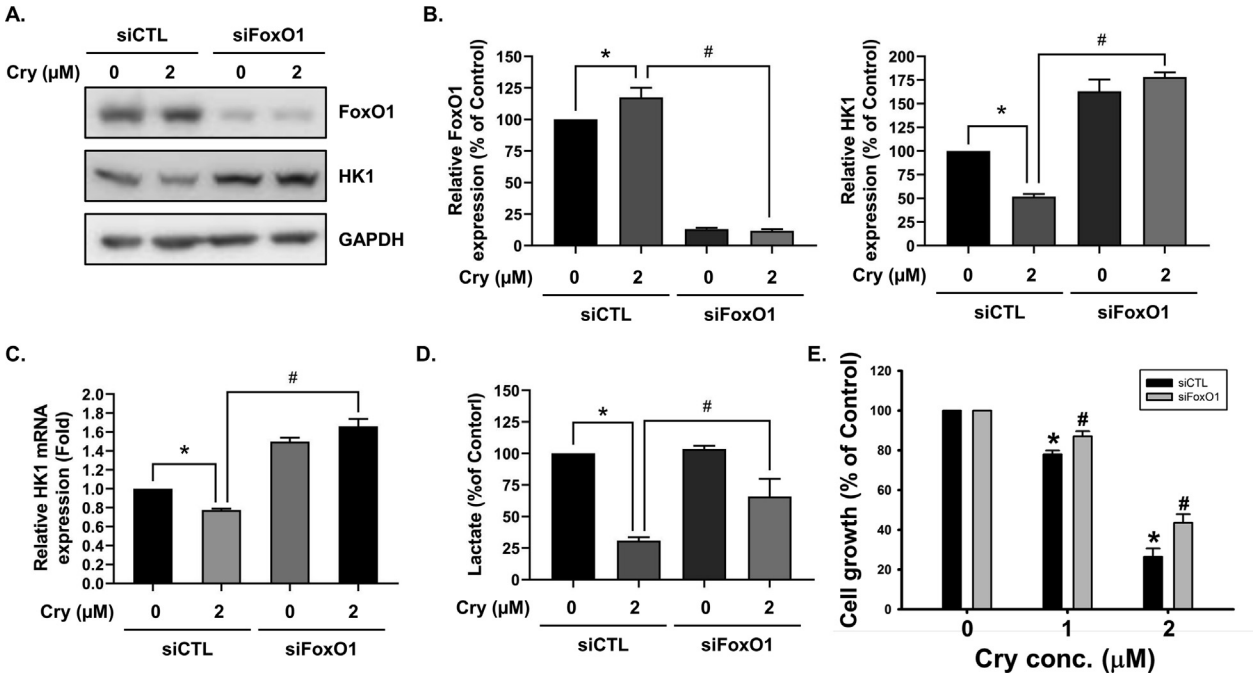
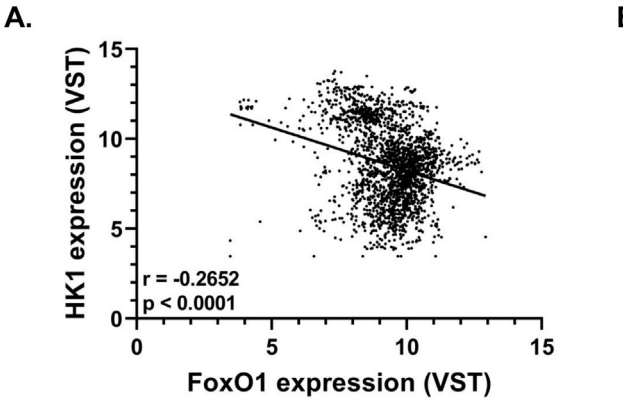
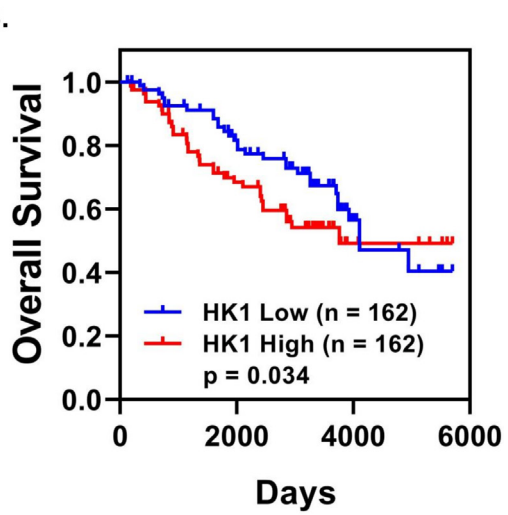


Fig. 6. ***Cryptocaryone impedes HK1 expression and cell growth via FoxO-1 modulation in human HCC cells.*** *SK-Hep-1 cells-transfected with control siRNA (siCTL) or FoxO1 specific siRNA (siFoxO1) were treated with or without cryptocaryone (Cry) for 24 h. (A&B) Cell lysates were subjected to Western blot analysis for FoxO1 and HK1. Quantified levels of indicated proteins were determined using ImageJ software. GAPDH was employed as an internal control. (C) Total RNA was subjected to quantitative real time PCR and normalized by GAPDH. (D) The lactate production was measured by assay kit. (E) The cell growth was determined using SRB assay. Data are shown as means ± standard deviation of at least three independent experiments. *p < 0.05, significantly different from the control. #p < 0.05, significantly different from the siCTL group treated with Cry.*





		HK1		χ^2 Value	P Value
		High	Low		
FoxO1	High	457	603	40.26	< 0.05
	Low	602	456		

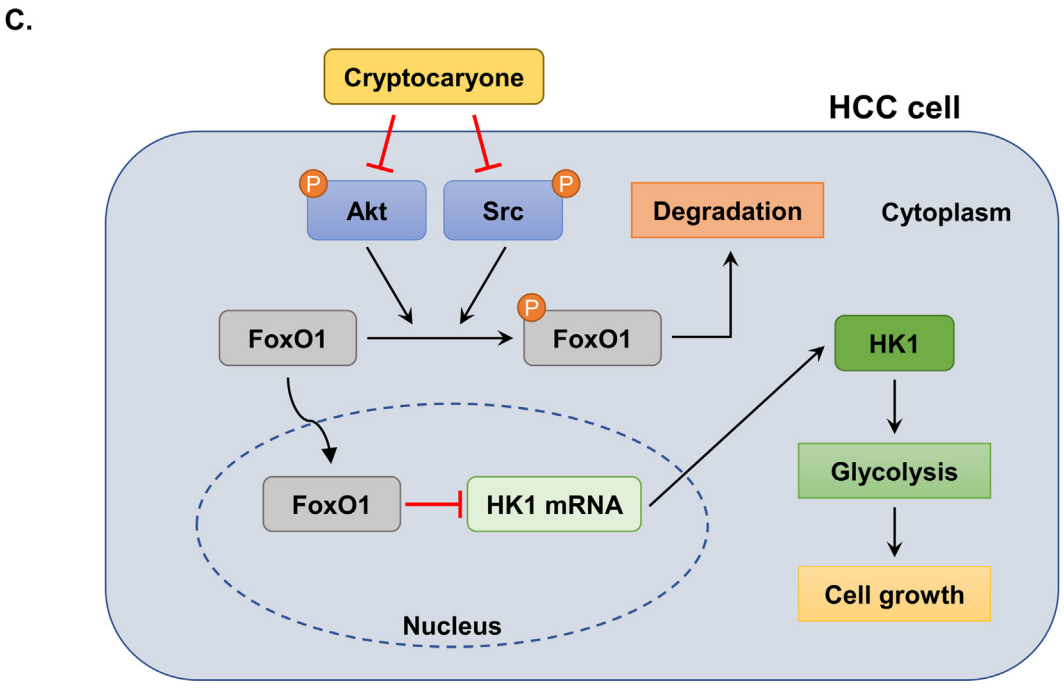


Fig. 7. ***Clinical database analysis for glycolytic enzymes and survival of HCC patients, and the apoptotic mechanism of cryptocaryone in human HCC cells.*** *(A) The correlation between HK1 and FoxO1 was analyzed by using Correlation AnalyzerR and Pearson's χ^2 test. (B) Comparison of the overall survival between HCC patients with low and high expression of HK1 from the GSE20140 database. (C) A schematic diagram showing how cryptocaryone induces apoptosis and reduces aerobic glycolysis in HCC cells.*

expression have a significantly higher overall survival rate (Fig. 7B). Altogether, these observations suggest that HK1 plays a negative role in HCC patients and targeting FoxO1/HK1 axis is a promising strategy for systemic therapy of HCC.

4. Discussion

Previous studies have reported the anti-cancer property of cryptocaryone against several lines of cancer cells. However, only some of these studies addressed the underlying mechanism of cryptocaryone, especially the impact of cryptocaryone on human HCC cells remained unclear. In prostate cancer cells, Chen et al. showed that the anti-cancer activity of cryptocaryone is related to the activation of death receptors-related apoptosis with an IC_{50} between 1.6 and 3.4 μM [31]. In ovarian and oral cancer, the authors discovered that the induction of DNA damage via oxidative stress is the fundamental

mechanism of the anti-cancer effect of cryptocaryone. Furthermore, cryptocaryone displayed a selective cytotoxic effect on oral cancer cells instead of normal oral cells. In these studies, the IC_{50} of cryptocaryone against ovarian and oral cancer cells was 1.5–9.5 μM and 3.9–11.6 μM, respectively [32,45]. In the present study, we found that cryptocaryone exhibited promising anti-cancer activity in three human HCC cell lines with the IC_{50} range at 1–2 μM (Fig. 1B), the most potent effects observed in SK-Hep-1 cells ($IC_{50} = 1.37 \pm 0.03$ μM). Our data showed that cryptocaryone induced the cleavage of Caspase 3 and PARP (Fig. 1C-D) and increased DNA fragmentation in SK-Hep-1 cells (Fig. 1E). In addition, cryptocaryone did not affected the expression of LC3-II, indicating that cryptocaryone acts anti-cancer effect against HCC cells via the autophagy-independent cascade (Fig. 1C). These findings confirmed that apoptosis is involved in the growth-inhibitory effect of cryptocaryone in human HCC cells.

Akt and c-Src pathway are known for their anti-apoptosis role in many cancers. Furthermore, elevated activation of Akt and c-Src pathway are both associated with poor prognosis in HCC patients [46,47]. In this study, we discovered that cryptocaryone inhibited the phosphorylation of Akt and c-Src signals, whereas the activation of MAPK pathway was not significantly reduced by cryptocaryone (Fig. 2A-B). Furthermore, transfection of Myr-Akt or co-treatment with Src activator significantly prevented the apoptotic effects caused by cryptocaryone (Figs. 3 and 4). These data suggest that cryptocaryone induces cell apoptosis via targeting both Akt and c-Src pathways in human HCC cells. In addition to their roles in apoptosis regulation, PI3K/Akt, MAPK and c-Src pathway are also known to modulate the aerobic glycolysis in cancer cells [41,42]. In accordance, we also found that the expression of FoxO1, a crucial suppressor of glycolysis in liver [48], was dramatically increased by cryptocaryone treatment in HCC cells. Furthermore, this effect was accompanied by a significant decrease in the phosphorylation of FoxO1 (Fig. 2C-D). As shown in previous studies, both Akt and c-Src can control the transcription of FoxO1 by phosphorylating FoxO1. This phosphorylation will lead to the nuclear exclusion and the subsequent degradation of FoxO1 [43]. We have confirmed that cryptocaryone regulates the expression of FoxO1 in a similar manner. By transfecting of Myr-Akt or co-treatment with Src activator, the expression of FoxO1 was significantly decreased, accompanied by the increase of FoxO1 phosphorylation (Fig. S1 https://www.jfda-online.com/journal/vol31/iss4/10/). Besides the glycolysis-regulating role of FoxO1, many studies reveal that FoxO1 dominantly dictates apoptotic cell death in a variety of tumor cells [43]. Based on the findings herein, we propose that cryptocaryone may increase FoxO1 expression by suppressing the activation of Akt and c-Src signaling cascades, and subsequently promotes apoptosis in human HCC cells.

As one of the hallmarks of cancer, aerobic glycolysis provides not only the necessary energy for the rapid proliferating cancer cells but also the acidic microenvironment in favor for the immune evasion of cancer cells [49]. In HCC, aerobic glycolysis has been reported to facilitate cancer progression in tumor microenvironment, including angiogenesis, metastasis, drug resistance and immune evasion [50]. Recent study has demonstrated that compound K, a metabolite of ginsenosides, induces apoptosis by suppressing glycolysis through AKT-related signaling pathway and their downstream signals, such as HK2 and PKM2, in HCC cells [51]. In addition, Kunag et al. also indicates that oxaloacetate promotes cell apoptosis via inhibition of glycolysis by targeting glycolytic enzymes [52]. Hence, targeting aerobic glycolysis is an emerging therapeutic approach for the treatment of HCC. In a previous study, Ren et al. reported that cryptocaryone induces cytotoxicity by inhibiting glucose transport in cancer cells [34]. However, the precise mechanism of such an effect is still unclear. Therefore, we sought to verify whether the glucose metabolism altering effect of cryptocaryone can be observed in human HCC cells and to elucidate the underlying mechanism of this action. Our data showed that the ATP production and the lactate production were significantly suppressed by cryptocaryone (Fig. 5A-B). Additionally, Seahorse bioenergetic analysis further confirmed the inhibitory effect of cryptocaryone against glycolysis in HCC cells (Fig. 5C-E). Furthermore, we also found that cryptocaryone significantly inhibited the expression of glycolytic enzyme HK1 (Fig. 5F-G). Taken together, these findings support that cryptocaryone may induce apoptosis via inhibition of glycolysis by targeting HK1 in human HCC cells.

It has been reported that FoxO1 negatively regulates glycolysis in human HCC cells, and high expression of FoxO1 is associated with a better survival rate in HCC patients [23]. Importantly, FoxO1 has been shown to control the expression of HK1 in many tissues [53,54]. Here, we employed FoxO1-specific siRNA to verify the importance of the FoxO1/HK1 axis in the cryptocaryone-induced apoptosis. By inhibiting the expression of FoxO1, we are able to rescue the protein and mRNA expression of HK1, the

glycolysis activity and the cell growth of cryptocaryone-treated SK-Hep-1 cells (Fig. 6). To further confirm this hypothesis in the clinical setting, we analyzed clinical database analysis and revealed a negative correlation of between HK1 and FoxO1 mRNA expression (Fig. 7A). Moreover, GEO database found that high level of HK1 expression is related to poorer survival in HCC patients (Fig. 7B). In previous studies, HK1 was shown to inhibit apoptosis by interacting with voltage-dependent anion channel-1 and other pro-apoptosis BCL family proteins on mitochondria [55,56]. Furthermore, previous study has shown that by reducing hexokinase 2, resveratrol and dioscin are able to inhibit glycolysis and induce apoptosis in HCC cells and colorectal cancer cells, respectively [57,58]. Suggesting that targeting the FoxO1/HK1 axis during glycolysis metabolism may represent a potential therapeutic strategy against human HCCs. However, the utilization of cryptocaryone is constrained by its limited availability, primarily due to the diverse processes required for structural characterization and its relatively low yield during natural product isolation, our experiments were confined to *in vitro* settings. It's worth noting that natural products often present challenges in terms of obtaining substantial quantities for research purposes. Nevertheless, the incorporation of clinical data analysis should provide compelling evidence for our proposed mechanism. Furthermore, the precise mechanism by which FoxO1 suppresses HK1 expression, along with the downstream effects on glycolysis metabolism, contributing to cryptocaryone-mediated apoptosis in HCCs, remains unresolved and warrant further research. In conclusion, the present study provides evidence for the anti-HCC effect of cryptocaryone. Firstly, cryptocaryone inhibits the phosphorylation of Akt and c-Src, subsequently suppressing the phosphorylation of FoxO1. This reduction in FoxO1 phosphorylation is expected to lead to its upregulation (due to decreased proteasomal degradation) and nuclear translocation within HCC cells. Consequently, cryptocaryone hampers HK1 expression and aerobic glycolysis via FoxO1 modulation, ultimately resulting in the induction of HCC apoptosis (Fig. 7C). This is the first demonstration that cryptocaryone exhibits anti-cancer property against human HCCs via inhibiting aerobic glycolysis. Cryptocaryone might serve as a promising therapeutic agent and novel approach to the treatment of HCC.

Author contributions

J.J. Lee, C.H. Tsai, and S.W. Wang designed and conceived the study. C.L. Yu, Y.W. Lai, and C.C. Huang performed the experiments. J.J. Chen and T.H. Chou isolated and purified the cryptocaryone. S.C. Liu and G.W. Chen analyzed the data. C.L. Yu, Y.W. Lai., and S.W. Wang wrote the paper. All the authors did final approval of the version to be submitted.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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