

Gambogic acid regulates the migration and invasion of colorectal cancer via microRNA-21-mediated activation of phosphatase and tensin homolog

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Received January 14, 2018; Accepted June 22, 2018

DOI: 10.3892/etm.2018.6421

Abstract. Gambogic acid (GA) has been reported to inhibit cancer cell proliferation and migration and enhance apoptosis. Several signaling pathways were identified to be involved in GA function, including PI3K/Akt, caspase-3 apoptosis and TNF- α /NF- κ B. However, to the best of our knowledge, the association between miRNA and GA has not been explored. The present study initially demonstrated that GA could inhibit HT-29 cancer cell proliferation using an MTT assay. In addition, a Transwell assay and a wound-healing assay respectively indicated that GA inhibited HT-29 cancer cell invasion and migration, which was also confirmed by the increased MMP-9 protein expression. Furthermore, GA induced the apoptosis of HT-29 cancer cells in an Annexin V and PI double staining assay. Moreover, treatment with GA significantly decreased miR-21 expression in these cells. Additionally, western blot analysis demonstrated that GA treatment enhanced the activation of phosphatase and tensin homolog (PTEN) along with the suppression of PI3K and p-Akt. Furthermore, miR-21 mimics reversed all the aforementioned activities of GA, which indicated that miR-21 was

the effector of GA and blocked PI3K/Akt signaling pathway via enhancing PTEN activity. In summary, GA induced HT-29 cancer cell apoptosis via decreasing miR-21 expression and blocking PI3K/Akt, which may be a useful novel insight for future CRC treatment.

Introduction

Colorectal cancer (CRC) is one of the most common types of cancer worldwide, which is comparable to lung, liver and stomach cancers (1). Each year, there are ~2 million newly diagnosed cases in China, thereby making CRC the third most common cancer and the fourth leading cause of cancer-associated death (2). In recent decades, although improvements have been made in health care and screening programs (3), the number of new cases of CRC and associated deaths is increasing. The high incidence of CRC suggests that it is urgent to investigate the mechanism of CRC in order to improve the current status of CRC therapy (4).

Patients with CRC are classified into four risk groups based on their tumor metastases, progression and biomarkers (5). Briefly, group 0 patients have no metastasis or lack signs of a poor prognosis. Group 1 patients exhibit borderline resectable metastasis. Group 2 patients are detected with disseminated and unresectable CRC. Patients with unresectable CRC and a lack of intensive or sequential treatment are classified as into group 3. The metastasis level and TNM stage guide the therapeutic decision and the treatment purpose along with the modified treatment strategy (6). Generally, the first-line chemotherapy approach is a cytotoxic agent alone or in combination with a biological targeted drug, as cytotoxic agents have been shown to increase the response rate of CRC and biological targeted drugs to reduce the toxicity of the treatment (7). The most commonly used agents are fluorouracil (5-FU) alone or combined with leucovorin (LV) (8). Due to the limitations in

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Key words: microRNA-21, gambogic acid, colorectal cancer, PTEN, apoptosis

the understanding of CRC biology, chemotherapy has been challenged with chemo-resistance.

Recently, a series of small non-coding RNAs, which are called microRNAs, has come to the attention of researchers. MicroRNAs are short RNAs with about 18-24 nucleotides, which regulate translation and the stability of the target mRNA (9). Decades ago, microRNAs were first discovered in chronic lymphocytic leukemia and showed the antitumor activity (10). Since then, microRNAs have been investigated and found to be involved in the initiation, development and progression of numerous cancer types, as a tumor suppressor or oncogene (11). Extensive research is now aimed at determining if microRNAs can be used as diagnostic biomarkers and therapeutic targets for cancer. Genome-wide profiling demonstrated that miRNA expression in both CRC cell lines and CRC tumors was regulated by methylation, which might lead to a reduced expression of miRNAs in CRC, including let-7, miR-34, miR-342, miR-345, miR-9 and miR-129 (12,13). In addition, it is well known that miR-21 is commonly upregulated in a variety of cancers, including lung, gastric, breast, colorectal, esophageal, pancreas and hepatocellular carcinoma (14-22). As the biological function of miR-21 has been well studied, it has been reported as a robust and reproducible prognostic marker of CRC (17,18). It has been reported that the potential mechanisms of miR-21 as oncogenic microRNA include downregulation of phosphatase and tensin homolog (PTEN), a decrease in Bax/Bcl-2/caspase-3 activity, repression of PDCD4 and downregulation of TIMP3 (23-25). Additionally, in combination therapy, miR-21 inhibitors enhanced the chemo-response to 5-FU (26).

Gambogic acid (GA) is a naturally occurring molecule, which is commonly extracted from *Garcinia hanburyi* trees (27). It has been reported that GA showed numerous activities involving cell cycle arrest, programmed cell death, autophagy, anti-proliferation, antioxidant, anti-metastatic and anti-inflammation (28). Currently, the molecular targets of GA have been well studied and numerous molecular pathways have been reported to be involved in the actions of GA, including PI3K/Akt, caspase-3 apoptosis, ATR-Chk1, TNF- α /NF- κ B and MET pathways (29-32). In addition, Huang *et al* (33) has reported that GA induced HT-29 cell apoptosis through the mitochondrial pathway. However, there are few reports regarding miRNA and GA, regardless of the mechanism of GA anti-CRC. The present study demonstrated that GA downregulated miR-21 in CRC and thus increased PTEN expression causing migration inhibition and resulted in CRC cell apoptosis. Taken together, these findings might provide strong evidence of GA antitumor activity and a new insight for future CRC investigation and treatment.

Materials and methods

Cell culture. Human CRC HT-29, SW480 and HCT116 cell lines were obtained from Stem Cell Bank of Chinese Academy of Sciences (Shanghai, China) and were cultured in complete RPMI-1640 medium (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) with 10% fetal bovine serum (Thermo Fisher Scientific, Inc., Waltham, MA, USA), penicillin (100 U/ml) and streptomycin (100 μ g/ml; both Sigma-Aldrich; Merck KGaA). Cells were maintained at 37°C with 5% CO₂

in a humidified cell culture incubator (Sanyo, Tokyo, Japan). For all experiments, five biological groups were designed according to the concentrations of GA (0, 0.33, 1, 3.3 and 10 μ M), which was purchased from Sigma-Aldrich; Merck KGaA. For each indicated time points (24, 48, and 72 h), cells were sampled for the following assay.

MTT assay of cell viability. An MTT assay kit (Beyotime Institute of Biotechnology, Shanghai, China) was used to measure cell growth for indicated time points following the protocol of manufacturer. Briefly, HT-29 cells at the logarithmic growth phase were seeded into 96-well plate at 5×10^3 /well. 200 μ l GA with dose escalation (0, 0.33, 1, 3.3 and 10 μ M) was added into each well in triplicate when cells were totally adhered at 24 h. Cells were cultured at 37°C (5% CO₂) and sampled at 24, 48 and 72 h. MTT (5 mg/ml, Sigma-Aldrich; Merck KGaA) solution was added (20 μ l/well) at each sampling time point and cells were further incubated for additional 4 h in a cell incubator. At the end of incubation, the supernatants were removed with a pipette. Before reading under the microplate reader, 150 μ l DMSO (Sigma-Aldrich; Merck KGaA) was added to each well. The proliferation rate was calculated with the absorbance value (OD) at a wavelength of 480 nm. The cell viability percentage was measured with the following formula: [(drug treated group/control group) x100]. Each assay was performed triplicate and the results are presented as the mean \pm SD.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. After incubation with GA (0, 1 or 3.3 μ M) for 24, 48 or 72 h, CRC cells were collected and total RNA was isolated from these cells using a PicoPureTM RNA Isolation kit (Arcturus, Sunnyvale, CA, USA) according to the manufacturer's instructions. Subsequently, 1 μ g isolated RNA was transcribed into cDNA using SuperScript III RNase H Reverse Transcriptase (Thermo Fisher Scientific, Inc.). Following the reverse transcription reactions, amplification was performed using a ViiTM 7 system (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. Transcript quantities were compared by relative Ct numbers, and the miR-21 expression level was normalized to an endogenous reference gene, GAPDH, and then the relative miR-21 mRNA levels to control sample was calculated using the 2^{- $\Delta\Delta$ Ct} method. Both primers for miR-21 and GAPDH were purchased from Thermo Fisher Scientific, Inc.

Flow cytometric analysis of cell apoptosis. Apoptotic cells were analyzed according to the previously described method (34). Briefly, HT-29 cells were exposed to 3.3 μ M GA, GA combined with miR-21 mimics or vehicle for 72 h. Subsequently, these three groups of HT-29 cells were harvested and resuspended in PBS. Apoptotic cells were identified with dual-staining of Annexin V-FITC and propidium iodide (PI; Thermo Fisher Scientific, Inc.). For each group, the experiments were performed in triplicate.

Wound healing assay of cell migration. For the wound healing assay, the procedure was performed according to the previous reports by Ni *et al* (35). HT-29 cells were seeded into 6-well plates (6×10^4 cells/well) and treated with GA, GA combined

with miR-21 mimics or a vehicle. When the cells grew to 90% confluence, vertical scratches were induced down through the monolayer cell surfaces using 1,000 μ l pipette tips. The media and cell debris were carefully aspirated before further culture with serum-free RPMI-1640 medium at 37°C. At 24 h, images of each sample were captured under a microscope at magnification, x100 (Leica DM500; Leica Buffalo Grove, IL, USA). The images were used to analyze the distance of one side of the wound to the other side using a scale bar.

Luciferase activity assay. MiR-21 mimics, scramble, wild-type and mutant 3'UTR PTEN vectors were provided by Ambion (Thermo Fisher Scientific, Inc.). Vectors carried the PTEN sequence, which contained the predicted miR-21 binding sites with wild-type or mutant 3'UTR. 5×10^3 HT-29 cells were plated into 24-well plates, which were then transfected with PTEN wild-type or mutant vector for 4 h using Lipofectamine® 2000 transfection reagent (Thermo Fisher Scientific, Inc.). Subsequently, the cells were treated with 100 nM miR-21 mimics or scramble miRNA. 48 h later, the cells were lysed using 0.2% trypsin at 37°C and a dual luciferase assay kit was used to detect the luciferase activities (Promega Corporation, Madison, WI, USA).

Western blot analysis. Following treatment with GA, GA combined with miR-21 mimics or vehicle for 72 h, HT-29 cells were collected and lysed in RIPA buffer (Sigma-Aldrich; Merck KGaA). The total protein concentration was quantified using a BCA Protein Assay kit (Beyotime Institute of Biotechnology), and 20 μ g protein from each group was separated with 10% Tris-SDS gel. After the electrophoresis, the gel was electro-transferred onto polyvinylidene fluoride membranes (Thermo Fisher Scientific, Inc.). For the subsequent blocking, washing and antibody incubation at room temperature, an iBind kit (Thermo Fisher Scientific, Inc.) was used according to the manufacturers' instructions. The following primary antibodies were used: anti-PTEN (1:250), anti-PI3K (1:300), anti-p-Erk (1:300), anti-matrix metalloproteinase (MMP2; 1:300), anti-MMP9 (1:300; all Cell Signaling Technology, Inc., Danvers, MA, USA) and anti-GAPDH (1:1,000; Santa Cruz Biotechnology, Inc., CA, USA). Horseradish peroxidase-conjugated secondary antibodies (1:3,000; anti-rabbit or anti-goat IgG; Santa Cruz Biotechnology, Inc.) were used. The luminescent signal was detected by adding super sensitive reagent (Thermo Fisher Scientific, Inc.) and quantified using the Image Lab™ system from Bio-Rad Laboratories.

Transwell assay of cell invasion. The online protocol published by Justus *et al* (36) was used in order to validate the effect of GA on HT-29 invasion. Briefly, 100 μ l HT-29 cells (1×10^4 cells/well) mixed with 0.33 μ M GA, GA combined with miR-21 mimics or vehicle were added to the upper chamber of Transwell chambers (Corning Life Science, Tewksbury, MA, USA) and 100 μ l RPMI-1640 medium with 30% FBS was added to the lower chamber. Following incubation for 72 h at 37°C, cells on the upper surface of the microporous membrane were carefully removed with cotton swabs, whereas cells on the lower surface of the membrane were fixed and stained with crystal violet. The cells in five selected views were counted under a microscope (Leica DM500; Leica) at

magnification, x100. The average sum of the cells was used to calculate the invasion rate.

Statistical analysis. All experiments were performed in triplicate and all data are presented as the mean \pm SD. One-way ANOVA followed by Dunnett's test was used to determine the difference between groups with $P < 0.05$ being considered to indicate a statistically significant difference (* $P < 0.05$, ** $P < 0.01$).

Results

GA inhibited CRC cell proliferation and downregulated miR-21 expression. The viability of HT-29 cells was detected at indicated time points with dose escalation GA (Fig. 1A) from 0.33 to 10 μ M using the MTT assay. As shown in Fig. 1B, GA inhibited the growth of HT-29 cells in a time- and dose-dependent manner. GA at all doses significantly inhibited HT-29 cell proliferation compared with vehicle at 72 h ($P < 0.01$). miR-21 was mostly found to be overexpressed in CRC. In order to explore the effect of GA on miR-21 expression, the cells treated with 1 or 3.3 μ M GA for 24, 48 and 72 h were subject to qPCR. The results indicated that GA downregulated miR-21 RNA expression at both doses, even at 24 h (Fig. 1C; $P < 0.05$). Moreover, we used the other two CRC cell lines, SW480 and HCT116, to investigate the involvement of miR-21 on the effects of GA. The results were consistent with the data obtained using HT-29 cells (Fig. 1D-F). All these results indicated that GA exerted anti-proliferation effects on HT-29 cancer cells through downregulation of miR-21 expression.

GA induced apoptosis of HT-29 cancer cells. To further investigate how GA inhibited HT-29 cancer cell growth and whether miR-21 was the effector of GA, apoptotic cells were detected with FACS by double staining with Annexin V and PI. In addition to the vehicle and GA (3.3 μ M) treatment groups, the GA with miR-21 mimics group was added to determine whether miR-21 mimics could interfere with the antitumor effect of GA. The results in Fig. 2A and B demonstrated that the majority of HT-29 cells were subject to apoptosis following GA treatment compared with the control group ($P < 0.01$). Moreover, when miR-21 mimics were added to the GA 3.3 μ M group, the apoptotic rate of the HT-29 cells was reduced to a lower level, which was similar to that of the control group and was significantly less than that of the GA 3.3 μ M group (Fig. 2B; $P < 0.01$). These results indicated that GA inhibited HT-29 cancer cell growth by inducing cell apoptosis via downregulation of miR-21 expression.

GA inhibited HT-29 cancer cell migration. In order to validate the effect of GA on HT-29 cell migration, a wound healing assay was performed. The results in Fig. 3A indicated that cell migration was significantly inhibited by 0.33 μ M GA compared with control group. Additionally, in the miR-21 mimics treated group, the wound was similar to that in the control group, which further confirmed that the anti-migration effect of GA was exerted through miR-21. Quantification of the migration activity revealed significant differences among the control, GA and GA with miR-21 groups (Fig. 3B, $P < 0.01$, $P < 0.05$). Thus,

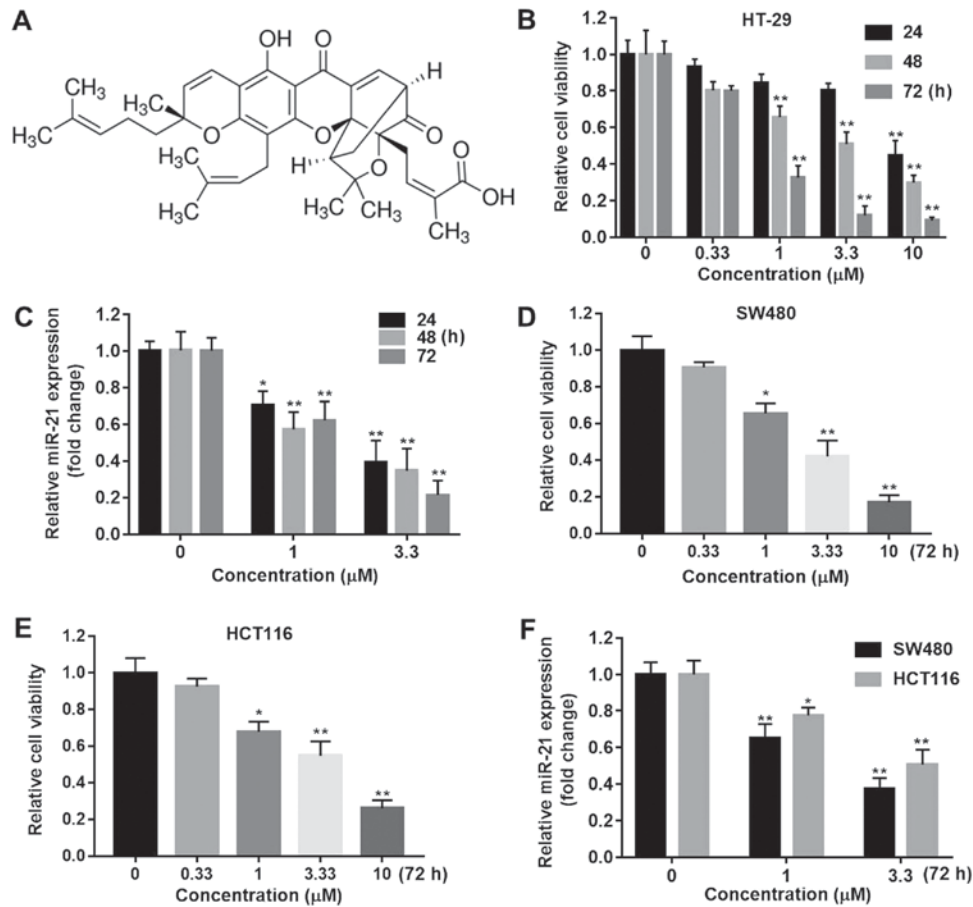


Figure 1. Gambogic acid decreased CRC viability and miR-21 expression. (A) The structure of GA. (B) Cell viability was determined using an MTT assay in HT-29 cells treated with GA (0, 0.33, 1, 3.3 or 10 μM) for 24, 48 or 72 h (** $P < 0.01$ compared with the 0 μM GA groups). (C) Expression of miR-21 in HT-29 cells treated with GA (0, 1 or 3.3 μM) at the indicated time points was determined by qPCR. The miR-21 expression level was normalizing to the GAPDH level (* $P < 0.05$, ** $P < 0.01$ compared with the 0 μM GA groups). Cell viability was determined using an MTT assay in (D) SW480 and (E) HCT116 cells treated with GA (0, 0.33, 1, 3.3 or 10 μM) for 72 h (* $P < 0.05$, ** $P < 0.01$ compared with the 0 μM GA group). (F) Expression of miR-21 in SW480 and HCT116 cells treated with GA (0, 1 or 3.3 μM) at 72 h was determined by RT-qPCR (* $P < 0.05$, ** $P < 0.01$ compared with the 0 μM GA group). CRC, colorectal cancer; GA, gambogic acid.

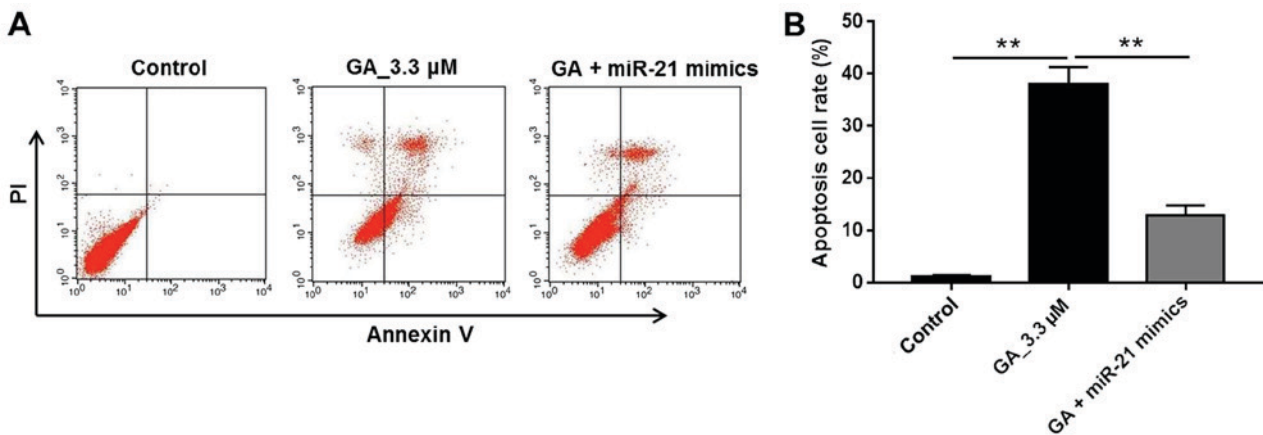


Figure 2. GA induced cell apoptosis by Annexin V/PI dual staining assay. (A) HT-29 cells were exposed to 3.3 μM GA or GA combined with miR-21 mimics for 72 h. Apoptotic cells were indicated with Annexin V and PI double staining. (B) The apoptosis cell rates were calculated (** $P < 0.01$ as indicated). GA, gambogic acid; PI, propidium iodide.

this result suggested that GA inhibited HT-29 cell migration via blocking miR-21 activity.

GA enhanced PTEN expression and blocked the PI3K/Akt pathway. In the present study, miR-21 gene expression

was inhibited by GA (Fig. 1B) and, as Zhang *et al* (15) had previously reported that miR-21 was an onco-miRNA that downregulated the tumor suppressor gene, PTEN in gastric cancer, we needed to determine whether GA could affect PTEN expression in CRC. From the luciferase assay, we

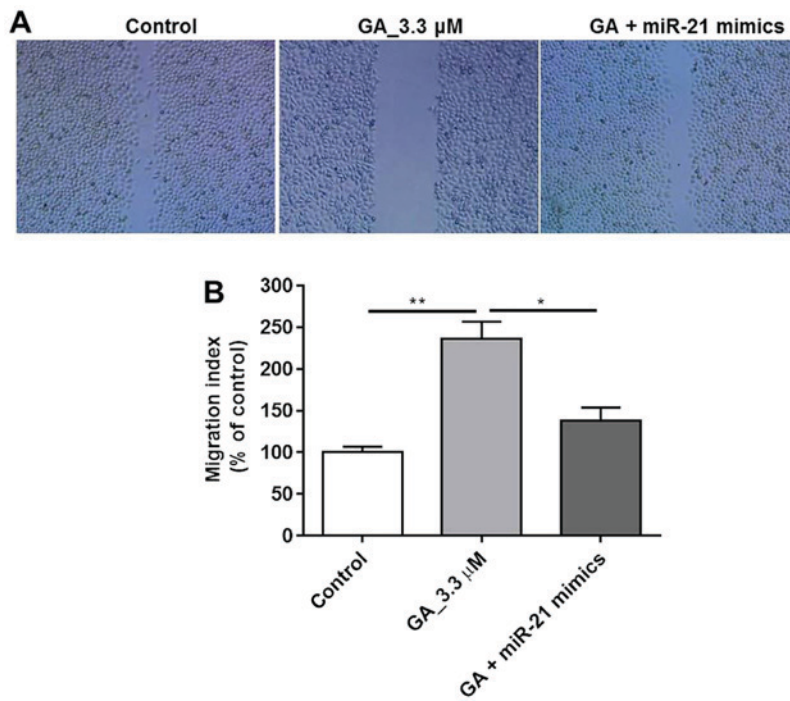


Figure 3. GA inhibited CRC migration. (A) Images of wound at the magnification, x100 in the HT-29 cell monolayers treated with vehicle, 0.33 μM of GA or GA combined with miR-21 mimics for 24 h. (B) The migration index indicated as a percentage of the control group (**P<0.05, ***P<0.01 as indicated). GA, gambogic acid; CRC, colorectal cancer.

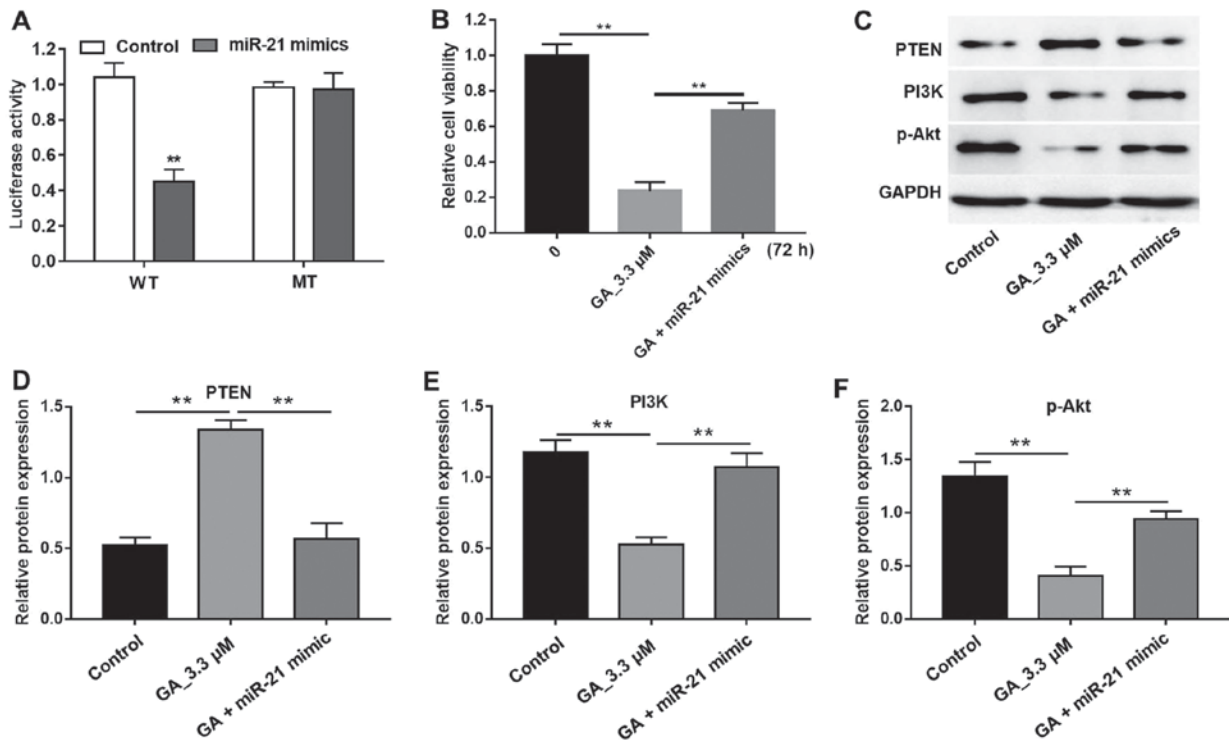


Figure 4. GA downregulated PI3K and p-Akt, and enhanced PTEN protein expression. (A) HT-29 cells were transfected with PTEN wild-type or mutant vector for 4 h. Later on, the cells were treated with 100 nM miR-21 mimics or scramble. (B) Cell viability was determined using an MTT assay in HT-29 cells treated with 3.3 μM GA or GA plus miR-21 mimics for 72 h (**P<0.01 as indicated). (C) PTEN, PI3K and p-Akt protein expression was detected by western blot analysis in HT-29 cells treated with vehicle, 3.3 μM GA or GA combined with miR-21 mimics for 72 h. Quantification of the (D) PTEN, (E) PI3K and (F) p-AKT protein expression level was determined by normalizing to the internal reference protein, GAPDH (**P<0.01 as indicated). GA, gambogic acid; CRC, colorectal cancer; PTEN, phosphatase and tensin homolog.

found a statistically significant decrease in firefly luciferase activity in the cells transfected with miR-21 mimics and

wild-type 3'UTR PTEN vectors (Fig. 4A). In addition, GA-induced cell growth inhibition was reversed by miR-21

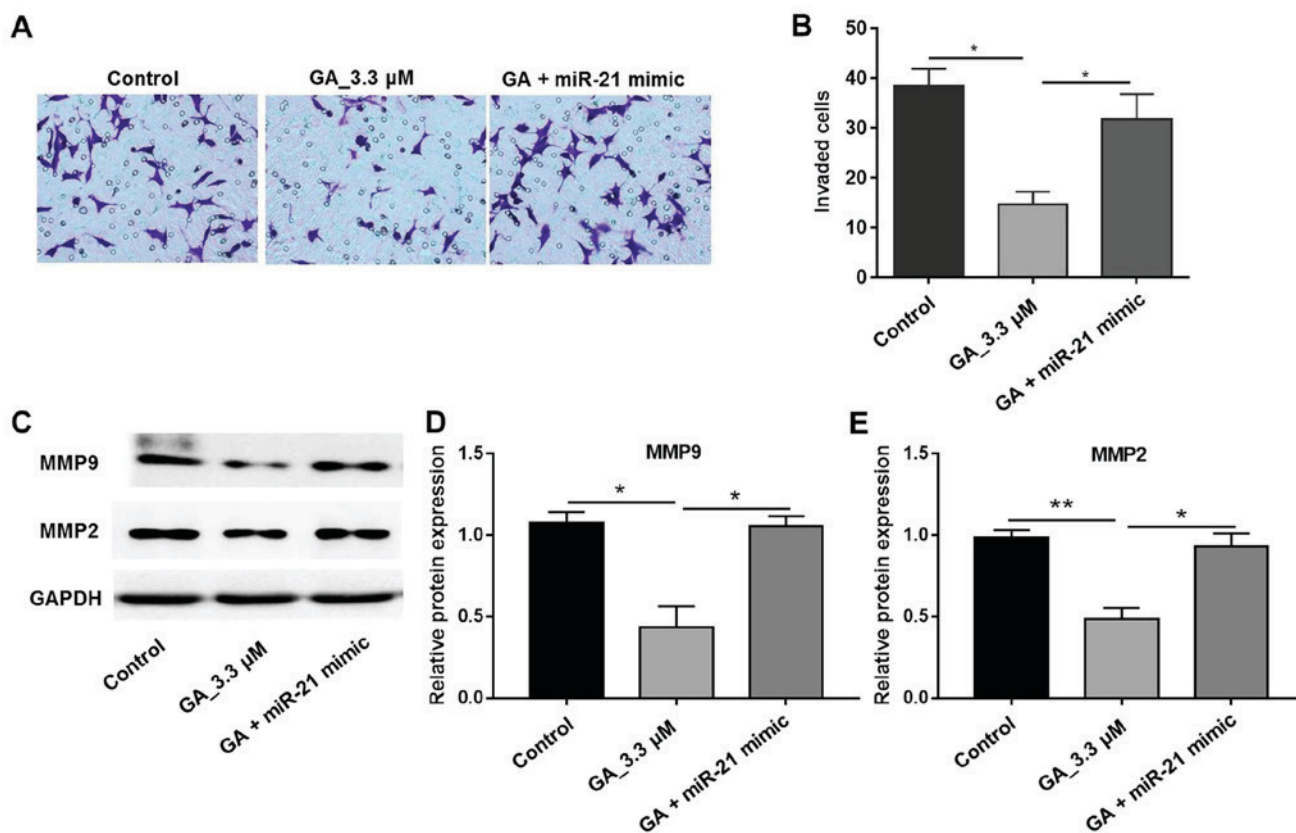


Figure 5. GA inhibited CRC invasion and the expression of MMP-9. (A) Images of the HT-29 cells stained with crystal violet in a Transwell invasion assay (magnification, x100). (B) Quantification of invasive cells ($P < 0.05$ as indicated). (C) MMP2 and MMP9 expression was detected by western blot analysis. Quantification of (D) MMP2 and (E) MMP9 protein levels via normalizing to the endogenous protein, GAPDH ($P < 0.05$ and $**P < 0.01$ as indicated). MMP, anti-matrix metalloproteinase; GA, gambogic acid; CRC, colorectal cancer.

mimics (Fig. 4B). The western blot results further confirmed that GA significantly increased the PTEN protein level and miR-21 mimics suppressed the enhancement induced by GA (Fig. 4C and D). Moreover, PTEN is a suppressor of the PI3K/Akt pathway and therefore, the enhanced expression of PTEN induced by GA would have definitely blocked the PI3K/Akt signaling pathway. The western blot results shown in Fig. 4C, E and F indicated that GA significantly decreased PI3K and p-Akt protein expression ($P < 0.01$). All these findings demonstrated that GA showed antitumor activity through targeting miR-21 and, in turn, blocking of the PI3K/Akt signaling pathway.

GA inhibited the invasion of HT-29 cells. A Transwell assay was used in order to further investigate the biological function of GA on HT-29 cell invasion. The invasion of HT-29 cells treated with $0.33 \mu\text{M}$ GA was markedly less than that in the control group under the microscope view (Fig. 5A). The invaded cell number was quantified and is shown in Fig. 5B, which reveals a significantly smaller number of invading cells compared with the vehicle group. Moreover, in the GA and miR-21 mimics treated group, the cell number was comparable with that in the control group (Fig. 5B). As MMP2 and MMP-9 are closely associated with cell migration and invasion, the expression of these factors was also detected. The results revealed that MMP-2 and MMP-9 expression was significantly decreased following treatment with GA (Fig. 5C-E). These results indicated that GA inhibited the activity of MMP2 and

MMP-9 and thus showed its anti-invasion activity in HT-29 cancer cells.

Discussion

CRC has become a global public health problem with its high incidence and mortality rate worldwide (4). One review published recently has summarized discoveries and newest findings in CRC diagnosis and treatment (5). Based on genetic screening tests, most of the major cancer genes involved in CRC have been well characterized, such as APC, KRAS, PI3K and TP53 (37-39). Epigenetic instability, mainly with respect to CpG island methylation, was also a common feature in CRC (38). Non-coding RNAs (ncRNA) are RNA molecules without an open reading frame and are not translated into proteins. This results in the deregulation of miRNAs associated with CRC. Previously, researchers have reported that miRNAs function as potential diagnostic and prognostic biomarkers in CRC (9). For instance, 12 miRNAs showed higher expression levels in CRC patient samples than in those from healthy controls, such as miR-7, -17, -20a, -21, -92a, -96, -106a, -134, -183, -196a, -199a-3p and -214. Meanwhile, 8 miRNAs were shown to be downregulated, including miR-9, -29b, -127-5p, -138, -143, -146a, -222 and -938 (40). At present, an extensive amount of research regarding CRC is focused on the development of new less aggressive and more effective therapies than conventional ones.

It has been shown that GA inhibited the growth of a wide variety of cancers, including hepatocarcinoma, gastric

carcinoma, lung cancer and breast cancer (41-45). The mechanisms of GA antitumor activity have been well studied. However, few studies about the effect of GA on miRNA expression have been reported. In this article, we not only validated the antitumor effect of GA via MTT assay for proliferation, wound healing assay for migration, Transwell assay for invasion and FACS for cell apoptosis, but we also first demonstrated that miR-21 was down regulated by GA in CRC. In addition, miR-21 expression was suppressed by GA in HT-29 cells, as determined by RT-qPCR. Based on this knowledge, we proposed that miR-21 might be an effector of GA in HT-29 cells. For the potential mechanisms of miR-21 as an onco-miRNA, several reports have been published. Meng *et al* (46) has found that PTEN was regulated by miR-21 in hepatocellular carcinoma. Similarly, PTEN was repressed by miR-21 in non-small cell lung cancer (47).

PTEN was revealed to be an essential tumor suppressor and modulator of cell growth and proliferation (48). PTEN was reported to be under the control of various transcription factors involving EGR-1, p53, ATF2 and PPAR γ (48). miRNA-guided degradation would control the process of PTEN mRNA splicing, which is translocated to the cytosol (48). The prominent miRNAs of PTEN-targeting included miR-19, miR-21 and miR-221 (46). Based on these, we investigated the protein expression level of PTEN, PI3K and p-Akt in GA-treated HT-29 cells. As expected, PTEN was increased by GA, while PI3K and p-AKT proteins were decreased in HT-29 cells.

Taken together, the anti-proliferation effect of GA on HT-29 cancer cells may be mediated via decreasing miR-21 expression and blocking the PI3K/Akt pathway. Therefore, our article might open up a new pathway toward CRC pharmacological intervention.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

GG, YB, HQ, MY, JH and LL collaborated in the experiment design, sample collection and experiments execution. GG, LY and BL analyzed and interpreted the patient data and were the major contributors in developing the first draft of the manuscript. BL reviewed and edited the manuscript. XQ analyzed and interpreted the data and reviewed and approved the final draft of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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