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Original Article

Inhibitory effects of Ganoderma lucidum triterpenoid on the growth and metastasis of hepatocellular carcinoma

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Abstract: Objective: To investigate the inhibitory effects and mechanisms of triterpenoids from Ganoderma lucidum (G. lucidum triterpenoids) on the growth and metastasis of hepatocellular carcinoma (HCC) both *in vitro* and *in vivo*. Methods: In *in-vitro* experiments, the inhibitory effects of G. lucidum triterpenoids on human HCC SMMC-7721 cell lines were investigated by observing the proliferation, apoptosis, migration and invasion phenotypes of the cell line and assessing the cell cycles as well as the cell apoptosis and proliferation. In *in-vivo* experiments, nude mouse SMMC-7721 tumor models were established and divided into control group, treatment group A (low concentration group) and treatment group B (high concentration group) according to the treatment models received. Magnetic resonance imaging (MRI) was performed 3 times on each mouse model to calculate their tumor volumes. The liver and kidney functions of the models were evaluated. Tissues harvested from their solid organs were subjected to HE staining, and the tumor tissues were subjected to HE staining and immunohistochemical staining (E-cad, Ki-67, and Tunel), respectively. Results: i. In *in-vitro* experiments, G. lucidum triterpenoids could inhibit the growth of human HCC SMMC-7721 cell lines via regulating their proliferation and apoptosis phenotype. ii. In *in-vivo* experiments, the comparison of tumor volumes of mouse models obtained from the second and third MIR scanning was found to be statistically significant between the control group and treatment group A ($P<0.05$); and statistically significant differences were also found in the tumor volumes from the second and third MRI scanning between the control group and treatment group B ($P<0.05$). iii. No significant acute injuries or adverse effects were observed in the liver or kidney of the nude mice. Conclusion: G. lucidum triterpenoids could inhibit the growth of tumor cells via blocking their proliferation, accelerating apoptosis, and inhibiting migration and invasion, without marked toxic effects on normal organs and tissues in the body.

Keywords: Hepatocellular carcinoma, G. lucidum triterpenoids, nude mouse model, pathology, MRI

Introduction

Hepatocellular carcinoma (HCC) is a common liver malignancy whose incidence ranks 5th among all malignancies and the 2nd in mortality [1]. There are over 700,000 new HCC patients and about 500,000 to 1000,000 HCC deaths each year in the world, among which more than half of the deaths are from China [2]. The morbidity and annual mortality of HCC have been significantly increasing over the past 20 years, with a high prevalence in Asian and African regions [3]. Currently, surgical removal is the main treatment approach for HCC patients, but the low 5-year survival rate after the surgery is an issue that hasn't been addressed yet [4]. Ganoderma lucidum (G. lucidum) is a kind of plant that can extend peoples' life span and promote our health with a long history of record in Traditional Chinese Medicine (TCM) [5, 6]. According to TCM records, G. lucidum can enhance peoples' immunity and has already been used as an herbal medicine for thousands of years. G. lucidum bears ideal effects on inhibiting all types of malignant tumors, such as prostate cancer [7], lung cancer [8-10], breast cancer [11, 12], colon cancer [13] and cervical cancer. One study on HCC suggested that polysaccharide from G. lucidum could improve intestinal

flora imbalance in HCC mouse models [14], and another study showed that the polysaccharide was able to inhibit the proliferation and migration of HCC cell lines Hep G2 by down-regulating the protein contents in the vascular endothelial growth factors [15].

Polysaccharide and triterpenoids are two major compositions that can be extracted from G. lucidum to treat tumors [16]. Polysaccharide normally functions as an immunomodulator or an antioxidant to fight against carcinomas, while triterpenoids primarily play their part by inhibiting the proliferation and metastasis of cancer cells [17]. Triterpenoids and polysaccharide, however, are not in a fixed proportion in G. lucidum extract, probably for the differences in the types, cultivation, growth area and extracting approach of the G. lucidum. Even in the same G. lucidum, its different composition has different triterpenoid levels. Usually, spores contain more triterpenoids than other compositions in G. lucidum.

G. lucidum Triterpenoids play a crucial role in the prevention and treatment of diseases in human beings. In addition to their anti-carcinogenic effect, they also play a role in anti-aging [18], liver protection [19], anti-atherosclerosis [20, 21], lowering blood glucose and lipid [22], anti-inflammation [23], anti-androgen [24], antibacteria [25], immuno-regulation [26], heart protection [27], and improving one's physical weakness [28], etc. Moreover, G. lucidum triterpenoids can prevent and treat cardio-cerebrovascular diseases via regulating patient's blood pressure, blood lipid and glucose [29, 30].

It has currently been reported that G. lucidum triterpenoid compounds kill tumor cells primarily through the following two approaches: one is to induce the apoptosis of tumor cells by damaging the completeness of cell membrane; the other is to inhibit the proliferation of tumor cells [10, 13, 31-34]. Currently, many studies have confirmed that G. lucidum triterpenoid compounds can lead to cell cycle arrest primarily by down-regulating the expressions of proteins related to cell cycles [35, 36]. Latest studies have also suggested that G. lucidum triterpenoids play a role in inhibiting the metastasis of tumor cells. And many studies have also revealed the effectiveness of G. lucidum triterpenoids in the treatment of common cancers such as pulmonary cancer [9, 37, 38], liver cancer [31, 39], cervical cancer [40], prostate cancer [32, 34, 41], breast cancer [10, 11] and colon cancer [13, 33].

Even though studies have reported the therapeutic effects of G. lucidum on liver cancer, the mechanism of which still remains unclear. Therefore, our study carried out both *in-vitro* and *in-vivo* experiments to investigate the mechanism of G. lucidum on HCC.

Materials and methods

Reagents and instruments in the cell experiment

Reagents: All chemical reagents were purchased from business suppliers, which can be used directly without further isolation and purification. All solvents were purified prior to use.

Cells and G. lucidum triterpenoids used in the experiment: Human HCC SMMC-7721 cell lines were purchased from the cell bank of the Chinese Academy of Science, and G. lucidum triterpenoids from Prof. MENG Xiangxian's research group at Hu'nan University.

Grouping and cell treatment

Grouping and CCK-8 treatment: Human HCC SMMC-7721 cell lines were divided into four groups: blank control group exempting from the treatment of G. lucidum triterpenoids, and low, medium and high dose groups receiving 2.5 μg/mL, 5 μg/mL, and 10 μg/mL G. lucidum triterpenoids, respectively. After the addition of G. lucidum triterpenoids, the cells were cultured for 12 h, 24 h and 48 h. Subsequently, 10 μL CCK-8 solution (Dojindo, CK04) was added to each well for incubation under 37°C for 4 h. Absorbance was determined with Microplate Reader (Bio-Rad, iMark) set at 450 nm. This process was repeated three times.

Effects of different concentrations of G. lucidum triterpenoids on HCC cell cycle: Cells that were growing logarithmically were added with different concentrations of G. lucidum triterpenoids after culture. Then, the cell culture medium was discarded to collect the cells, whose number was controlled between 1×10^5 and 1×10^6. After that, the cells were filtered with a 400-mesh screening net, with the use of Flow Cytometry (BD Biosciences, FACS Calibur) following standard procedures to be detected.

Then, the results were analyzed. The process was repeated three times.

Effects of different concentrations of G. lucidum triterpenoids on HCC cell apoptosis: Cells that were growing logarithmically were selected, digested and counted. Subsequently, the selected cells were cultured overnight until the cell density reached 80-90%, added with medium (Gibco, 11965092) containing different concentrations of G. lucidum triterpenoids (0 μg/mL, 2.5 μg/mL, 5 μg/mL, 10 μg/mL), and incubated for 24 h. After that, the cells were digested and collected. The process was repeated 3 times. Flow Cytometry (BD Biosciences, FACS Calibur) was used to detect the Annexin V/PI activity, and the results were analyzed using FlowJo software.

Effects of different concentrations of G. lucidum triterpenoids on HCC cell apoptosis (Tunel): HCC cells were smeared, cultured overnight until their density reached 80-90%, and added with medium containing different concentrations of G. lucidum triterpenoids (0 μg/mL, 2.5 μg/mL, 5 μg/mL, 10 μg/mL) for experimenting. The treated cells were placed under an optical microscope (Olympus, CX41) for observation and photographing. This process was repeated for 3 times.

Detection of cell migration ability by wound healing assay: Wound healing assay was employed to determine the migration ability of HCC cells. For starters, the HCC cells were digested, counted, made into suspension and inoculated evenly into a 6-well plate. Then the plates were added with mediums containing different concentrations of G. lucidum triterpenoids (0 μg/mL, 2.5 μg/mL, 5 μg/mL, 10 μg/mL) to be studied. Samples were taken at 0 h and 24 h and photographed. The process was repeated 3 times. The migration area of cells was measured with Image J and the results were recorded.

Detection of cell invasion ability with the transwell assay: Cells that were growing logarithmically were selected, digested and counted to determine their invasion ability. The treated cells were then centrifuged at 1000 rpm under room temperature and added with serum-free DMEM medium for re-suspension and adjustment of cell density to 2×10^5 cells/mL. Next, cell suspension of 100 μL was added to the upper chamber and DMEM medium containing 10% fetal bovine serum to the lower chamber. Mediums containing different concentrations of G. lucidum triterpenoids (0 μg/mL, 2.5 μg/mL, 5 μg/mL, 10 μg/mL) were subsequently added for experimenting. The process was repeated 3 times. In the end, the cells were observed carefully under an optical microscope and random visual fields were selected for photography. The cells were again counted and the results recorded.

Animal experiment methods

Experimental animals and cells: All animal experiments were performed sticking to the 3R principles in an effort to minimize the suffering of animals. A total of 45 female BALB/c nude mice, aged 8-10 weeks and weighed 20±2 g, were purchased successively from an experimental animal center. All nude mice were fed with standard forage and sufficient water. They received standard light treatment, which was light exposure for 14 h and the remaining 10 h in a dark environment. They were kept under 22°C in an environment with stable humidity. HCC SMMC-7721 cell lines for model construction were obtained from the cell bank of the Chinese Academy of Science (Shanghai, China). This study was approved by Experimental Animal Ethics Committee, the Second Xiangya Hospital, Central South University (2020496).

Establishment of nude mouse SMMC-7721 models: The prepared SMMC-7721 cells of 100 μL (approximately 2×10^6 cells) were injected into the right armpit of the 15 nude mice. Once the tumor was formed, it was harvested and inoculated into the rest 30 nude mice through their armpits.

The tumor size of the mice that had been inoculated with SMMC-7721 cells was assessed daily using a vernier caliper and received their first MRI one week after the inoculation to ensure the success of modeling. According to the first MRI results, 26 nude mice were observed to have significant tumors, the other 4 without. Next, 18 nude mice that had almost the same tumor volume (about 39.15 mm^3) were selected out of the 26 and divided into control group, treatment groups A and B, with 6 nude mice in each group. In a preliminary experiment, we found that G. lucidum triterpenoids, which were diluted to 200 μg/mL and

400 μg/mL, had inhibitory effects on tumors. According to this result, the G. lucidum triterpenoid extract was also diluted to a low concentration (200 μg/mL) for nude mice in treatment group A and a high concentration (400 μg/mL) for those in treatment group B.

Treatment of nude mouse models with different concentrations of G. lucidum triterpenoids: In the preliminary experiment, because a gradually increased stress response was observed in some nude mice one week after the intragastric injection of 0.5 mL G. lucidum triterpenoids per day using a 1 mL syringe, the dose of 0.3 mL per day was decided in the subsequent experiments to avoid unnecessary death. Nude mice in the two treatment groups received 0.3 mL G. lucidum triterpenoids intragastrically using the 1-mL syringe with a No. 10 gavage needle at the same time every day, and those in the control group received normal saline in the exact same way. The needle was disinfected with alcohol before each injection. Once the needle was placed in the stomach of the mice, the triterpenoid extract was injected drop by drop at first to ensure no unexpected accident was observed. Such an unexpected accident could be the transfer of the extract into the lung via a hiccup, leading to an accidental death caused by pneumonia, etc.

MRI scanning for nude mouse models at different time points: On the day before gavage (day 0) and days 7 and 14 after gavage, the nude mice received MRI scanning using 3.0T magnetic resonance (uMR 790, Shanghai United Shadow Medical Technology Co., Ltd., China) and customized animal coils (Zhongzhi Medical Technology Co., Ltd., Suzhou, China) to obtain MRI images. Conventional sequences were as follows: T1-weighted fast spin-echo (FSE) T2-weighted single-shot fast spin-echo (SSFSE). Before MRI, all mice were injected intraperitoneally with 50 mg/kg pentobarbital sodium solution to get anesthetized.

During MRI scanning, unconscious movements were observed visually via a real-time closed-circuit television. All images were imported into the RadiAnt DICOM Viewer (64-bit) software for data analysis. Tumors were clearly observed on conventional T1- and T2-weighted sequence images. Two radiologists who have over 10-year's working experience (YHL and CM) and one with more than 15 years (QLS) analyzed the images. All three radiologists didn't have the knowledge of the histopathological results beforehand. However, they confirmed a tumor in the mice according to the MRI images.

Sacrifice, blood and tissue samplings of the mouse models: After the third MRI scanning, the nude mice were anesthetized by intraperitoneal injection of anesthetic, and sacrificed through cardiac puncture. The mice were prepared for further use after their eyeballs were removed and blood collected. The prepared mice, after the harvest of their tumors, were cut in their belly to obtain the heart, liver, spleen, lung and kidney. These organs and tumors of mice in different groups were fixed in 10% formalin solution within 24 h after being harvested, embedded in paraffin, sectioned, and then stained. Meanwhile, an immunohistochemical S-P method was used to evaluate the proliferation and metastasis abilities of these tumor cells.

Blood test: After the third MRI scanning, the nude mice were sacrificed, their eyeballs removed and blood collected. About 1 mL of blood was drawn from each nude mouse and centrifuged at 3000 rpm for 15 min to get the supernatant for the test. However, due to the limited amount of blood samples, liver and renal functions were the focus of the test. Their indicators were alanine aminotransferase (ALT), aspartate aminotransferase (AST), direct bilirubin (DBIL), total bilirubin (TBIT), albumin (ALB), alkaline phosphatase (ALP), urea (BUN), creatinine (CR) and uric acid (UA). Data were subjected to one-way ANOVA using Graphpad Prism software (GraphPad company).

Pathological and immunohistochemical examinations: The tumors, hearts, livers, spleens, lungs and kidneys of the nude mice in the three groups were made into parafin sections and stained regularly with HE. As only the proliferation, apoptosis and migration phenotype were chosen for the determination of anti-carcinogenic effects of G. lucidum triterpenoids in the cell experiments, the very same phenotype was selected in the animal experiment for the immunohistochemical analysis of the tumor sections, which were Ki-67 for proliferation, Tunel for apoptosis, and E-cad for migration. Each tumor section underwent Ki-67, E-cad and Tunel immunohistochemical analysis. At the

same time, both positive and negative control assays for the detection of primary antibodies were completed.

An optimal microscope (DP72, Olympus Corporation, Tokyo, Japan) and ImageJ software (Open Source, NIH Image, USA) were used for observation and data analysis. The results of HE, E-cad, Ki-67 and Tunel from microscopic observation under the same light exposure were all written down. Morphological changes were observed by an experienced pathologist (5-year experience), and the ratio of the positive areas of E-cad, Ki-67, and Tunel to the whole tumor section was calculated [42].

Statistical analysis

GraphPad Prism 8.0 software was used for data analysis. Continuous variables were expressed as mean ± standard deviation ($\bar{x}$ ± sd). One-way ANOVA was used to detect differences for further analysis and the Turkey method was used for between-group comparison. Enumeration data were expressed as cases/100 (n/%), and analyzed with the Pearson chi-square test, expressed as chi-square. *P* value <0.05 was considered statistically significant.

Results

Cell experiment results

Regulation of cell viability by G. lucidum triterpenoids: CCK-8 assay was conducted for HCC SMMC-7721 cell lines. Compared with the control group, the inhibition rate of SMMC-7721 in mice in the treatment groups rose as the concentration of G. lucidum triterpenoids increased (P<0.001). See **Figure 1A**.

Effects of G. lucidum triterpenoids on cell cycles: Compared with the control group, the number of cells in phase G1/G2 was reduced following the treatment of 2.5 μg/mL, 5 μg/mL and 10 μg/mL triterpenoids (all P<0.001), and no significant difference was found in cells in phases between the groups. See **Figure 1B**.

Effects of G. lucidum triterpenoids on cell apoptosis: Cells exhibited significantly increased apoptosis in the 2.5 μg/mL, 5 μg/mL and 10 μg/mL G. lucidum triterpenoids treatment groups than those in the control group (all P<0.001). See **Figure 1C**.

Effects of G. lucidum triterpenoids on cell apoptosis (Tunel): Cells exhibited significantly increased apoptosis (Tunel) in the 2.5 μg/mL, 5 μg/mL and 10 μg/mL G. lucidum triterpenoids treatment groups than those in the control group (all P<0.001). See **Figure 2A**.

Results of the wound healing assay: Compared with the control group, cell migration reduced in nude mice in the 2.5 μg/mL triterpenoids extract treatment group, but without statistical difference. However, cell migration markedly diminished in the 5 μg/mL and 10 μg/mL triterpenoids extract treatment groups in comparison to the control group (all P<0.001). See **Figure 2B**.

Results of the transwell assay: Cell migration was markedly reduced in the 2.5 μg/mL, 5 μg/mL and 10 μg/mL triterpenoids extract treatment groups than that in the control group (all P<0.01). See **Figure 2C**.

Animal experiment results

Calculation and analysis of tumor volume: No statistical significance was found in the comparison of tumor volumes between the control group and treatment groups A and B after the first MRI scanning (t value: 0.403, P>0.05). The tumor volumes in the treatment groups A and B were smaller than those in the control group from the second and third MRI scannings (all P<0.05). Tumor volumes were smaller in treatment group B than those in the treatment A, with a statistically significant difference. For details see **Figure 3A**, **3B** and **Table 1**.

Blood test analysis: No significant difference was found in the comparison of blood test results among the three groups.

Pathological results: HE staining was performed for all tumor and solid organ sections of nude mice in the three groups. Meanwhile, the results of HE staining suggested that no marked acute injuries or adverse events were observed in the solid organs (heart, kidney, spleen, lung and kidney). See **Figure 3C**. Also, much more necrotic cells were found in the HE staining sections of nude mice in the treatment groups A and B than those in the control group. See **Figure 3D**.

Results of immunohistochemical analysis: The results of the immunohistochemical analysis

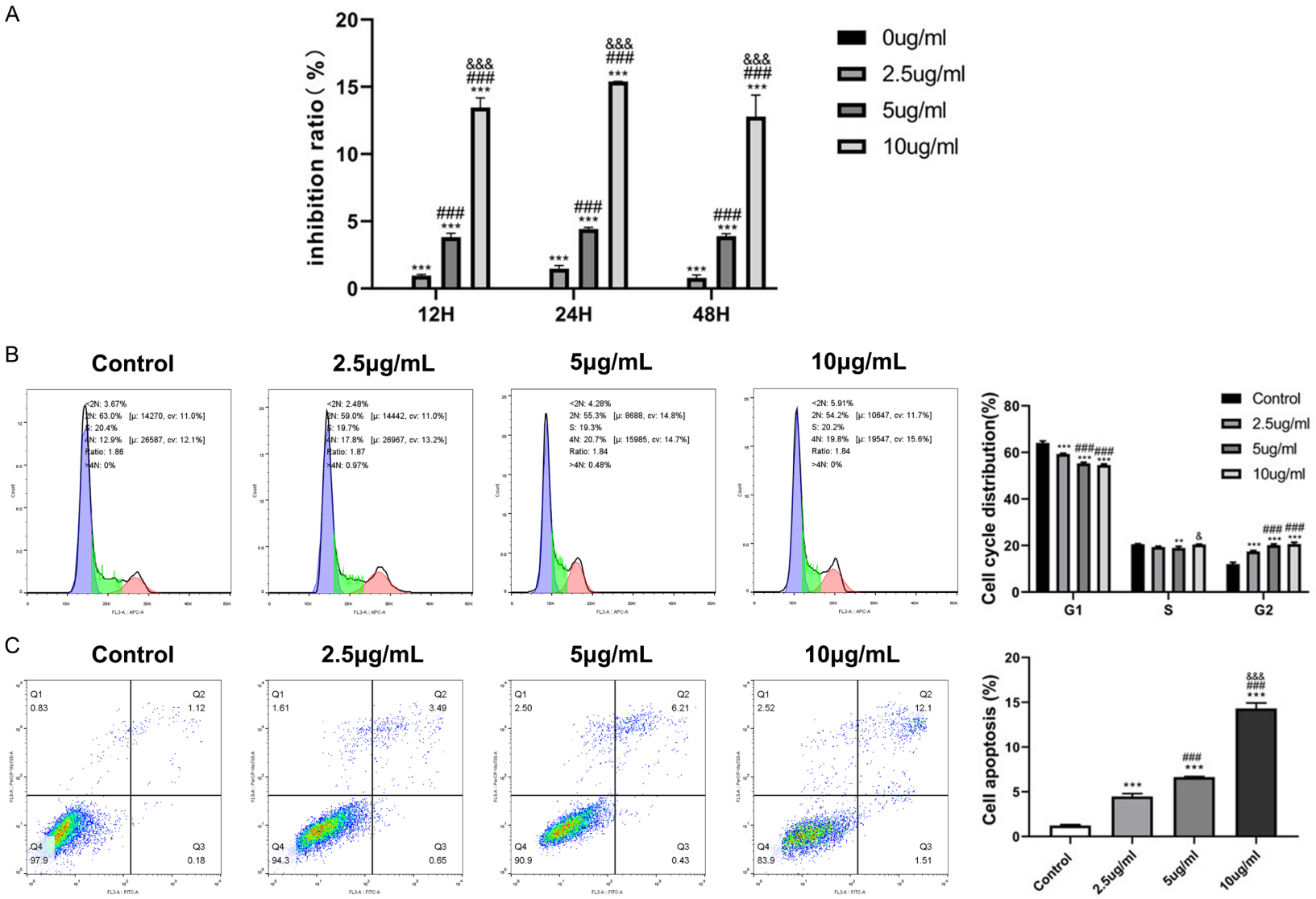

Figure 1. Experimental results. A. Effects of different concentrations of G. lucidum triterpenoids on the activity and cell cycles of HCC cells detected by CCK-8 at different time points (***P<0.001, vs. 0 ug/mL, ###P<0.001, vs. 2.5 ug/mL, &&&P<0.001, vs. 5 ug/mL). B. Bar charts showing the effects of different concentrations of G. lucidum triterpenoids on HCC cell cycles detected by flow cytometry (**P<0.01, ***P<0.001, vs. Control, ###P<0.001, vs. 2.5 ug/mL, &P<0.05, vs. 5 ug/mL). C. Bar charts and the flow cytometry figure showing the effects of different concentrations of G. lucidum triterpenoids on HCC apoptosis (***P<0.001, vs. Control,

###P<0.001, vs. 2.5 ug/mL, &&&P<0.001, vs. 5 ug/mL). One-way ANOVA was used to detect the differences for further analysis and Turkey method was used for between-group comparison.

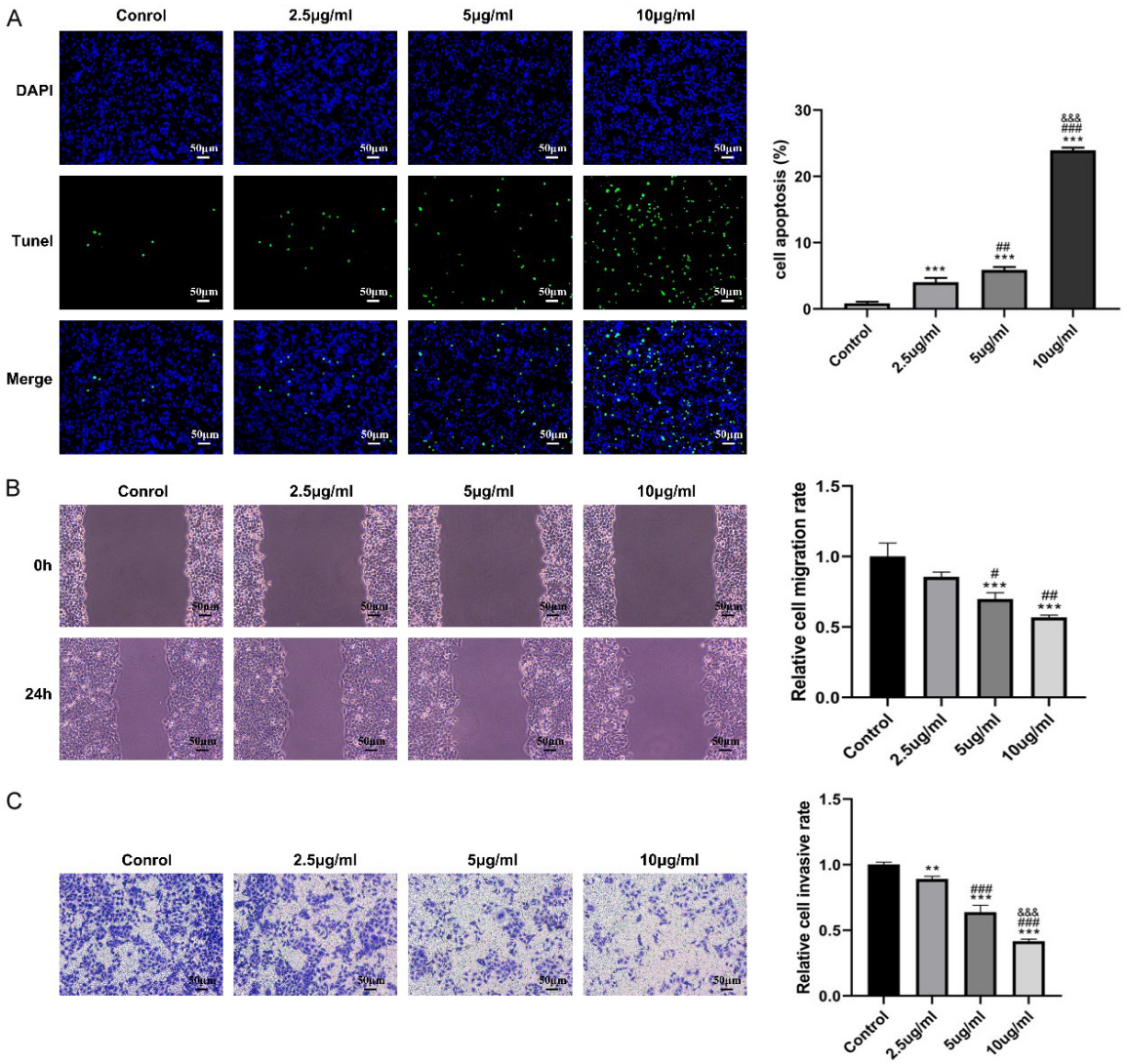

Figure 2. Effects of different concentrations of G. lucidum triterpenoids on HCC cell apoptosis, metastasis and invasion. A. Effects of different concentrations of G. lucidum triterpenoids on HCC cell apoptosis, as shown in the TUNEL fluorescent images and bar charts (***P<0.001, vs. 0 ug/mL, ##P<0.01, ###P<0.001, vs. 2.5 ug/mL, &&&P<0.001, vs. 5 ug/mL). B. The migration ability of HCC cells after the treatment of different concentrations of G. lucidum triterpenoids by wound healing assay (***P<0.001, vs. Control, #P<0.05, ##P<0.01, vs. 2.5 ug/mL). C. The invasion ability of HCC cells after the treatment of different concentrations of G. lucidum triterpenoids by Transwell assay (**P<0.01, ***P<0.001, vs. Control, ###P<0.001, vs. 2.5 ug/mL, &&&P<0.001, vs. 5 ug/mL). One-way ANOVA was used to detect the differences for further analysis and Turkey method was used for between-group comparison. All figures were zoomed in 200 times.

showed that the positive areas of Ki-67 and E-cad were smaller in the treatment groups A and B than those in the control group, and the area was smaller in treatment group B than that in treatment group A. However, the positive area of Tunel was slightly bigger in the treatment groups than that in the control group. No statistical difference was observed in the comparison between the treatment groups A and B. See **Figure 3E**.

Discussion

In our experiments, the inhibitory effects of G. lucidum triterpenoids were explored both *in vivo* and *in vitro* with the use of HCC SMMC-

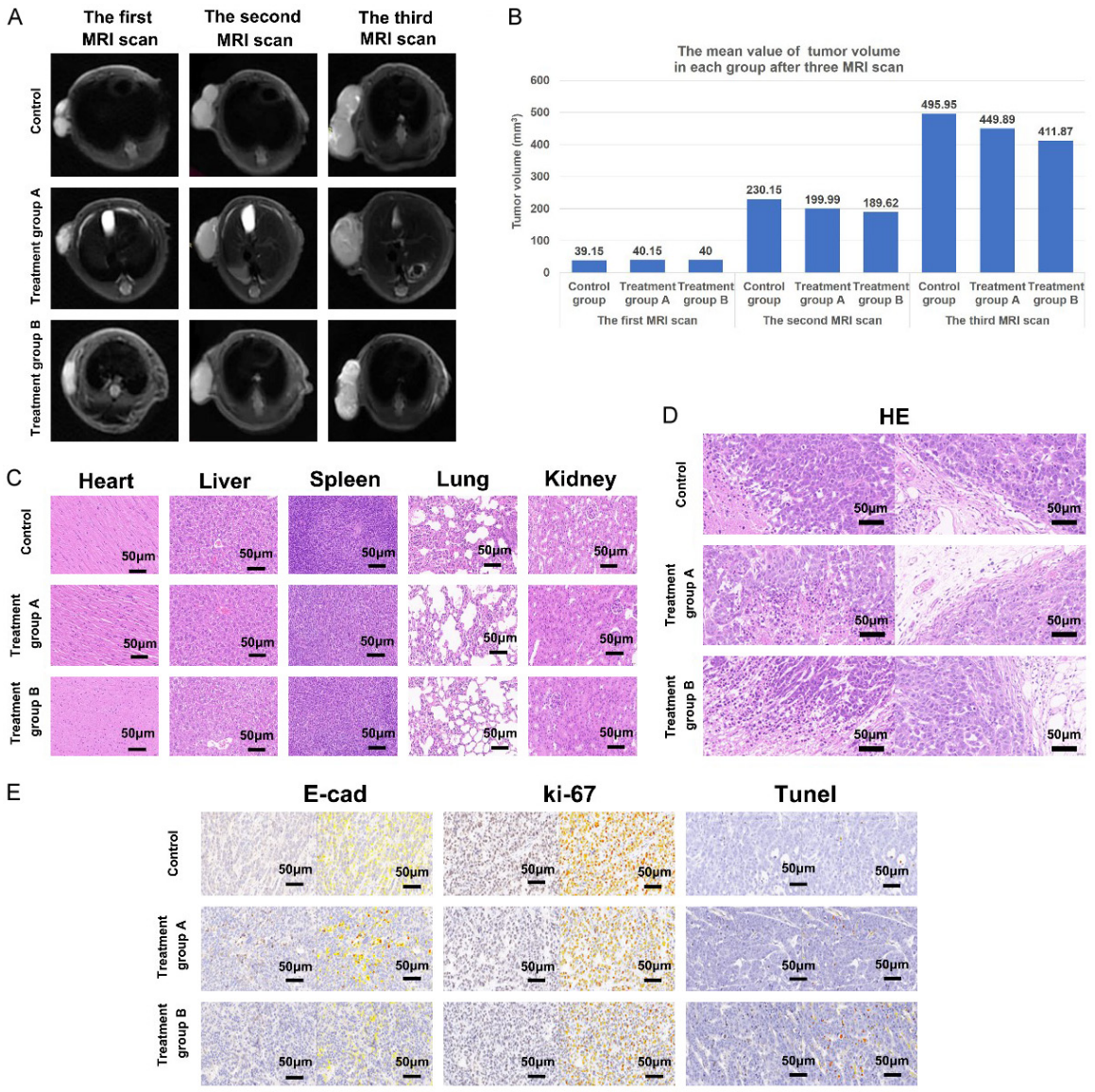

Figure 3. Comparisons of the tumor volume and immunohistochemical staining results of each organ and tumor tissue. A. Three MRI results of the tumor tissues of nude mice in each group. B. The average tumor volumes of nude mice in each group after three MRI scans. C. HE staining images of the heart, liver, spleen, lung, and kidney tissues of nude mice in each group. D. HE staining images of the tumor tissues of nude mice in each group. E. E-cad, Ki-67 and Tunel staining images of tumor sections of nude mice in each group. Note: All figures were zoomed in 400 times. n=6 in all three groups.

7721 cell lines and nude mice models built using the same cell lines. All our results suggested that G. lucidum triterpenoids could inhibit the growth of HCC cells as well as their metastasis.

Our study suggested that the inhibitory effects on the proliferation of tumor cells grew as the concentration of G. lucidum triterpenoids rose. From the experiment on HCC cell cycles, it was concluded that G. lucidum triterpenoids inhibited HCC cell growth via regulating the cycles of SMMC-7721 cells. Moreover, with experiments on the apoptosis, wound healing and invasion of HCC cells, it was demonstrated that G. lucidum triterpenoids bore potentials for inhibiting HCC cell metastasis.

G. lucidum triterpenoids were reported to inhibit the growth of HepG2 cells to some extent

Table 1. Comparison of tumor volumes from MRI scanning among the three groups

Item	Control group (n=6)	Treatment group A (n=6)	Treatment group B (n=6)	F	P
Tumor volume at week 0 after injection	39.15±3.07	40.15±5.25	40.00±4.75	0.088	0.916
Tumor volume at week 1 after injection	230.15±18.87	199.99±17.76*	189.62±10.96**	10.082	0.002
Tumor volume at week 2 after injection	495.95±22.89	449.89±24.72**	411.87±26.20***,#	17.517	0.000

Note: According to the one-way ANOVA, no statistical significance was found in the comparison of tumor volumes from the first MRI scanning among the three groups (P>0.05), while significant difference was observed in the comparison of tumor volumes from the second and third MRI scannings among the three groups (P<0.05). MRI: Magnetic resonance imaging. *P<0.05, **P<0.01, ***P<0.001, vs. the control group; #P<0.05, vs. the treatment group A.

[31]. A study showed that G. lucidum triterpenoids could inhibit the proliferation and induce apoptosis of HepG2 cells [43]. G. lucidum triterpenoids were also found to enhance the sensitivity of HepG2 to chemotherapy and exacerbate the cytotoxicity of cisplatin by inhibiting JAK-1 and JAK-2 in JAK-STAT-3 signaling coproteins [44]. G. lucidum triterpenoids could have cytotoxic effects on HCC SMMC-7721 cell lines, with different degrees of cytotoxic effects on different tumor cells [45, 46]. Hampering different cell cycle phases by G. lucidum triterpenoids has been verified in different cells. It was reported that HT-29, MCF-7, and MDA-MB-231 cells were arrested in phase G1, HUCPC and Huh-7 cells in phase G2/M and H69 cells in phase S [47, 48]. Interestingly, if different concentrations of G. lucidum triterpenoids were used to intervene the macrophage cells RAW264.7 of mice, then the cell cycle arrest could be changed from phase G0/G1 to phase G2/M. Such change might suggest that G. lucidum triterpenoids have functioned as a regulator for transitioning the cell cycle arrest from phase G1 to phase G2/M. Protein responses in the arrest of cell cycle phases G1 and G2/M were significantly different following G. lucidum triterpenoids treatment. Arrest in phase G1 was associated with a decrease in the expressions of cyclin D1 and cyclin dependent kinase (CDK) 4 and an increase in expressions of p21 and p53 [35, 36]. Arrest in phase G2/M was probably related to the inhibited expressions of cyclin B1 [49]. Apoptosis induced by G. lucidum triterpenoids may be mediated by the up-regulation of Bax and activation of caspase-7, caspase-9, caspase-3 and PARP, suggesting an intrinsic intervention in the process through mitochondria [35, 36]. Currently, no extrinsic cell apoptosis induced by death receptors after the treatment of G. lucidum triterpenoids has been reported.

In our experiments, G. lucidum triterpenoids were found to have imposed significant damages to HCC SMMC-7721 cell lines and were capable of inhibiting the proliferation and inducing apoptosis of SMMC-7721 cells. Moreover, the presence of cells in phase G0/G1 was decreased and those in phases remained mostly the same in a cell cycle experiment, demonstrating that G. lucidum triterpenoids played a role in both damages and cell cycle controls over mouse HCC HepG2 cell lines and human HCC SMMC-7721 cell lines. Various studies have suggested that the anti-carcinogenic effects and mechanisms of G. lucidum triterpenoids are associated with cell apoptosis and cell cycle arrest [50, 51], which is in line with our study results.

Apart from the cell experiments, we also conducted several animal experiments. In these experiments, human HCC SMMC-7721 cell lines were selected to establish nude mice models. Once the model establishment succeeded, the models were treated with G. lucidum triterpenoids intragastrically for some time, and underwent several MRI scans for the calculation of tumor volumes. The results indicated that nude mice in treatment group A (low concentration) and treatment group B (high concentration) had markedly smaller tumors than those in the control group, strong evidence for the inhibitory effects that G. lucidum triterpenoids have on tumor growth, with better effects in the high concentration group than those in the low concentration group. In addition, G. lucidum triterpenoids of different concentrations had no marked impacts on the liver and kidney of nude mice. And it was further suggested by the immunohistochemical results that G. lucidum triterpenoids could effectively hamper the growth and metastasis of tumors.

Similar studies were carried out in exploring the inhibitory effects of G. lucidum triterpenoids on lung cancer A549 cell lines in mouse models, and the results suggested the presence of anti-carcinogenic effects of G. lucidum triterpenoids [37]. So far, no studies on G. lucidum triterpenoids for the treatment of liver cancer models were found. However, G. lucidum polysaccharide, another extract from G. lucidum, was reported to bear the ability to significantly inhibit the growth of tumor cell H22 in mice with liver cancer. Moreover, the tumor volumes kept decreasing as the concentrations of the given drug increased [52]. This result just conformed to our result that triterpenoids, also an extract from the G. lucidum, could effectively inhibit the growth of the human HCC SMMC-7721 tumor cells. According to relevant studies in China and other countries around the world, G. lucidum has exhibited ideal results and has become a valuable approach for the prevention and treatment of cancers. In current animal studies, mouse liver or other cancer cells HepG2 have been primarily used for constructing tumor models. The reason is that the human liver or other cancer cells couldn't form tumors in regular mice, but that can be done in nude mice, which are more expensive than the regular ones. Knowing that the ultimate goal of an experiment is for the result to be applied in clinical settings, human HCC cell lines were selected and used to build tumor models in nude mice in our experiments, which, we believed, have increased the credibility of such experiments in some aspects compared with those using mouse HCC HepG2 cell lines to construct the tumor models.

It was concluded from our several *in-vivo* and *in-vitro* experiments that G. lucidum triterpenoids were anti-carcinogenic via prolonging cell cycles, inhibiting the metastasis of tumor cells and the formation of vessels, and inducing the apoptosis of tumor cells. Therefore, we summarized three possible targets of G. lucidum triterpenoid molecules here. In 2011, Hanahan and Weinberg refined the article entitled *The Hallmarks of Cancer* and expanded the hallmark research of cancers. After the refinement, the article determined 10 hallmarks of cancers and proposed 10 targets for cancer treatment. And a conclusion was drawn from our study that at least 7 targets were involved in the anti-carcinogenic process of G. lucidum triterpenoids. They were prolonging the cell cycles by targeting at steering clear of growth suppressors, down-regulating ERK, JNK, MAPK, FAK and AKT at maintaining proliferation signals, inducing apoptosis and autophagy at fighting cell death, inhibiting cell migration and invasion at activating invasion and metastasis, inactivating telomerase and DNA topoisomerase by targeting at replicating immortal, inhibiting inflammation at promoting carcinogenic inflammation, and exerting anti-angiogenic activity at inducing angiogenesis. Other than these targets, triterpenoid GA-Me enhances immune activation (to avoid immunity destructing targets) by stimulating the secretion of IL-2 and IFN-γ [53]. Another triterpenoid GA-Me also promotes immune responses by increasing the presence of type II HLA in melanoma cells [54].

Apart from the targets mentioned above, G. lucidum triterpenoids could also target several other molecules. First, NF-κB pathway is the regular channel for G. lucidum triterpenoids to induce cell cycle arrest, and inhibit cell invasion and inflammation. NF-κB is widely expressed and constitutively activated in cancer cells and tumor tissues to regulate the expressions of a large number of genes through binding with specific promoters and enhancing responses to many stimuli. Down-regulation of NF-κB and its pathway is conducive to the development and progression of the tumor inhibition process [55]. One in five myeloma patients had altered genes caused by the activation of NF-κB. Therefore, inhibition of NF-κB is also helpful in the treatment of hematologic malignancies [56]. An *in-vitro* experiment on 26 human cancer cell lines showed that hematological tumor cell lines were highly sensitive to G. lucidum extracts. However, no *in-vivo* studies have elucidated the mechanism of such optional effects of cancers. How to transduce the signals from receptors of G. lucidum triterpenoids or other responders to NF-κB remains to be a mystery.

Although studies have revealed some signaling pathways and targets of G. lucidum triterpenoid molecules, the specific signaling pathways and key targets for G. lucidum triterpenoids are still unclear. Many issues remain to be addressed. For example, how do G. lucidum triterpenoids get into human cells? What are essential receptors in the human body to recognize the G. lucidum triterpenoids? What spe-

cific pathways do G. lucidum triterpenoids regulate to inhibit the tumor cell growth and metastasis? Also, we know very little about the treatment effects of G. lucidum triterpenoids in combination with other drugs, which should be the focus of future studies. Technological advances in "omics" research, including genomics, proteomics, and metabolomics, as well as network pharmacology, are conducive to the study on such topics [57]. Two proteomics studies using purified triterpenoids revealed possible targets of G. lucidum triterpenoids [58, 59].

In recent years, although considerable achievements have been made in discovering the targets of the molecules of triterpenoid compounds, their more detailed anti-carcinogenic mechanisms and activated components should be revealed. Some pre-clinical and clinical studies are also necessary for discovering the potential effects of G. lucidum triterpenoids. Future clinical studies should focus on a large number of characterized extracts of active compounds. And these extracts ought to be investigated repeatedly in both *in-vivo* and *in-vitro* studies and their relevant chemical characterization should be revealed. Through these studies, the exact number of specific compounds investigated in experiments can be further determined.

Disadvantages and prospects: i. In our cell experiments, more concentrations of G. lucidum triterpenoids, such as 15 μg/mL, 20 μg/mL or even higher, ought to be selected to further explore their anti-carcinogenic effects, and to discover the turning point of the concentrations. ii. Although the phenotype of proliferation, apoptosis and migration were investigated in our study, in-depth responses and mechanisms should be explored for supporting the study. iii. Apart from the human HCC SMMC-7721 cell lines, other liver cancer cell lines should be used for further study.

In summary, our study carried out both cell and animal experiments to explore the effects of G. lucidum triterpenoids on HCC. And it was concluded that G. lucidum triterpenoids had significant inhibitory effects on the growth of tumor cells, and the potentials to block cell metastasis without marked toxic effects on solid organs.

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Disclosure of conflict of interest

None.

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