Ginkgolic acid suppresses the invasion of HepG2 cells via downregulation of HGF/c-Met signaling

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Abstract. Liver cancer is one of the most devastating types of cancer worldwide. Despite years of improvements in treatment, the prognosis of patients with this type of malignancy remains poor due to frequent recurrence and metastasis after surgical resection. Ginkgolic acid (GA) is a botanical drug extracted from the seed coat of *Ginkgo biloba* L. that possesses a wide range of bioactive properties. However, to the best of our knowledge, whether GA can inhibit the invasion of liver cancer cells and the underlying mechanisms remains unknown. The aim of the present study was to investigate the effects of GA on the migration and invasion abilities of liver cancer cells and the underlying molecular mechanism. The results revealed that GA suppressed the migration and invasion abilities of HepG2 cells. In addition, GA treatment inhibited the expression of invasion-related molecules (MMP-2 and MMP-9) and prevented the epithelial-mesenchymal transition (EMT) of HepG2 cells. Further experiments revealed that GA-reduced hepatocyte growth factor (HGF) production and suppressed c-Met phosphorylation may be the underlying mechanisms. Exogenous recombinant HGF supplementation improved the cell invasion ability impaired by GA. Moreover, the *in vivo* experiment revealed that GA inhibited the tumor growth of liver cancer and prevented EMT. Collectively, these data indicated that GA effectively suppressed the invasion and EMT of HepG2 cells via downregulation of HGF/c-Met signaling, thus GA may serve as a novel chemotherapeutic agent for the treatment of HCC.

Introduction

Liver cancer is one of the most common causes of cancer-related deaths worldwide. Due to the high incidence in sub-Saharan Africa and eastern Asia, liver cancer has become an important medical problem in these areas (1). Despite advances in liver cancer prevention, detection, diagnosis and treatment, the prognosis of liver cancer remains unsatisfactory (2). There are still a considerable number of liver cancer cases that are diagnosed at a stage with distant metastasis, at which point it is not possible to perform radical surgery (2). The targeted therapeutic drug sorafenib can significantly improve progression-free survival and overall survival of patients with advanced liver cancer (3). However, the expensive cost and occurrence of drug resistance limit the wide application of sorafenib. Thus, identification of novel and effective drugs to manage this disease is urgently required.

Hepatocyte growth factor (HGF) and its tyrosine kinase receptor (RTK) c-Met have been revealed to exert diverse physiological effects during embryogenesis and morphogenesis (4). Normally, activation of c-Met by HGF induces a variety of cellular responses, which are critical for liver development and regeneration (5). However, dysregulation of HGF/c-Met signaling is implicated in liver cancer carcinogenesis and progression (6). Previous studies reported that c-Met was overexpressed in liver cancer tissue and was closely associated with portal vein invasion and early recurrence of liver cancer (7,8). Binding of the HGF to c-Met induced phosphorylation of c-Met and activation of multiple downstream signaling pathways, including mitogen-activated protein kinase and phosphoinositide-3 kinase pathways, resulting in enhancement of motility and invasiveness of a variety of types of tumor cell (9). Therefore, identification of novel agents that target the HGF/c-Met axis may present a promising therapeutic strategy for liver cancer treatment (10).

Ginkgo biloba L., also known as ginkgo, is an ancient gymnosperm species that is widely distributed in China. Ginkgolic acid (GA) is the main botanical component extracted from the seed coat of *Ginkgo biloba* L. As a mixture of phenolic acids, GA possesses a wide range of bioactive properties and can exert diverse pharmacological activities (11,12). Several monomer structures of GA have been identified (13). C15:1 is one of the most abundant GAs in *Ginkgo biloba* L. extract (14). Recently, a number of studies have indicated the anticancer activity of GA in a variety of cancer types and the less toxic reaction on non-cancerous cells (15-18). However, to the best of our knowledge, whether GA can inhibit the invasion of liver cancer cells and the underlying mechanisms remains unknown. The aim of the present study was to investigate the effects of GA on the migration and invasion abilities of liver cancer cells and identify the underlying molecular mechanism.

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Materials and methods

Cell culture and reagents. The human liver cancer cell line HepG2 was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium containing 10% FBS (HyClone Laboratories; GE Healthcare Life Sciences, Logan, UT, USA) and 1% penicillin/streptomycin. Experiments were conducted with cells at <25 passages. GA (C15:1; $C_{22}H_{34}O_3$) was obtained from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany) and initially dissolved in pure methanol as a stock solution of 1 mM. Working dilutions of GA were performed with culture medium immediately before use. Recombinant human HGF was purchased from R&D Systems (Minneapolis, MN, USA). Primary antibodies against E-cadherin (cat. no. 3195, rabbit), N-cadherin (cat. no. 13116, rabbit), ZO-1 (cat. no. 13663, rabbit), vimentin (cat. no. 5741, rabbit), HGF (cat. no. 52445, rabbit), c-Met (cat. no. 8198, rabbit) and phosphorylated c-Met (p-c-Met) (cat. no. 3077, rabbit) were purchased from Cell Signaling Technology (Danvers, MA, USA); mouse anti-MMP-2 (cat. no. ab86607), mouse anti-MMP-9 (cat. no. ab58803) and mouse anti-β-actin (cat. no. ab8226) were obtained from Abcam (Cambridge, MA, USA); and secondary antibodies (goat anti-rabbit IgG-HRP; cat. no. sc-2004; goat anti-mouse IgG-HRP; cat. no. 2005) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell proliferation assays. The effect of GA on HepG2 cell proliferation was assessed by an MTT assay. HepG2 cells were seeded in 96-well plates (3,000 cells/well) and incubated for 12 h. After treatment with GA at different concentrations (0, 5, 10, 25, 50 and 100 μM) at the indicated time-points (12, 24, 36 and 48 h), 20 μl of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, Merck KGaA) was added to each well and cells were incubated for an additional 4 h at 37˚C (19). Then 150 μl DMSO (Sigma-Aldrich; Merck KGaA) per well was added to dissolve the crystals. Cell proliferation was assessed by measuring the absorbance at 490 nm using a 96-well plate spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Wound healing migration assays. Wound healing migration assays were conducted in order to assess the migration ability of HepG2 cells. HepG2 cells were seeded in 6-well plates and grown to 80% confluence in complete medium. After serum-starvation for 12 h, the cells were then treated with GA (0, 25 and 50 μM) for 24 h. Subsequently, a sterile 200-ml pipette tip was used to make a wound across the cell culture monolayer. Discarding the medium and washing three times with PBS removed floating cells. Multiple images of the matched-pair wound regions were obtained immediately after wounding (0 h) and after 36 h using a light microscope (Nikon Corp., Tokyo, Japan) at a magnification of x100. The migration distance was determined by calculating the area of the cell gap at the indicated time-points (0 and 36 h).

Cell invasion assays. Cell invasion assays were performed using Matrigel-coated Transwell inserts (8-μm pore size; BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's recommendation. Cells ($5x10^4$) were added to the upper chamber and incubated in the presence of GA (0, 25 and 50 μM) or GA plus HGF (5 ng/ml) in serum-free medium. Complete medium (500 μl) was added to the lower chamber. After 48 h in culture, cells in the upper chamber were carefully removed with a cotton-tipped swab. The invaded cells were fixed and stained with 0.1% crystal violet solution. Quantification of invasion was calculated by counting stained invaded cells in at least 10 randomly selected fields using a light microscope (Nikon Corp.) at a magnification of x200. Each experiment was performed in triplicate to confirm the reproducibility of the data.

Western blot assays. Tissues or cells were lysed in RIPA Lysis Buffer (Beyotime Institute of Biotechnology, Guangzhou, China). Following protein quantification with BCA (Pierce; Thermo Fisher Scientific, Inc.), samples were subjected to 10-12.5% SDS-PAGE and transferred to PVDF membranes. After blocking with 5% BSA, the membranes were incubated with the primary antibodies (dilutions for antibodies: MMP-2, 1:800; MMP-9, 1:750; E-cadherin, 1:1,500; ZO-1, 1:1,000; vimentin, 1:750; N-cadherin, 1:1,000; β-actin, 1:1,000; c-Met, 1:800; p-c-Met, 1:1,000) and then with species-specific secondary antibodies (1:5,000). β-actin was used as an internal loading control. Protein bands were detected on a ChemiDoc XRS imaging system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using enhanced chemiluminescence (EMD Millipore, Billerica, MA, USA).

Quantitative PCR. After the designated treatment, total cell RNA was isolated using TRIzol reagent (Invitrogen, Thermo Fisher Scientific, Inc.) and cDNA was synthesized using a PrimeScript RT reagent kit (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's instructions. Quantitative PCR was then performed with an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.) using a SYBR Green reagent (Takara Biotechnology Co., Ltd.). The cycling conditions were as follows: Denaturing at 95˚C for 10 min followed by 35 cycles of denaturing at 95˚C for 10 min, 38 cycles of denaturing at 95˚C for 20 sec, annealing and extension at 60˚C for 1 min. The primer sequences used in this study are listed in Table I. Relative expression of the sample genes was calculated using the ΔΔCq method (20) with β-actin as the endogenous control.

Immunofluorescence analysis. After the designated treatment, cells were fixed with 4% ice-cold methanol and permeabilized with 0.5% Triton X-100. Cells were then blocked with 1% bovine serum albumin (BSA) followed by incubation with a primary antibody against HGF (dilution 1:200) at 4˚C overnight. After washing with PBS, cells were incubated with a goat anti-rabbit FITC (green) IgG antibody (cat. no. ZF-0311; ZSGB-BIO Inc., Beijing, China) at 1:200 dilutions for 1 h at room temperature and then washed with PBS again. Subsequently, the cells were stained with DAPI in order to visualize the nuclei. Images were captured with a fluorescence microscope (Nikon Eclipse Ti-s; Nikon Corp.) using the appropriate excitation wavelength at a magnification of x200.

In vivo study. Animal experimental protocols were approved by the Ethics Committee of The Second Affiliated Hospital of Medical College, Xi'an Jiaotong University (Xi'an, Shaanxi, China). Twenty 6-week-old male BALB/c nude mice were supplied by and housed in the Animal Center at the Medical

Table I. Primers for real-time PCR.

Genes	Forward primer (5'-3')	Reverse primer (5'-3')
MMP-2	GATGATGCCTTTGCTCGTGC	CAAAGGGGTATCCATCGCCA
MMP-9	GAGACCGGTGAGCTGGATAG	TACACGCGAGTGAAGGTGAG
HGF	TTTGCCTTCGAGCTATCGGG	TGATCCCAGCGCTGACAAAT
E-cadherin	ATTCTGATTCTGCTGCTCTTG	AGTCCTGGTCCTCTTCTCC
N-cadherin	ACAACAGACCTGAGTTCTTACAC	TTGGAGCCTGAGACACGATT
Vimentin	AATGACCGCTTCGCCAAC	CCGCATCTCCTCCTCGTAG
ZO-1	GAGATGAACGGGCTACGC	GAGACTGCCATTGCTTGG
β-actin	CATCACTATCGGCAATGAGC	GACAGCACTGTGTTGGCATA

College, Xi'an Jiaotong University. The mice were housed in a ventilated, temperature-controlled (22-24˚C) and standardized sterile animal room with a 12-h light/dark cycle, and free access to food and water. A single-cell suspension (30 μl) of HepG2 cells (suspended in HBSS, containing 1x10^6 cells) was inoculated subcutaneously into the back of the BALb/c nude mice. One week after inoculation, mice were randomly divided into two cohorts with 10 mice in each group. One cohort received the vehicle (100 μl saline) by oral gavage and the other was orally administrated GA (suspended in saline, 50 mg/kg) daily for 5 weeks. The tumor volume was monitored throughout the experiment and calculated using the following formula: V (tumor volume)=S (shorter diameter)^2 x L (longer diameter) x0.5. At the end of the experiment, the mice were euthanized by CO_2 asphyxiation (the CO_2 flow rate used for euthanasia was 30% of the chamber's volume/min) and the tumor samples were harvested. Tissue protein was prepared and subjected to western blot assays as previously described.

Statistical analysis. Data are presented as the mean ± standard deviation. Each experiment was performed at least three times. All quantitative data were analyzed using SPSS (version 15.0; SPSS, Inc., Chicago, IL, USA). Student's t-test was performed to assess the difference between two groups. Two-way analysis of variance was used to analyze data between groups and post-hoc Tukey's test was used for multiple comparisons, and P<0.05 was considered to indicate a statistically significant difference.

Results

GA suppresses the proliferation, migration and invasion abilities of HepG2 cells. Firstly, the effect of GA on the migration ability of HepG2 cells was investigated. The concentration of GA used was determined according to a previous study (15) and by cell proliferation experiments. The proliferation of HepG2 cells was suppressed by GA treatment at a concentration of more than 25 μM (Fig. 1). HepG2 cells were pre-treated with GA (25 and 50 μM) for 24 h and then wound healing migration assays were performed. As shown in Fig. 2A, the migration capacity of HepG2 cells was impaired by 25 and 50 μM GA intervention compared to that of the control cells (0 μM GA), as determined by the cell migration distance. To further assess the effect of GA on the invasion capacity of HepG2 cells, a Matrigel invasion assay was conducted. The results revealed that there was a significant reduction in the number of invaded cells after the HepG2 cells were treated with 25 or 50 μM GA (Fig. 2B). Altogether, the data demonstrated that GA inhibited the migration and invasion of HepG2 cells *in vitro*.

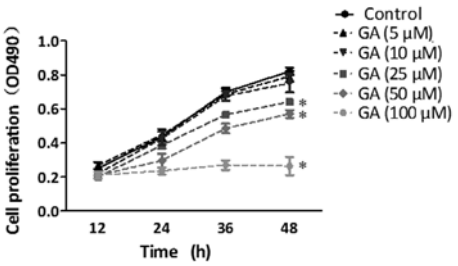

Figure 1. Effects of GA on HepG2 cell proliferation. HepG2 cells were seeded in 96-well plates. After treatment with GA at different concentrations at indicated time-points, cell proliferation was assessed by an MTT assay. Data represent the results of three independent experiments. Column, mean; bar, standard deviation. *P<0.05 compared with the control group (0 μM). GA, ginkgolic acid.

GA inhibits the expression of invasion- and EMT-related molecules in HepG2 cells. Epithelial-mesenchymal transition (EMT), which endows tumor cells with enhanced motility and invasion capacities, is a prerequisite for tumor infiltration and metastasis (21). In order to investigate the effect of GA on the expression of invasion-related genes (MMP-2 and MMP-9) and EMT-related genes (E-cadherin, ZO-1, vimentin and N-cadherin), HepG2 cells were treated with GA (25 and 50 μM) for 24 h and then the total cell RNA was extracted and subjected to RT-qPCR analysis. As revealed in Fig. 3A, the mRNA expression levels of MMP-2 and MMP-9 were significantly reduced in the HepG2 cells after treatment with GA. In addition, increased expression levels of epithelial markers (E-cadherin and ZO-1) and decreased expression levels of mesenchymal markers (vimentin and N-cadherin) were detected in the HepG2 cells with GA intervention. These observations were confirmed at the protein level by the results of the western blot assays (Fig. 3B). Collectively, these results indicated that GA inhibited the expression of invasion-related molecules and prevented the EMT process in HepG2 cells.

GA downregulates HGF/c-Met signaling activity in HepG2 cells. A previous study has demonstrated that HGF/c-Met signaling plays critical roles in the promotion of cell invasion and the EMT process (8). To determine whether GA has an influence on HGF/c-Met signaling activation in liver cancer

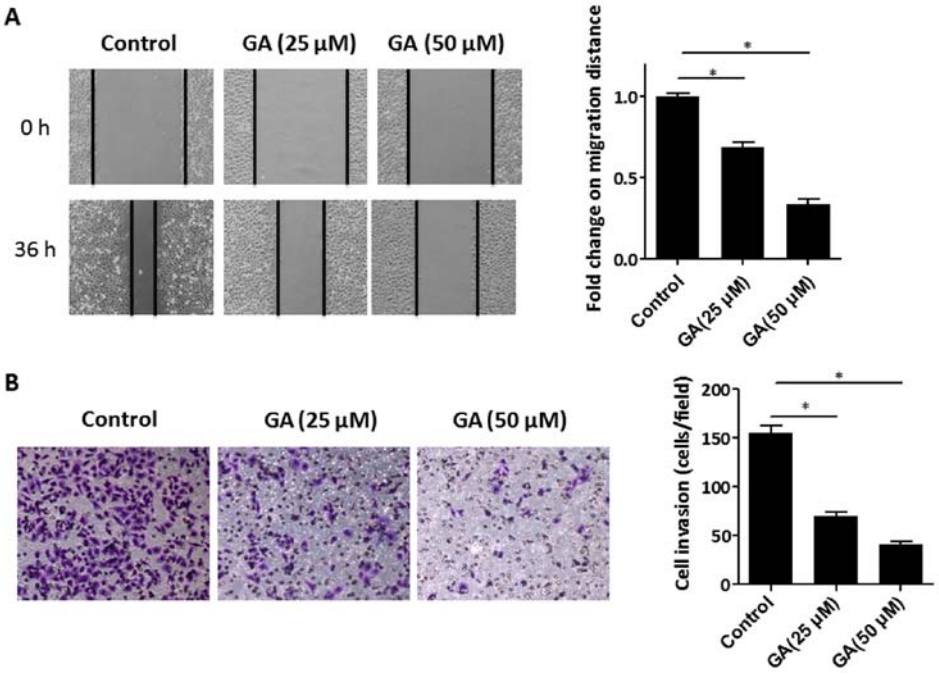

Figure 2. GA suppresses the migration and invasion of HepG2 cells. (A) HepG2 cells were plated in triplicate and treated with GA (25 or 50 μM), then wound healing migration assays were conducted in order to detect the migration ability of the HepG2 cells. The representative images revealed the same area at 0 and 36 h, using a microscope at a magnification of x100. (B) Matrigel invasion assays were performed in order to assess the effect of GA on the invasion ability of HepG2 cells. Images are representative of three independent experiments (magnification, x200). *P<0.05 vs. the control group (0 μM GA). GA, ginkgolic acid.

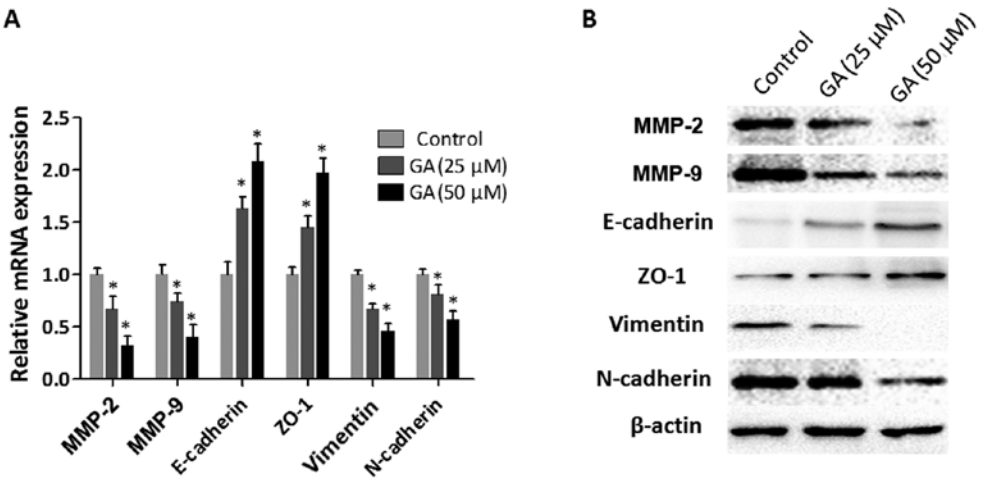

Figure 3. Effect of GA on the expression of invasion- and EMT-related molecules. (A) HepG2 cells were incubated with GA (25 or 50 μM) for 24 h and then total RNA was extracted and examined for expression of invasion-related molecules (MMP-2 and MMP-9) and EMT markers (E-cadherin, ZO-1, N-cadherin and vimentin) at the mRNA level by RT-qPCR. β-actin was used as an internal control. *P<0.05 vs. the control group (0 μM GA). (B) The protein expression of invasion-related molecules (MMP-2 and MMP-9) and EMT markers (E-cadherin, ZO-1, N-cadherin and vimentin) was assessed by western blot assays after HepG2 cells were treated with GA (25 or 50 μM) for 48 h. GA, ginkgolic acid; EMT, epithelial-mesenchymal transition.

cells, HepG2 cells were treated with GA (25 and 50 μM) for 48 h, and then the phosphorylation level of c-Met (p-c-Met) was determined by immunoblotting. Exogenous recombinant HGF (5 ng/ml) was used as a positive control. As observed in Fig. 4A, the total c-Met (t-c-Met) remained unchanged in HepG2 cells after GA treatment. However, treatment with GA resulted in a dose-dependent decrease of p-c-Met expression in HepG2 cells. The binding of HGF to its corresponding RTK c-Met is necessary for c-Met phosphorylation and triggering of downstream events. To further confirm that GA-prevented c-Met phosphorylation is mediated by a reduction in HGF production, the expression of HGF after GA intervention was detected. As revealed in Fig. 4B, the RT-qPCR results demonstrated that the mRNA expression of HGF was suppressed by GA treatment. This was further confirmed by immunofluorescence against HGF (Fig. 4C). These findings indicated that GA suppressed the activity of HGF/c-Met in HepG2 cells via reduction of HGF production.

Exogenous HGF supplementation improves GA-impaired cell invasion ability. To further confirm that the GA-mediated migratory/invasive response in HepG2 cells was due to HGF

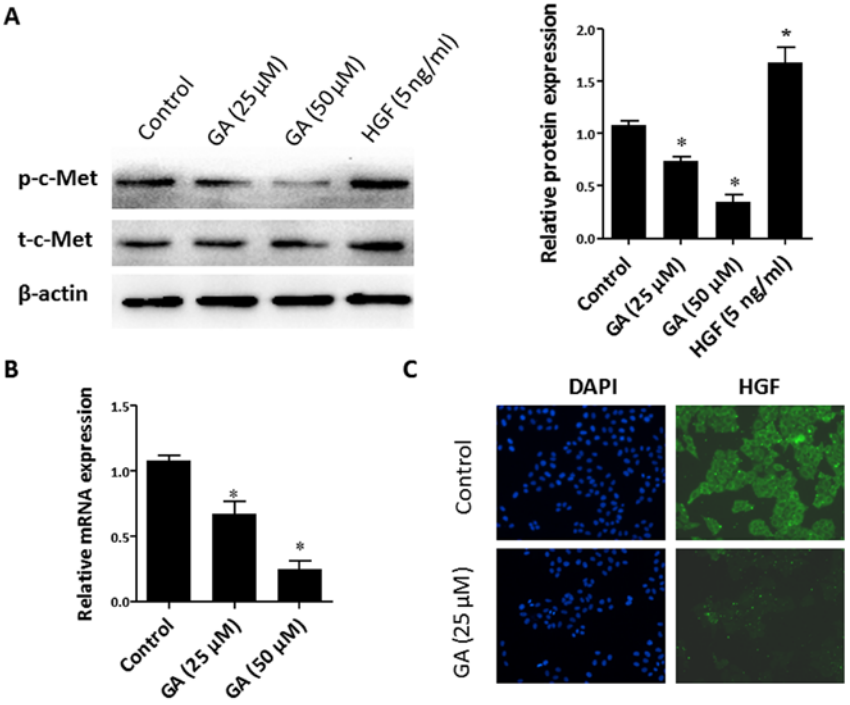

Figure 4. GA suppresses HGF expression and c-Met phosphorylation in HepG2 cells. (A) HepG2 cells were treated with GA (25 or 50 μM) for 24 or 48 h, then the protein expression of t-c-Met/p-c-Met was analyzed using western blot assays with the 0 μM GA intervention group as the control group. *P<0.05 vs. the control group. (B and C) The mRNA and protein expression of HGF in the HepG2 cells were detected by RT-qPCR and immunofluorescence after cells were incubated with GA (25 or 50 μM) for 24 and 48 h. β-actin was used as an internal control. *P<0.05 vs. the control group. GA, ginkgolic acid; HGF, hepatocyte growth factor.

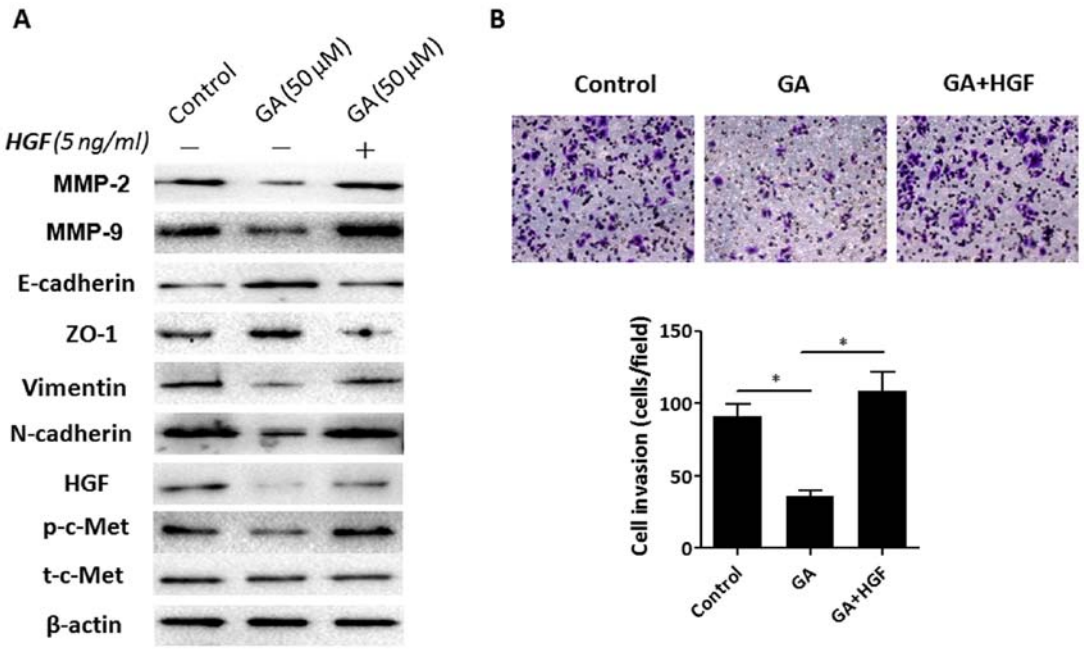

Figure 5. Recombinant HGF improves GA-suppressed invasion and EMT changes in HepG2 cells. (A) HepG2 cells were treated with 50 μM GA or GA plus 5 ng/ml HGF for 48 h, then the protein expression levels of invasion-related molecules (MMP-2 and MMP-9) , HGF, t-c-Met, p-c-Met and EMT markers (E-cadherin, ZO-1, N-cadherin and vimentin) were assessed using western blot assays. (B) Matrigel invasion assays were conducted in order to detect the invasion ability of HepG2 cells after 50 μM GA or GA plus 5 ng/ml HGF intervention. Images are representative of three independent experiments (magnification, x200). *P<0.05 vs. the control group (0 μM GA). HGF, hepatocyte growth factor; GA, ginkgolic acid; EMT, epithelial to mesenchymal transition.

suppression, recombinant HGF was used to treat HepG2 cells. As revealed in Fig. 5A, consistent with the previous data, 50 μM GA treatment resulted in reduced expression of p-c-Met, HGF, MMP-2, MMP-9, vimentin and N-cadherin and increased expression of E-cadherin and ZO-1 in HepG2 cells. However, exogenous HGF (5 ng/ml) supplementation reversed these effects that were mediated by GA. The inhibited expression of p-c-Met, MMP-2, MMP-9, vimentin and N-cadherin

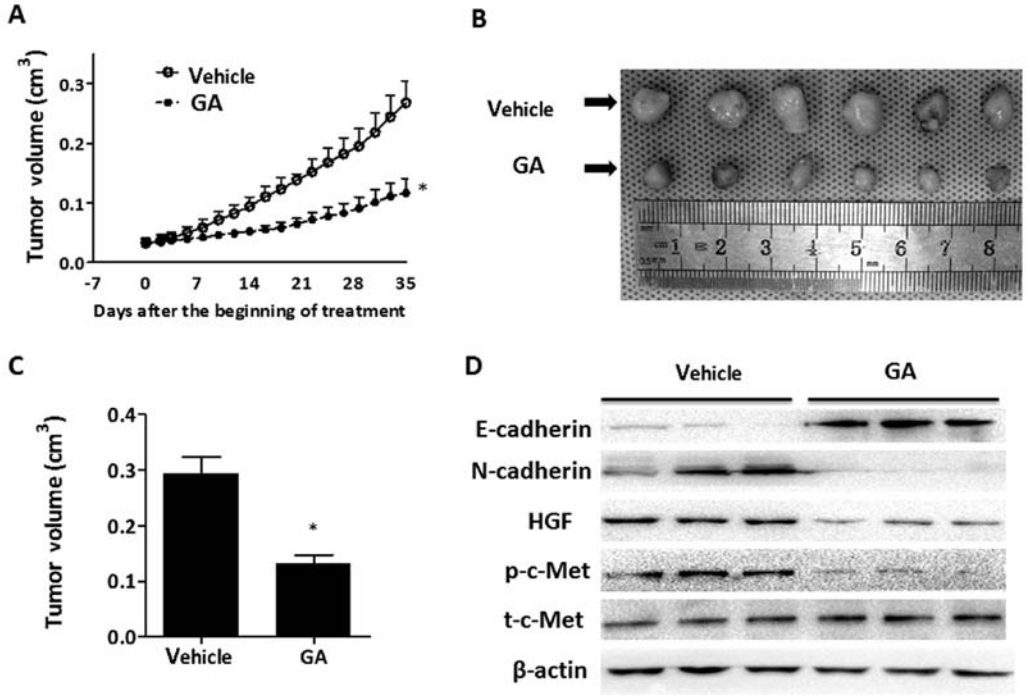

Figure 6. GA prevents tumor growth and EMT in a subcutaneous model of liver cancer in nude mice. (A) The tumor volume in mice treated with vehicle or GA was monitored throughout the experiment. *P<0.05 vs. the vehicle. (B) Representative images revealed the macroscopic tumor specimens from vehicle- or GA-treated subcutaneous models of liver cancer in nude mice at the end of the experiment. (C) Tumor volume in mice treated with the vehicle or GA was assessed at the end of the experiment. *P<0.05 vs. the vehicle. (D) The expression of E-cadherin, N-cadherin, HGF, t-c-Met and p-c-Met in tumor tissue from vehicle- or GA-treated subcutaneous models of liver cancer was detected by western blotting. GA, ginkgolic acid; EMT, epithelial to mesenchymal transition.

by GA was improved in the presence of HGF. Furthermore, the enhanced expression of E-cadherin and ZO-1 by GA was markedly suppressed by HGF supplementation. In addition, the Matrigel invasion assay results revealed that exogenous HGF supplementation restored the inhibitory effect of GA on the invasiveness of HepG2 cells (Fig. 5B). These results indicated that reduced HGF expression was responsible for the GA-suppressed invasion response in HepG2 cells.

GA suppresses tumor growth in vivo. Animal experiments were conducted in order to assess the effect of GA on the growth of HepG2 cells *in vivo*. A single-cell suspension (30 μl) of HepG2 cells (containing 1x10^6 cells) was inoculated subcutaneously into the back of BALb/c nude mice. One week after inoculation, mice were randomly divided into two cohorts, one of which received the vehicle and the other was orally administrated with GA. Through monitoring the tumor volume, tumor growth was found to be significantly suppressed by GA intervention as compared to the mice in the vehicle group (Fig. 6A). The volumes of the tumors from the GA-treated mice were reduced by >50% compared with the control mice at the end of the experiment (Fig. 6B and C). Western blotting was performed in order to detect the expression of E-cadherin, N-cadherin and p-c-Met in the tumor tissue. As shown in Fig. 6D, treatment with GA decreased the expression of HGF and the phosphorylation level of c-Met. Levels of E-cadherin were markedly increased, while levels of N-cadherin were markedly decreased in tumor tissue from the GA-treated mice. We did not observe any metastases both in the vehicle and GA-treated group at the end of the experiment. These *in vivo* findings demonstrated that GA effectively suppressed tumor growth, and inhibition of the activation of c-Met in tumor cells and prevention of EMT may be the underlying mechanism of GA.

Discussion

Liver cancer is one of the most devastating cancers, with the majority of patients being diagnosed at late stages where intrahepatic or extrahepatic metastasis is present and curative surgical treatments are not possible. Tumor relapse and metastasis remain the main obstacles for long-term survival even after curative partial hepatic resection or orthotopic liver transplantation (22,23). Thus, inhibition of cancer invasion and metastasis may be of great clinical significance in improving the survival of patients with liver cancer. The present study revealed that GA suppressed migration, invasion and EMT-related gene expression of HepG2 cells, and targeting of HGF/c-Met signaling may be the underlying mechanism.

Aberrant HGF/c-Met signaling has been implicated in the acquisition of an aggressive phenotype with metastatic potential in several types of malignancy by promoting tumor cell migration, invasion, EMT and angiogenesis (9,24). Upon binding with its high-affinity ligand HGF, c-Met undergoes dimerization, auto-phosphorylation of its tyrosine residues and formation of the multifunctional docking site for adaptor protein binding, resulting in activation of downstream signal transduction pathways (25). The role of the HGF/c-Met axis in liver cancer has been systematically reviewed in the past, and pharmacological c-Met inhibition is a promising therapeutic strategy for liver cancer (10,19). It has been reported that c-Met activation is significantly associated with vascular invasion, neoangiogenesis and poor outcomes in liver cancer (26). In

addition, overexpression of c-Met in liver cancer tissue has been revealed to be correlated with early tumor recurrence or metastasis after hepatectomy (27). Tivantinib (ARQ 197), a selective oral inhibitor of c-Met, has exhibited promising anticancer activity both *in vitro* and *in vivo* (28). Results from a phase II trial (29) revealed that tivantinib statistically significantly improved the time to progression and overall survival versus a placebo among patients with unresectable liver cancer, indicating that inhibition of the c-Met pathway by tivantinib may provide an effective and safe second-line option for patients with advanced liver cancer, particularly for those with c-Met overexpression. Collectively, these results indicated that targeting c-Met may serve as a promising therapeutic strategy for the treatment of liver cancer.

EMT is a process by which epithelial cells lose their cell polarity and intercellular adhesion, and acquire mesenchymal features with migratory and invasive properties (30). EMT has been revealed to play critical roles in embryonic development, wound healing, tissue regeneration, organ fibrosis and malignant transformation (31). Previous studies indicated that the EMT process exerted pleiotropic functions in cancer progression (31,32). Cancer cells undergoing EMT are endowed with enhanced migratory, invasive and metastatic properties. In addition, there is some evidence that EMT is associated with chemoresistance and immunosuppressive tumor microenvironment formation in cancer (33,34). Loss of the epithelial adhesion protein E-cadherin and gain of mesenchymal markers such as N-cadherin and/or vimentin are a hallmark of EMT (35). HGF has been shown to be an effective inducer of EMT in liver cancer (36). The present study revealed that the expression of E-cadherin and ZO-1 was upregulated, while that of N-cadherin and vimentin was downregulated in HepG2 cells after GA intervention, accompanied by weakened invasion and migration properties. However, these changes were reversed by exogenous HGF supplementation.

The anticancer function of GA has attracted interest. The inhibitory effects of GA on cancer cells have been verified by a series of studies (15,17,18). It was reported that GA can serve as a safe and potent anticancer agent against pancreatic cancer by inducing AMPK activation and inhibiting the signaling pathway and genes involved in lipogenesis (15). In addition, GA inhibited the migration of breast cancer cells without causing cytotoxicity to the non-cancerous cell line (18). In a recently published study, the anticancer effect of GA in colon cancer has been studied and both *in vitro* and *in vivo* data revealed that GA suppressed the proliferation, migration and invasion of colon cancer cells without toxicity (16). Induction of AMPK activation and inhibition of the expression of invasion-associated proteins was found to be responsible for GA-suppressed proliferation, migration and invasion of colon cancer cells (17). In the present study, we mainly focused on the effect of GA on the invasion ability and EMT changes of HepG2 cells, and our results indicated that GA could suppress the invasion ability and prevented EMT progression of HepG2 cells efficiently. These results were consistent with previous research on other cancer types (15,37). In addition, reduction of the HGF/c-Met axis activity may be the underlying mechanism. In this study, we did not investigate the effect of GA on the apoptosis of HepG2 cells. However, a previous study has shown that GA can induce apoptosis of human cancer cells by decreasing the Bcl-2/Bax ratio (38). In conclusion, these results indicated that GA may serve as a promising agent for liver cancer treatment. Whether GA has a promoting effect on liver cancer cell apoptosis and the underlying mechanisms warrants further study.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Author's contributions

HL and YL conceived and designed the study; XM, DZ and XX acquired the data. DZ and SL analyzed and interpreted the data; XX and HL wrote, reviewed, and revised the manuscript; XM and XX provided administrative, technical and material support; YL supervised the study. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The experimental protocols were authorized by the Ethics Committee of The Second Affiliated Hospital of Medical College, Xi'an Jiaotong University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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