Ginkgolic acid inhibits proliferation and migration of human hepatocellular carcinoma cells by inducing G0/G1 cell cycle arrest

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ABSTRACT: Hepatocellular carcinoma (HCC) is a common cancer worldwide with high morbidity and mortality. Ginkgolic acid (GA) is a natural compound obtained from leaves and seed coats of *Ginkgo biloba* L., and it has been reported to have various bioactivities. However, the effects of GA on HCC cell cycle distribution and the mechanisms involved are still unknown. By CCK-8 assay and Transwell assay, the cell viability and migration of HCC cells were shown to be inhibited significantly by GA in a concentration-dependent manner. By cell cycle analysis and western blot, the cell cycle arrest at G0/G1 phase was shown to contribute to the inhibitory effects of GA. Furthermore, the phosphorylation of p38 MAPK was found to be elevated upon GA treatment as analyzed by western blot. Thus, GA inhibited cell proliferation and migration of HCC cells by inducing G0/G1 cell cycle arrest via p38 MAPK activation, indicating GA as an agent candidate for HCC treatment.

KEYWORDS: ginkgolic acid, hepatocellular carcinoma, G0/G1 cell cycle arrest, p38 MAPK

INTRODUCTION

Liver cancer is the fourth common cause of cancer-related death around the world [1]. The cases of liver cancer continue increasing due to the growth of population and aging, despite the slowly decreased global age-standardized incidence rate (ASIR) from 1990 to 2015 [2]. Hepatocellular carcinoma (HCC) is a primary type of liver cancer, accounting for over 80% of the total [3]. The incidence rate and mortality of HCC are different in various parts of the world due to the diversity of environmental and medical conditions; it is serious in low-resource or middle-resource countries [3]. The prognosis of HCC is poor in all regions of the world. In the United States, 2 year survival rate is less than 50%, and 5 year survival rate is only 10% [4]. At present, there are several treatment options for HCC according to the size, number and the situation of

invasion, and metastasis, mainly including surgical therapies, tumor ablation, transarterial therapies, and systemic therapies [1]. So far, only two kinds of systemic drugs, Sorafenib and Lenvatinib, are used in first-line therapy; but the therapeutic effects are not satisfactory [5, 6]. It is necessary to discover novel agent candidates for HCC therapy.

Ginkgo biloba L. is an ancient gymnosperm species distributed around the world and widely used in traditional Chinese medicine [7]. Ginkgolic acid (GA) is an alkylphenol constituent extracted from the leaves and seed coats of *Ginkgo biloba* [8]. GA had the potential of anti-inflammation, anti-HIV, anti-diabetes, anti-bacterial, and anti-virus [9–13]. For cancer, GA presented inhibitory effects on the viability of pancreatic cancer, colon cancer, and lung cancer cells, with little cytotoxic effect on non-cancer cells [14–16]. Recently, GA was found to induce HCC cell death via apoptosis, autophagy

and mitochondrial pathway [17]. The inhibition of HGF/c-Met signaling was involved in GA-induced suppression of HepG2 cells invasion [18]. However, the specific mechanisms in HCC cells upon GA treatment have not been determined.

In this study, the anti-HCC effects of GA were determined by cell viability, migration, cell cycle distribution assays; and the activation of MAPKs signaling was detected by western blot in HCC cells upon GA treatment. This investigation might provide a novel agent candidate for the future therapy of HCC, as well as the underlying mechanisms.

MATERIALS AND METHODS

Cell culture and reagents

Human liver cancer cell lines HepG2 and Huh-7 were cultured in Dulbecco's Modified Eagle's medium (DMEM, high glucose, HyClone, Beijing, China) with 10% FBS (Biological Industries, Israel) and 1% Penicillin-Streptomycin solution (HyClone) at 37 °C with 5% CO_2 and 95% relative humidity. GA was purchased from MCE (Shanghai, China) and dissolved in 100 mM dimethyl sulfoxide (DMSO) and stored at −20 °C for use. Working solutions of GA were prepared in DMEM at the indicated concentrations.

CCK-8 assay

The cells were dissociated by trypsin-EDTA solution (Biological Industries) and seeded onto 96-well plates. After incubation for 24 h at 37 °C, the medium was replaced with new medium containing different concentrations of GA (0, 12.5, 25, 50, 100, 200 and 300 µM). Following incubation for another 24 h, 10 µl CCK-8 reagent (Meilun, Dalian, China) was added into each well and incubated for 30 min at 37 °C. The absorbance value at wavelength 450 nm was measured using a microplate reader (iMRAK, Bio-Rad, Hercules, CA, USA), with a reference wavelength at 630 nm.

Cell migration assay

After incubation with GA for 24 h, the cells were harvested and added into the upper chamber of Transwell (3422, Corning, NY, USA) at 2×10^5 cells/well in 200 µl serum-free DMEM medium. 500 µl DMEM with 10% FBS was added into the lower chamber. After incubation for 48 h, the cells residual in the upper chamber were scraped off by a cotton swab and other cells on the lower side of the chamber were fixed by 4% paraformaldehyde (Beyotime, Jiangsu, China) for 10 min and stained by 0.1% crystal violet (Beyotime) for 10 min. Subsequently, the cells were imaged under a microscope (Observer A1, Carl Zeiss, Oberkochen, Germany).

Cell cycle assay

HepG2 cells were harvested into centrifuge tube and washed by cold PBS. After fixing in 70% ethyl alcohol at 4 °C overnight, the cells were washed by cold PBS again and then stained with propidium iodide (PI) and RNAse mixture (Beyotime) at 37 °C for 30 min in the dark. The cell cycle distribution was analyzed by a Flow cytometer (Canto II, BD Bioscience, San Jose, CA, USA).

Real-time PCR

Total RNA was extracted with RNAiso Plus (TaKaRa, Dalian, China) according to the manufacturer's protocol. The RNA was analyzed and quantified by NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Total RNA was reverse-transcribed into cDNA using GoScript Reverse Transcription System (Promega, Madison, WI USA) according to the manufacturer's protocol. The mRNA relative expression level was determined in the Applied Biosystems 7500 Real-Time PCR system using PowerUp SYBR Master Mix (Applied Biosystems, Shanghai, China). The reaction conditions were as followed: holding at 50 °C for 2 min, 95 °C for 2 min; 40 cycles at 95 °C for 15 s, 60 °C for 1 min. The mRNA level was quantified using $\Delta\Delta Ct$ method and ACTB served as an internal control. The primer sequences are as follows: CCNA2, forward primer: 5′-AACACTCACTGGCTTTTCATCT-3′ and reverse primer: 5′-AACACTCACTGGCTTTTCATCT-3′; CCNB1, forward primer: 5′-TTGGTCTGACTGCT TGCTCTT-3′ and reverse primer: 5′-TTGGTCTG ACTGCTTGCTCTT-3′; CCNE1, forward primer: 5′-AAGGAGCGGGACACCATGA-3′ and reverse primer: 5′-ACGGTCACGTTTGCCTTCC-3′; ACTB, forward primer: 5′-TGGCACCCAGCACAATGAA-3′ and reverse primer: 5′-CTAAGTCATAGTCCGCCTAGAA GCA-3′.

Western blot

HepG2 cells were lysed with RIPA (Beyotime) supplemented with PMSF and Complete Protease Inhibitor Cocktail (Roche, Shanghai, China) and the concentration of total protein was measured using BCA Protein Assay Kit (TaKaRa) according to the manufacturer's protocol. The protein was separated by 10% SDS-PAGE and transferred to PVDF membrane. After blocking with 5% skim milk

for 1 h, the membranes were incubated with primary antibodies against CCNA2 (1:2000, Proteintech, Wuhan, China), CCNB1 (1:500, Proteintech), CCNE1 (1:2000, Proteintech), ERK (1:2000, CST, Shanghai, China), p-ERK (1:2000, CST), p38 MAPK (1:2000, CST), p-p38 MAPK (1:2000, CST), and GAPDH (1:10000, Proteintech) at 4 °C overnight followed by a wash with TBST for 3 times. The membranes were incubated with HRP-conjugated goat anti-rabbit IgG (1:5000, Proteintech) or goat anti-mouse IgG (1:5000, Proteintech) at room temperature for 1 h and then rinsed in TBST for 3 times. The protein expression levels were detected by Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare, Princeton, NJ, USA) using Tanon-5200 chemiluminescence detection system (Tanon, Shanghai, China). Protein gray values were analyzed by Image J software.

Statistical analysis

The data are presented as the mean ± standard deviation (SD). Differences among groups were analyzed by Student's t-test. $p < 0.05$ was considered to be statistically significant.

RESULTS

GA inhibits cell viability of HCC cells

In order to explore the effects of GA (Fig. 1A) on HCC proliferation, the cell viability was determined by CCK-8 assay. As shown in Fig. 1B and 1C, the cell viabilities of Huh-7 and HepG2 cells were significantly inhibited by GA (50–300 μM) versus DMSO group in a concentration-dependent manner. Consistent with this data, the cell number was obviously reduced by GA under phase-contrast microscope (Fig. 1D). These results proved the inhibitory effect of GA on HCC cell proliferation.

GA inhibits cell migration of HCC cells

Cell migration is crucial in the mediation of cancer metastasis [19]. In order to investigate the role of GA on the migration of HCC cells, Transwell assay was conducted. As presented in Fig. 2, the migrated cell number was significantly reduced by GA treatment at 50 and 100 μM in a concentration-dependent manner, indicating the remarkably suppressive effect of GA on HCC cells migration.

Cell cycle arrest at G0/G1 phase induced by GA

Cell cycle plays a key role in cancer treatment since the aberrant function of cell cycle regulators will result in uncontrolled cell proliferation [20]. To investigate whether GA can induce cell cycle arrest of HCC cells, we assessed the effect of GA on cell cycle distribution of HepG2 cells by PI staining followed by flow cytometry. The results revealed that GA increased the percentage of cells in G0/G1 phase (Fig. 3A and 3B). Furthermore, the mRNA levels of cell cycle regulator CCNA2, CCNB1 and CCNE1 were found to be inhibited by GA, and the decreased protein levels of the GA treated samples were verified by western blot analysis (Fig. 3C to 3E). These results indicated that GA could induce G0/G1 cell cycle arrest of HCC cells by reducing CCNA2, CCNB1 and CCNE1 expression.

p38 MAPK activation induced by GA

Mitogen-activated protein kinase (MAPK) signaling pathway plays critical roles in some cancer-related activities including proliferation, differentiation and migration [21, 22]. To investigate the potential mechanisms involved in the inhibitory effects of GA on HCC cells, ERK and p38 MAPK were analyzed by western blot. After treatment with GA, the phosphorylation of ERK and the total protein of ERK showed no significant differences compared to DMSO group. The phosphorylation of p38 MAPK was elevated and the total protein of p38 MAPK was decreased (Fig. 4), indicating that GA increased MAPK phosphorylation to inhibit proliferation and migration of HCC cells.

DISCUSSION

In this study, GA presented anticancer activity on HCC cells by arresting cell cycle at G0/G1 phase. Furthermore, the phosphorylation level of p38 MAPK was found to be significantly enhanced by GA. In previous studies, GA showed inhibitory effects on the growth of pancreatic cancer cells [14]. The invasion of colon cancer and lung cancer cells was inhibited through AMPK and PI3K/Akt signaling [15, 16]. For liver cancer, GA could inhibit invasion and cause cell death [17, 18], which are consistent with our results. Here, we have discovered the cell cycle regulation by GA and the involvement of MAPK signaling. Moreover, the cytotoxicity of GA in primary hepatocytes from rats was lower than that in HepG2 cells [23, 24], indicating GA as a prospective agent candidate for the treatment of HCC.

Cell cycle regulation plays important roles in various cellular events. Mistakes, such as mutation, overexpression or elimination in cell cycle regulation or its checkpoints, may lead to the development of malignant cells [25]. Here, we showed that
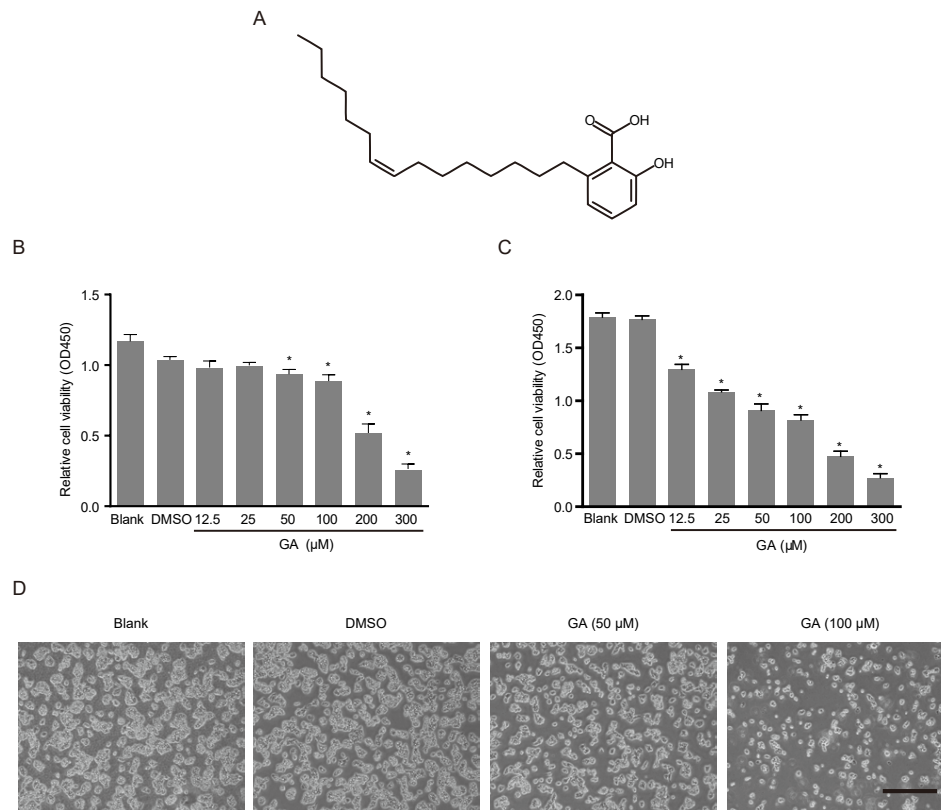
Fig. 1 HCC cell viability was inhibited by GA. (A) The chemical structure of GA. (B) The cell viability of Huh-7 cells was analyzed by CCK-8 assay. (C) The cell viability of HepG2 cells was analyzed by CCK-8 assay. * $p < 0.05$ compared with DMSO group. (D) The representative images of HepG2 cells treated with GA at 50 and 100 µM, under phase-contrast microscope. Blank indicates cells without treatment. Bar indicates 100 µm.
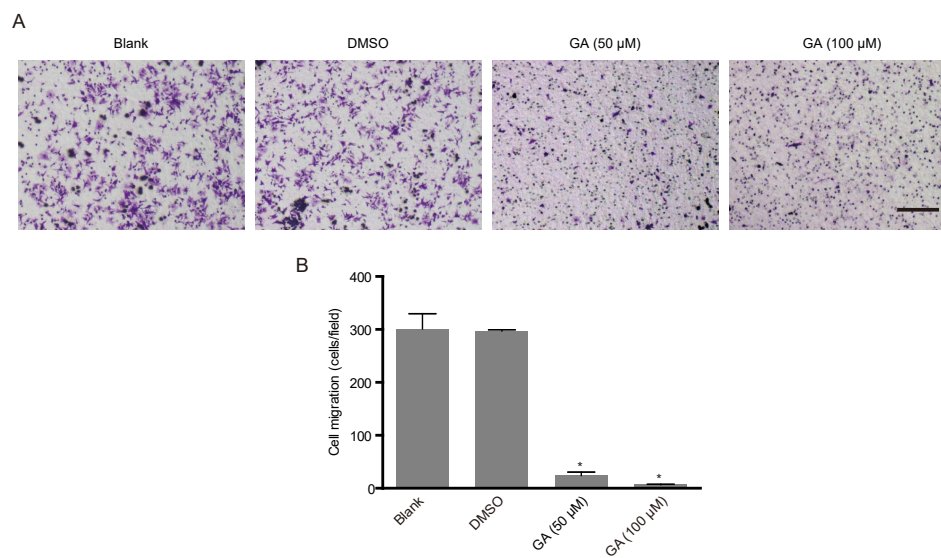

Fig. 2 HCC cell migration was inhibited by GA. (A) The HepG2 cells were treated with GA and the cell migration was determined by Transwell assay. Bar indicates 100 µm. (B) The migrated cell numbers were analyzed by Image J software. Blank indicates cells without treatment. * $p < 0.05$ compared with DMSO group.
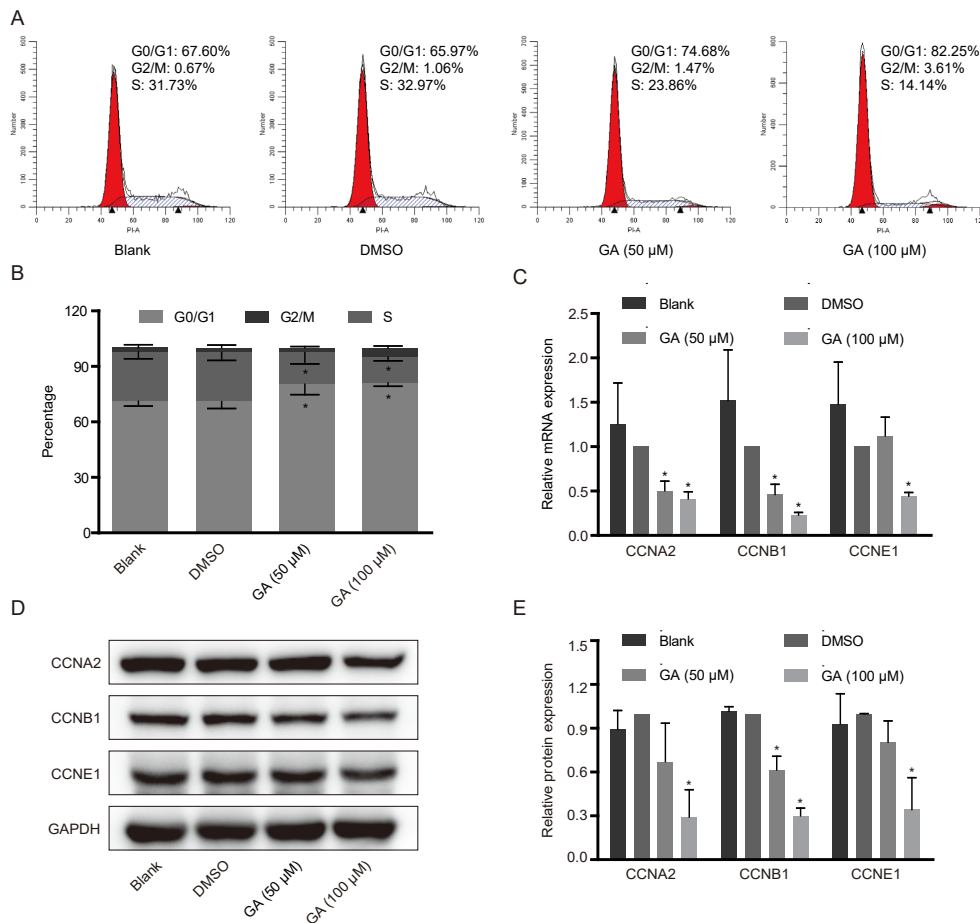
Fig. 3 GA induced cell cycle arrest in HCC cells. HepG2 cells were treated with GA at indicated concentrations. (A) The cell cycle distribution was analyzed by PI staining followed by flow cytometry. (B) The statistical analysis of cell cycle data. (C) The mRNA levels of CCNA2, CCNB1 and CCNE1 were analyzed by real-time PCR. (D) The protein levels of these genes were determined by western blot. (E) The images of western blot were analyzed by Image J software. Blank indicates cells without treatment. * $p < 0.05$ compared with DMSO group.
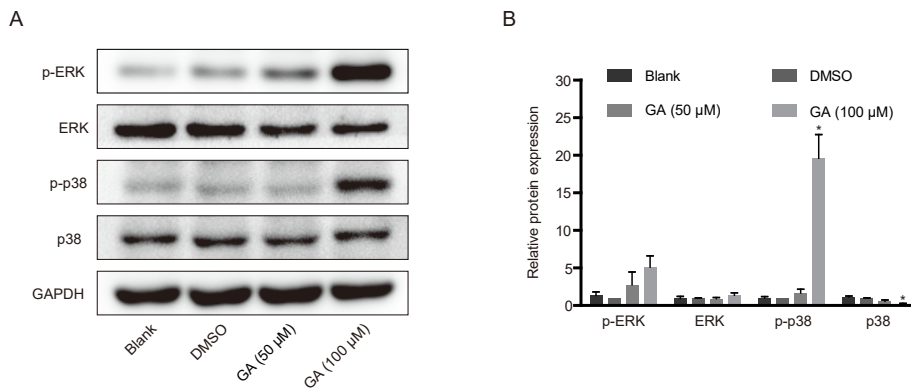

Fig. 4 The activation of p38 MAPK by GA treatment. HepG2 cells were treated with GA at indicated concentrations. (A) The total and phosphorylation protein levels of ERK and p38 MAPK were analyzed by western blot. (B) The images of western blot were analyzed by Image J software. Blank indicates cells without treatment. * $p < 0.05$ compared with DMSO group.

GA induced G0/G1 arrest in HepG2 cells, with the depression of mRNA and protein expression level of CCNA2, CCNB1 and CCNE1. During cell cycle progression, CCNE1 begins to accumulate in late G1 phase, reaches the maximum at G1/S transition and decreases during S phase [26], thus controlling the timing of G1/S transition [27]. For HCC, CCNE1 and CDK2 are crucial to the initiation instead of progression. The overexpression of CCNE1 will cause chromosome instability in liver cells, which makes it a high risk to lead to HCC in mouse [28, 29]. Besides, CCNA2 has been reported to induce the transition in both G1/S and G2/M and promotes invasion and migration of non-small cell lung carcinoma cells [30, 31]. In this study, the treatment with GA reduces the expression of CCNE1 and CCNA2 in HCC cells, leading to cell cycle arrest at G0/G1 phase, thus inhibiting cell proliferation and migration.

MAPK signaling pathways are essential for cell growth in physiological and pathological processes [32]. There are 3 main members of MAPK family in mammalian species: ERK, p38 MAPK and JNK [33]. The activation of p38 MAPK and JNK is related to apoptosis, inflammation and growth [34]. ERK could induce cell death [35]. Increasing phosphorylation levels of p38 MAPK in HCC tissues is positively associated with tumor size and the formation of satellite tumors; while JNK phosphorylation will antagonize the promoting effect of p-p38 MAPK in human liver cancer [36]. In contrast, p38α phosphorylation pathway targeting Hsp27 has been inversely correlated with tumor size, invasion, and tumor stages of human HCC [37]. Our study showed that GA increased p38 MAPK phosphorylation and inhibited the growth of HCC cells. Thus, we concluded that MAPKs signaling activation was involved in the anti-HCC activity of GA.

For HCC treatment, several agents targeting various pathways are under research. Sorafenib is the only approved targeted drug, but with serious side effects and limited efficacy [38]. γ-tocotrienol was proven to inhibit AKT/mTOR pathway to reduce angiogenesis in HCC mice model [39]. In a previous study, GA extracted from *Ginkgo biloba* leaves presented inhibitory effect on STAT3 activation, which was considered to be a potential therapeutic target in HCC [40–42]. Considering the MAPK activation found in this study, GA might also be a multi-targeting agent.

In summary, the presented data proved that GA inhibited HCC cell viability and migration by inducing cell cycle arrest at G0/G1 phase, partly through p38 MAPK signaling activation. GA is a novel agent candidate for HCC treatment.

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