

Gamma linolenic acid suppresses hypoxia-induced gastric cancer cell growth and epithelial-mesenchymal transition by inhibiting the Wnt/ β -catenin signaling pathway

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Abstract

Introduction. Gastric cancer is one of the most common malignancies in China and the fifth most common cancer in the world. Gamma linolenic acid (GLA) was reported to have anti-inflammatory and anti-cancer effects. The purpose of this research was to investigate the effect and mechanism of GLA on gastric cancer cell growth under hypoxic conditions.

Material and methods. The hypoxia models of SGC-7901 and MGC-803 cells were established, and then were exposed to different concentrations of 50, 100 or 200 μ M GLA. MTT assay, colony formation assay, wound healing assay and transwell assay were used to investigate the effects of GLA treatment on gastric cancer cell growth under hypoxia (1% O₂). The expression of apoptosis- and epithelial-mesenchymal transition (EMT)-related proteins was detected by qPCR and western blot.

Results. GLA treatment significantly decreased viability and inhibited colony formation ($p < 0.05$, $p < 0.01$) of SGC-7901 and MGC-803 cells under hypoxia. Western blotting analysis showed that GLA treatment decreased the expression of proliferating cell nuclear antigen (PCNA), microchromosome maintenance complex component 2 (MCM-2) and anti-apoptotic protein Bcl-2, while increased the expression of pro-apoptotic proteins (Bax and Cleaved Caspase-3) ($p < 0.05$ and $p < 0.01$). In addition, Wound healing analysis and Transwell assays showed that GLA treatment inhibited the migration and invasion of SGC-7901 and MGC-803 cells in a dose-dependent manner ($p < 0.01$). Western blotting analysis showed that GLA treatment increased the expression of epithelial marker proteins (γ -catenin and E-cadherin), while decreased the expression of stromal and extracellular matrix marker proteins (fibronectin, Snail and β -catenin) ($p < 0.01$). Further analyses showed that GLA treatment decreased the expression of β -catenin in Wnt/ β -catenin pathway ($p < 0.01$). Moreover, exogenous Wnt3a reversed the inhibitory effect of GLA on β -catenin expression, and further reversed the inhibitory effect of GLA on gastric cancer cell growth and EMT markers ($p < 0.05$, $p < 0.01$).

Conclusion. These findings suggest that GLA should be tested in animal models and in clinical studies as a potentially effective bioactive phytochemical substance for the treatment of gastric cancer. (*Folia Histochemica et Cytobiologica* 2020, Vol. 58, No. 2, 117–126)

Key words: Gamma linolenic acid; gastric cancer; SGC-7901 cells; MGC-803 cells; hypoxia; cell growth; Wnt/ β -catenin; EMT; apoptosis

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Introduction

Gastric cancer is one of the most common malignancies in China and the fifth most common cancer in the world [1, 2]. Although great progress has been achieved in the survival rate after surgery, the recurrence and metastasis rates are still high [3]. Chemotherapy plays an important role in the treatment of gastric cancer. However, patients with distant metastasis or recurrent advanced gastric cancer are still incurable, and the median overall survival time of patients with metastatic disease is still not ideal [4]. In addition, the sensitivity of conventional radiotherapy is poor, which makes the 5-year survival rate of gastric cancer patients still less than 30% [5, 6]. In general, since current therapeutic methods are not sufficient for the treatment of patients with gastric cancer [7] many drugs are currently used as complementary strategies for the treatment of this malignancy [8–11]. For example, it has been reported that, arginine, glutamine, and omega-3 PUFA significantly improved the three-month survival rate of patients with gastric cancer [12].

In particular, gamma linolenic acid (GLA) is an omega-6 polyunsaturated fatty acid (PUFA) that is present in plant seed oils, such as *Borago officinalis* [13], *Oenothera biennis* and *Ribes nigrum* [14]. As early as in 1980, PUFA has been shown to inhibit various cancer cells without affecting normal cells [15–17]. In the past few decades, extensive studies have confirmed the potential anticancer effect of these fatty acids [18–21]. For examples, *in vivo* experiments have shown that GLA exhibits a dose-dependent regulatory effect on an experimental study on hepatocellular carcinoma [22]. Further studies of underlying molecular mechanisms indicated that GLA may exert a chemical protective effect on DEN-induced liver cancer by altering hypoxic microenvironment, mitochondria-mediated death, apoptosis, and anti-inflammatory pathways [22]. In addition, Chas *et al.* used gas chromatography to establish a biochemical profile fatty acids (FA) in the adipose tissue and found that inflammatory breast cancer was associated with decreased levels of eicosapentaenoic acid (EPA) and GLA in breast cancer adipose tissue [23]. However, the role of GLA in the progression of gastric cancer remains unknown.

In this study, we investigated the effects of GLA on the viability, colony formation, migration and invasion *in vitro* in SGC-7901 and MGC-803 cells under hypoxia, and then comprehensively analyzed the epithelial-mesenchymal transition (EMT). The underlying mechanisms were also investigated. The results of our study suggest that GLA may be a potentially attractive bioactive phytochemical for the treatment of gastric cancer.

Materials and methods

Cell culture and treatment. The gastric epithelial cell line GES-1, human gastric cancer cell lines SGC-7901, and MGC-803 were purchased from Cancer Institute of Chinese Academy of Medical Sciences (Beijing, China). SGC-7901 and MGC-803 cells were seeded in RPMI 1640 (GIBCO) supplemented with 10% fetal bovine serum (FBS) (GIBCO, Grand Island, CA, USA), 100 $\mu\text{g}/\text{mL}$ streptomycin and 100 $\mu\text{g}/\text{mL}$ penicillin (Hyclone, Los Angeles, CA, USA), and cultured at 37°C and 5% CO_2 in a humid sterile incubator. Cells were seeded at a density of 1×10^6 cells into each 100 mm petri dish and cultured until the cells grew to approximately 90% confluence. SGC-7901 and MGC-803 cells hypoxia models were established as previously described [24]. Briefly, SGC-7901 and MGC-803 cells were exposed to 1% O_2 , 5% CO_2 , and 94% N_2 humidified atmosphere at 37°C under hypoxic conditions, respectively. SGC-7901 and MGC-803 cells were then treated with various concentrations of GLA (50, 100 and 200 μM ; Sigma Aldrich, St Louis, MO, USA) under hypoxia.

Cell viability assay. The effect of GLA on the viability of GES-1, SGC-7901 and MGC-803 cells was detected by MTT assay. In brief, GLA was dissolved in RPMI 1640 medium at a concentration of 1 M as a stock solution, which was then mixed with fresh medium to achieve the desired final concentrations (50, 100, 200 μM). Cells were seeded in a 96-well plate at 5×10^3 cells per well under hypoxic conditions. Subsequently, the cells were exposed to different doses of GLA (50, 100 or 200 μM) or 200 ng/mL WNT3a (Abcam, cat.no. ab153563, Cambridge, MA, USA) [25]. The cells in blank control were treated with RPMI 1640 medium only. After incubation of the cells for 48 h, 20 μL of diluted (1 mg/ml) MTT (stock solution was made by dissolving MTT powder in 10 mg/mL of phosphate buffered saline) was added to the cells which were further incubated at 37°C for 4 h. Finally, the absorbance of each well at a wavelength of 570 nm was measured on a Microplate Reader (ELX800, BIO-TEK Instruments, Inc., Doraville, GA, USA). Each treatment was performed in triplicate.

Colony formation assay. GES-1, SGC-7901 and MGC-803 cells were used for colony formation analysis. Briefly, 5×10^5 cells were seeded in 60 mm culture dishes under hypoxia and treated with GLA (50, 100 or 200 μM) or/and Wnt3a (200 ng/ml dissolved immediately before use). After two weeks, cell colonies were stained with crystal violet.

Wound healing assay. Cell migratory ability was tested by wound healing assay. Under hypoxic conditions, 5×10^5 cells were seeded in each well of a 6-well plate and cultured to 90% confluence. Cells were exposed to different doses of GLA (50, 100 or 200 μM) or/and Wnt3a (200 ng/ml) and

cultured to monolayer cells at 37°C. Then the cells were scraped with the tip of a 1 mL sterile pipette. After 24 h, wound recovery was assessed under light microscopy.

Transwell assay. Cell invasive ability was detected by Transwell assay. Cells (3×10^4) were seeded into the transwell upper cavity covered with matrigel and RPMI 1640 medium was added to the lower chamber. Then, cells were treated with different doses of GLA (50, 100 or 200 μ M) or/and Wnt3a (200 ng/ml) under hypoxic conditions. After 48 h, the cells in the lower chamber were fixed with methanol and stained with crystal violet. Finally, cell colonies were counted by taking pictures under a light microscope.

Western blotting. The levels of the studied proteins were measured by western blotting. After the cells were lysed in lysis buffer, a whole cell lysate was obtained. Total protein (50–90 μ g) was separated on a 10% polyacrylamide gel (SDS-PAGE). Subsequently, the proteins in the gel were transferred to a nitrocellulose membrane and blocked with blocking buffer (1% Tween-20 and 1% BSA, Ph 7.6 in 20 mM TBS) at room temperature for 60 min, and then incubated with antibodies (all from Abcam, Cambridge Science Park, Cambridge, UK) against PCNA (1:1000; cat. no. ab92552), MCM-2 (1:200; cat. no. ab31159), Bcl-2 (1:1000; cat. no. ab185002), Bax (1:500; cat. no. ab53154), Cleaved Caspase-3 (1:500; cat. no. ab49822) or γ -catenin (1:1000; cat. no. ab184919), E-cadherin (1:500; cat. no. ab15148), fibronectin (1:1000; cat. no. ab32419), Snail (1:1000; cat. no. ab229701), β -catenin (1:5000; cat. no. ab32572), GAPDH (1:10000; cat. no. ab181602;) overnight at 4°C or for 3 h at room temperature. The membrane was washed 3 times with TBST (10 min each), and then incubated with anti-mouse or anti-rabbit secondary antibody (1: 1,000; cat. no. umbers 7076 and 14708; Cell Signaling Technology, Danvers, MA, USA) at 37°C for 60 min. The membrane was washed twice with TBST and once with TBS for 15 min each wash. The membrane was then incubated with alkaline phosphatase until an appropriate signal level was visible. The protein band was photographed by FluorChem® Imaging Systems (Alpha Innotech Corp., San Leandro, CA, USA). Quantification was performed spectrophotometrically using ImageJ (National Institutes of Health, Bethesda, MD, USA).

Quantitative real-time polymerase chain reaction (qRT-PCR). The mRNA levels of were measured by qRT-PCR. Total RNAs were isolated using Trizol reagents from cells, and then RNA (1 μ g) was reverse transcribed into cDNA (a template for qPCR) using QuantScript RT Kit (Tiangen, Beijing, China). qPCR was conducted by FastFire qPCR PreMix (SYBR Green) (Tiangen) with GAPDH as a standard reference. The expression was calculated using $2^{-\Delta\Delta CT}$ method. The primers were as follows:

γ -catenin, F, 5'-GCTAACGTAACGTGACTT-3'; R, 5'-ACAACCTGCTAGCTGAAACGT-3'
E-cadherin, F, 5'-CCATTCGACCATAGCTCTC-3'; R, 5'-GGCAAATTCGGGTACGCCT-3'
Fibronectin, F, 5'-CTAGGTACGTAACGTGACG-3'; R, 5'-GCAATCAACTTACGTTAC-3'
Snail, F, 5'-GGCCCTTTACAGTGCACC-3'; R, 5'-TTCGTCGACGCTGCACACTC-3'
 β -catenin, F, 5'-TTTCAACGGGTACCCTGTC-3'; R, 5'-GCTAACTCGAATGCAACGT-3'

Statistical analysis. All data were expressed as mean \pm SD from at least three independent experiments. Statistical analysis was carried out using SPSS software (version 15.0; IBM Corp., Armonk, NY, USA). ANOVA followed by *post hoc* was performed to evaluate the significance of differences in the mean values. *p* values < 0.05 were considered statistically significant.

Results

GLA inhibits the viability and colony formation and promotes apoptosis of gastric cancer cells under hypoxia

As shown in Figures 1A–C, GLA, at all 3 concentrations used, did not inhibit the viability, colony formation or apoptosis of GES-1 cells. Since hypoxia is the basic feature of tumor microenvironment, the SGC-7901 and MGC-803 cells were cultured under hypoxia to study the effects of GLA (50,100 or 200 μ M) on cell growth, viability and colony formation. The results showed that, compared with the control group, GLA treatment for 72 h significantly decreased cell viability and inhibited colony formation (Fig. 1D and 1E, $p < 0.05$, $p < 0.01$). Western blot analysis showed that GLA treatment decreased the expression levels of cell proliferation-related proteins (PCNA and MCM-2) and anti-apoptotic protein (Bcl-2), while increased the expression of anti-apoptotic proteins, Bax and cleaved Caspase-3, in a dose-dependent manner (Fig. 1F, $p < 0.05$, $p < 0.01$).

GLA inhibits the migration and invasion of gastric cancer cells under hypoxia

The effect of GLA on the invasion and migration of gastric cancer cells was detected by transwell assay and wound healing assay, respectively. As shown in Figure 2A, the wound closure area in SGC-7901 and MGC-803 cells cultured under hypoxia was increased in a dose-dependent manner after GLA treatment compared with the control group (Fig. 2A, $p < 0.01$). The transwell assay showed that the invasion of SGC-7901 and MGC-803 cells treated with GLA was de-

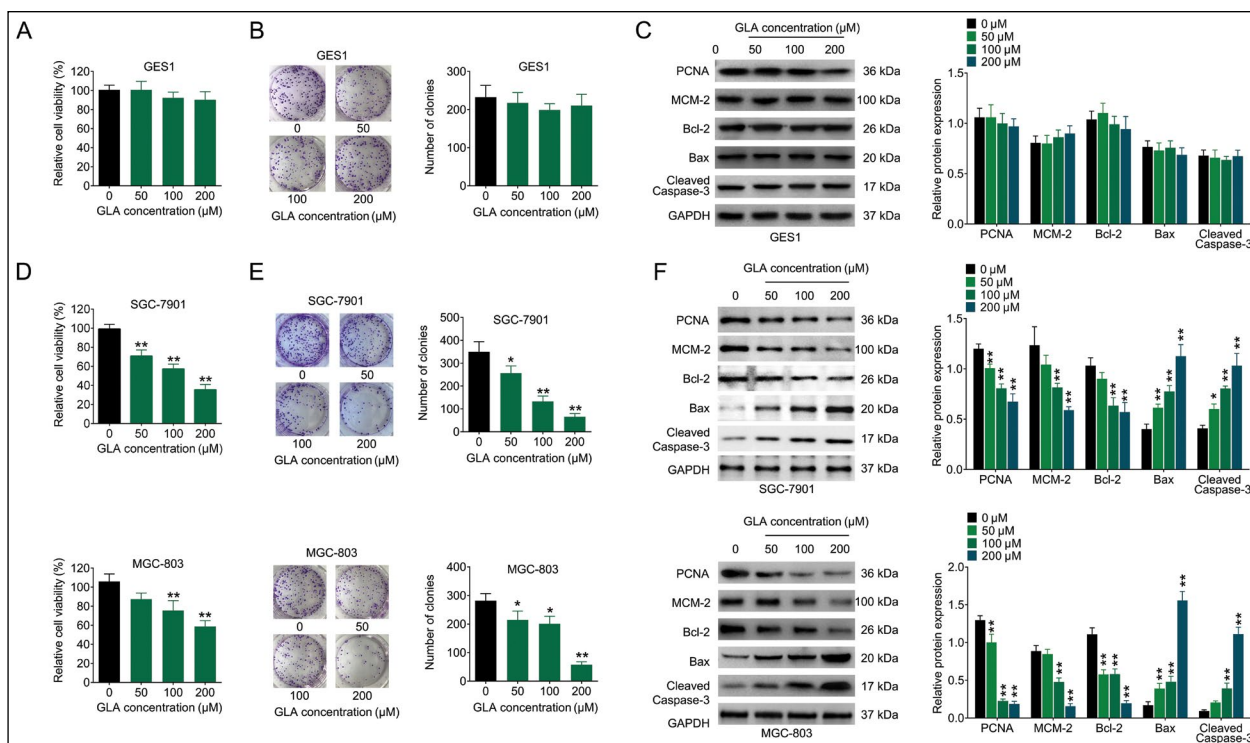


Figure 1. GLA inhibits the viability and colony formation and promotes apoptosis of gastric cancer cells under hypoxia. SGC-7901 and MGC-803 cells exposed to 1% O₂, 5% CO₂, and 94% N₂ humidified atmosphere at 37°C were treated with different doses of GLA (50, 100 and 200 μM), respectively. **A.** Viability of GES-1 cells was measured by MTT assay. **B.** The colony formation of GES-1 cells was detected by colony formation assay. **C.** The protein levels of PCNA, MCM-2, Bcl-2, Bax and Cleaved Caspase-3 in GES-1 cells were determined by Western blotting. **D.** Viability of SGC-7901 and MGC-803 cells was measured by MTT assay. **E.** The colony formation of SGC-7901 and MGC-803 cells was detected by colony formation assay. **F.** The protein levels of PCNA, MCM-2, Bcl-2, Bax and Cleaved Caspase-3 in SGC-7901 and MGC-803 cells were determined by Western blotting. n = 3, *p < 0.05, **p < 0.01 vs. control group (no GLA).

creased in a dose-dependent manner compared with the control group (Fig. 2B, $p < 0.01$).

GLA inhibits EMT and Wnt/β-catenin signaling pathways in gastric cancer cells

To reveal the effects of GLA on the invasion and migration of SGC-7901 and MGC-803 cells, cells were treated with different concentrations of GLA (50, 100 or 200 μM) under hypoxic conditions for 72 h. After GLA treatment, the protein levels of epithelial cell markers (γ-catenin and E-cadherin) increased significantly, while the protein levels of the mesenchymal cell markers (fibronectin and Snail) were decreased in SGC-7901 and MGC-803 cells. Besides, GLA treatment also reduced the protein level of β-catenin protein in a dose-dependent manner compared to the control group (Fig. 3, $p < 0.01$).

GLA inhibits hypoxia-induced EMT in gastric cancer cells by inhibiting Wnt/β-catenin signal pathway

To further explore the molecular mechanism, SGC-7901 cells were treated with GLA (200 μM)

and/or Wnt3a (200 ng/ml). As shown in Figure 4A, compared with cells that were treated only with GLA, Wnt3a significantly reversed the inhibitory effect of GLA on the protein levels of epithelial cells (γ-catenin, E-cadherin) and EMT markers (fibronectin and Snail) in SGC-7901 cells ($p < 0.05$, $p < 0.01$). Results from qRT-PCR analyses showed consistent results (Fig. 4B, $p < 0.05$, $p < 0.01$).

GLA inhibits the growth of gastric cancer cells under hypoxia through Wnt/β-catenin signal pathway

Finally, we analyzed the possible molecular mechanism underlying the regulatory role of GLA on the growth of the studied gastric cancer cells under hypoxia. As shown in Figure 5A, Wnt3a pre-treatment for 72 h significantly reversed the inhibitory effect of GLA on the viability of SGC-7901 cells ($p < 0.01$). Colony formation analysis showed that Wnt3a treatment also significantly reversed the inhibitory effect of GLA on the colony formation of SGC-7901 cells (Fig. 5B, $p < 0.01$). Wound healing assay and Transwell assay showed that compared with GLA treatment group,

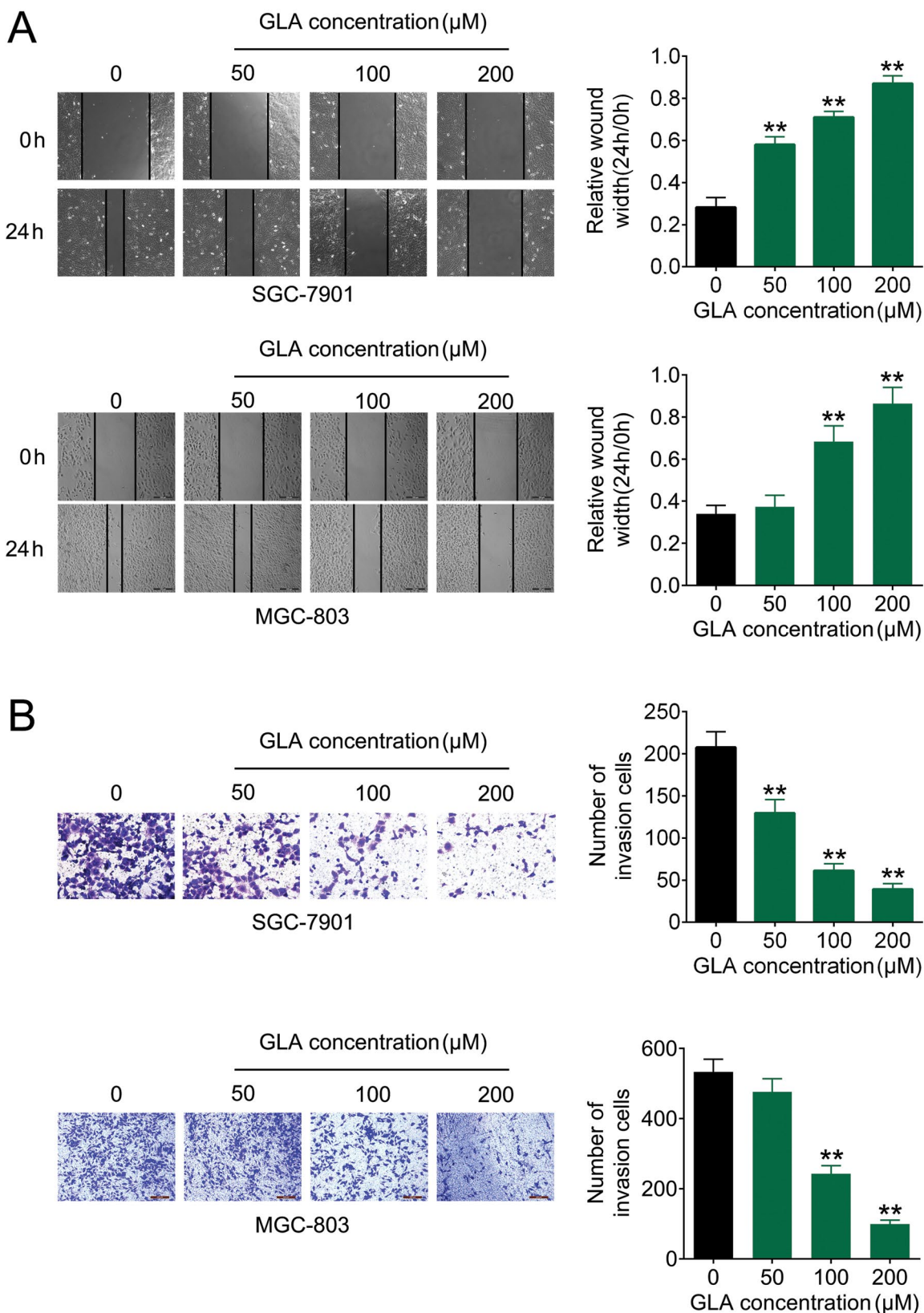


Figure 2. GLA inhibits the migration and invasion of gastric cancer cells under hypoxia. SGC-7901 and MGC-803 cells exposed to 1% O_2 , 5% CO_2 , and 94% N_2 humidified atmosphere at 37°C were treated with different doses of GLA (50, 100 or 200 μM). The migratory ability (A) and invasive ability (B) of SGC-7901 and MGC-803 cells were detected by wound-healing assays and transwell assay, respectively. $n = 3$, * $p < 0.05$, ** $p < 0.01$ vs. control group (no GLA).

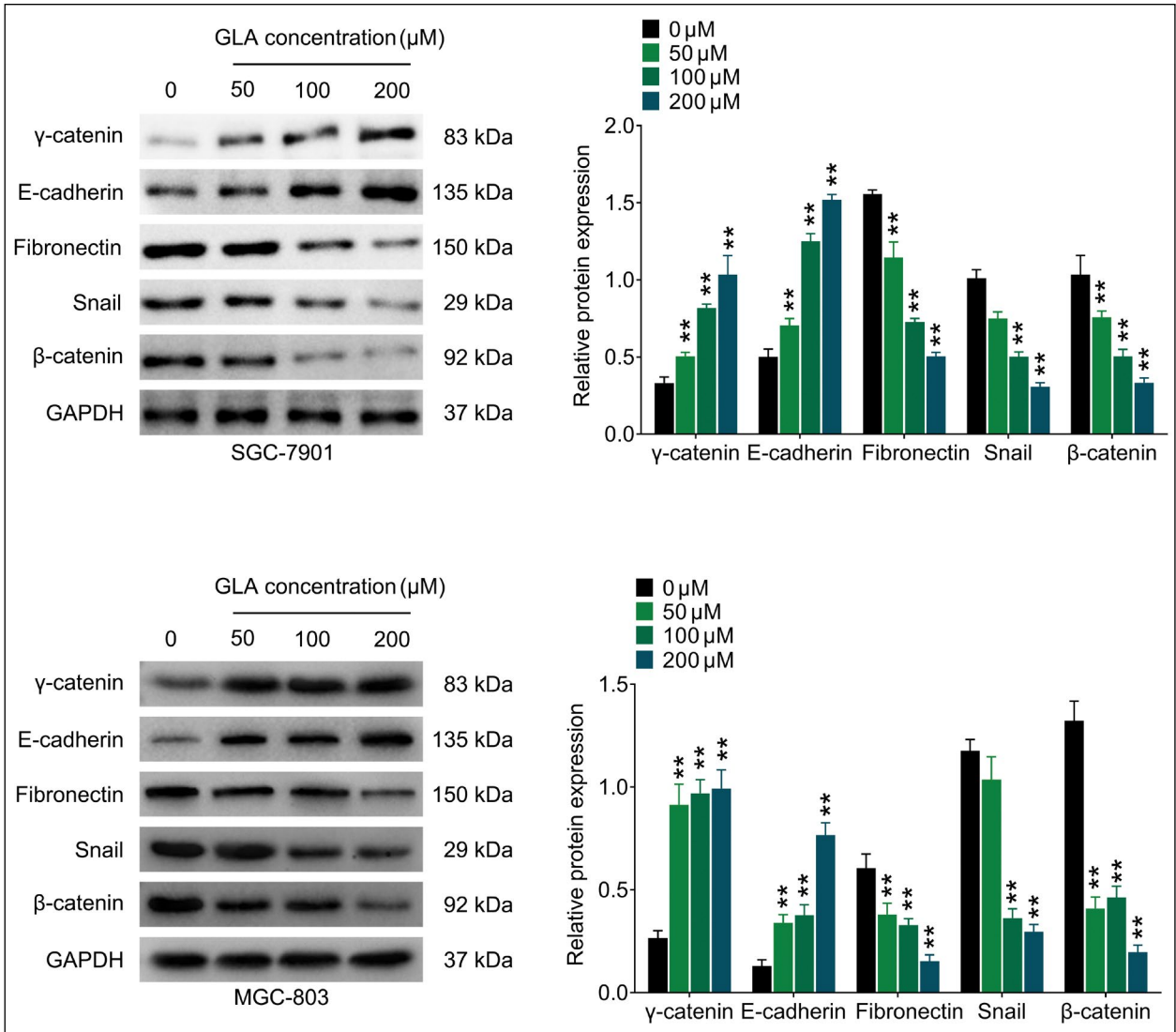


Figure 3. GLA inhibits EMT and Wnt/ β -catenin pathways in gastric cancer cells. SGC-7901 and MGC-803 cells exposed to 1% O₂, 5% CO₂, and 94% N₂ humidified atmosphere at 37°C were treated with different doses of GLA (50, 100 or 200 μM). The protein levels of γ -catenin, E-cadherin, fibronectin, Snail and β -catenin were determined by Western blotting in SGC-7901 and MGC-803 cells. n = 3, *p < 0.05, **p < 0.01 vs. control group (no GLA).

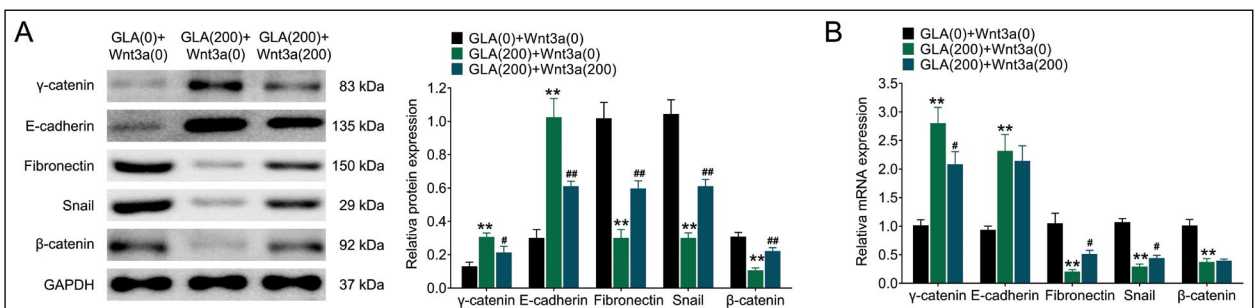


Figure 4. GLA inhibits hypoxia-induced EMT of gastric cancer cells by inhibiting Wnt/ β -catenin signal pathway. SGC-7901 and MGC-803 cells exposed to 1% O₂, 5% CO₂, and 94% N₂ humidified atmosphere at 37°C were treated with GLA (200 μM) or/and Wnt3a (200 ng/ml). The protein levels of γ -catenin, E-cadherin, fibronectin, Snail and β -catenin in SGC-7901 and MGC-803 cells were determined by Western blotting and qPCR. n = 3, **p < 0.01 vs. GLA (0 μM) + Wnt3a (0 μM); #p < 0.05 vs. GLA (200 μM) + Wnt3a (200 ng/ml).

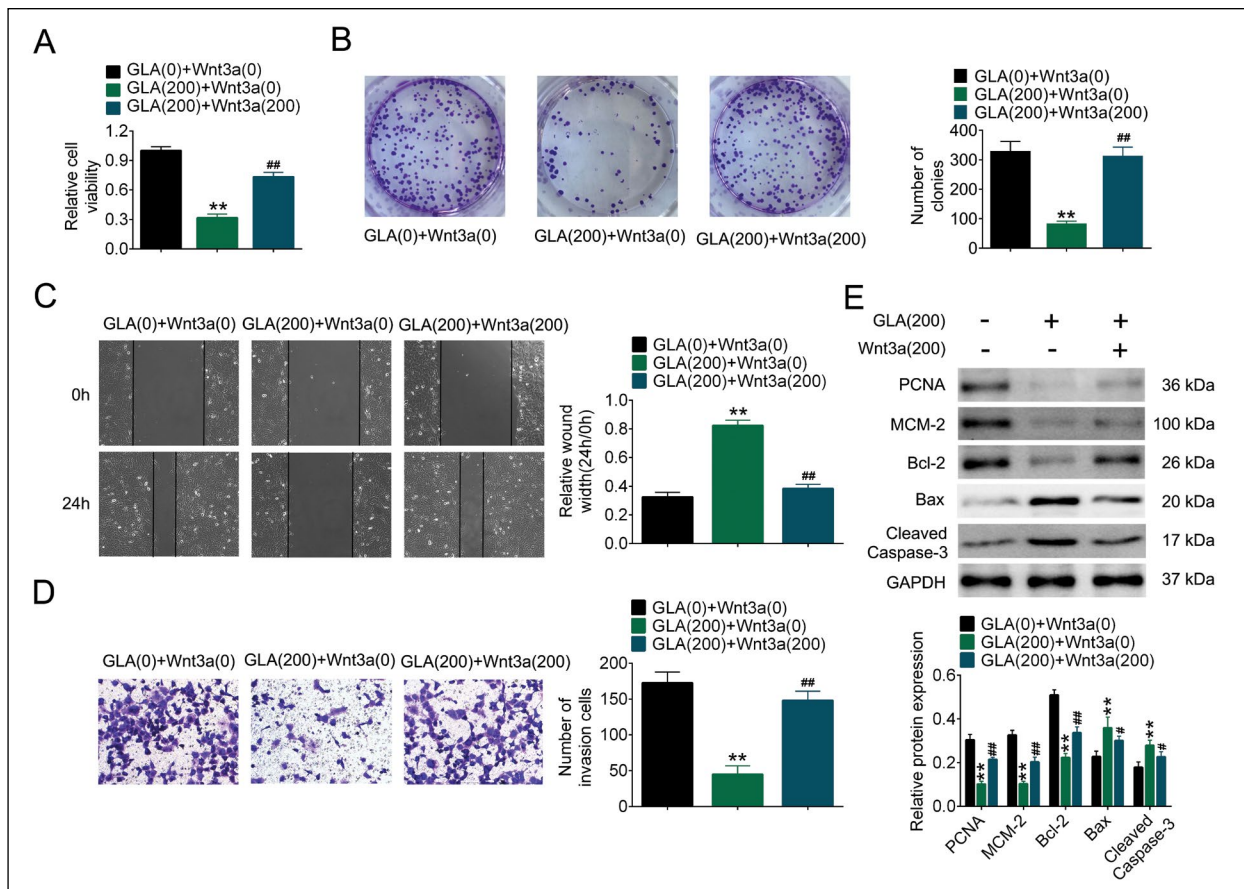


Figure 5. GLA inhibits the growth of gastric cancer cells under hypoxia through Wnt/ β -catenin signal pathway. SGC-7901 and MGC-803 cells exposed to 1% O₂, 5% CO₂, and 94% N₂ humidified atmosphere at 37°C were treated with GLA (200 μ M) or/and Wnt3a (200 ng/ml). **A.** Viability of SGC-7901 cells were measured by MTT assay. **B.** The colony formation assay of SGC-7901 cells was performed. Cell migratory ability (**C**) and cell invasive ability (**D**) of SGC-7901 cells were detected by wound-healing assays and Transwell assay, respectively. **E.** The protein levels of PCNA, MCM-2, Bcl-2, Bax and Cleaved Caspase-3 were determined by Western blotting. n = 3, **p* < 0.05, ***p* < 0.01 vs. GLA (0 μ M) + Wnt3a (0 μ M); #*p* < 0.05, ##*p* < 0.01 vs. GLA (200 μ M) + Wnt3a (200 ng/ml).

preincubation with Wnt3a significantly reversed the inhibitory effects of GLA on the invasion and migration of SGC-7901 cells (Fig. 5C and 5D, *p* < 0.01). In addition, Western blotting showed that compared with GLA treatment group, Wnt3a treatment significantly reversed the inhibition of GLA on the expression of PCNA, MCM-2 and Bcl-2 and the expression of Bax and cleaved Caspase-3 in SGC-7901 cells (Fig. 5E, *p* < 0.05, *p* < 0.01).

Discussion

Despite the achievements in surgical resection and chemotherapy for the treatment of gastric cancer, its recurrence rate and metastasis rate are still high, and the median overall survival (OS) time of patients with metastatic disease is still very poor [3, 4]. GLA is a promising bioactive molecule that has anticancer ac-

tivity in many types of cancer [18, 22, 26, 27], but its biological activity in gastric cancer is poorly understood. It has been reported that regular intake of GLA could reduce inflammation and inhibit the development of cancer [28–30]. Previous studies have also shown that GLA inhibited cell growth in the human neuroblastoma cell line [31], and decreased GLA levels are associated with the development of inflammatory breast cancer [23]. GLA inhibited DMBA-induced breast cancer by mediating mitochondrial death and apoptosis, reducing hypoxic microenvironment, hindering the synthesis of fatty acids from scratch, and mediating cholinergic anti-inflammatory pathway [32]. In this study, we found that GLA could inhibit the activity and growth of hypoxic-induced SGC-7901 and MGC-803 cells in a concentration-dependent manner, and reverse the migration, invasion and EMT SGC-7901 and MGC-803 cells induced by hypoxia. Further mo-

lecular mechanisms have shown that GLA treatment inhibited the progression of hypoxic gastric cancer by inducing the inactivation of Wnt/ β -catenin signaling pathway. This preliminary study suggested that GLA could inhibit the progression of gastric cancer.

Hypoxia is a common marker for several types of human solid tumors and an adverse prognostic factor that associated with the development of EMT [33–35]. Hypoxia-induced EMT decreased the expression of E-cadherin and other epithelial markers and increased the expression of N-cadherin, vimentin, and other mesenchymal markers [36]. Jo *et al.* found that hypoxia can induce EMT process by up-regulation of N-cadherin and snail/slug and down-regulation of PCNA in liver cancer cells [37]. Tam *et al.* explored the effects of different oxygen levels on EMT induction and stemness maintenance and attempted to elucidate the signal transduction pathways in colorectal cancer cells. It was found that 10% O₂ may be an EMT-induced condition, and inhibition of JNK can be used as a therapeutic method to inhibit the metastasis of colorectal cancer cells [38]. Consistent with the above results, we found that GLA induced epithelial to mesenchymal transformation of gastric cancer cells by increasing epithelial cell markers (γ -catenin and E-cadherin), and decreasing mesenchymal cell markers (fibronectin and Snail) under hypoxia. Interestingly, GLA exerted a protective role in hypoxic-induced EMT in gastric cancer cells, because dose-dependent treatment with GLA reversed the hypoxia-induced growth of gastric cancer cells.

It is well-known that the regulatory mechanism underlying hypoxic-induced EMT is complex and complicated. For example, GRP78 promoted hypoxia-induced EMT in A549 cells by activating the smad2/3 and Src/MAPK pathways [39]. *Celastrus orbiculatus* extract significantly inhibited tumor metastasis and EMT by inhibiting the HIF-1 α /Twist1 signaling pathway in hypoxia-induced HepG2 cells [40]. Scutellarin reversed hypoxia-induced EMT in breast cancer cells through the PI3K/Akt and MAPK pathways [34]. Besides, it is also reported that Wnt signaling pathway promotes tumor progression and metastasis in various cancers [41]. Previous studies have shown that the Wnt/ β -catenin pathway is activated in gastric cancer [42], and contributes to tumor malignancy in gastrointestinal stromal tumor [43]. In gastric cancer, a variety of biologically active substances have been shown to regulate the Wnt/ β -catenin pathway, such as lycopene [44], sinomenine [45] and cardamom [46]. However, whether GLA can also modulate the progression of gastric cancer by regulating the Wnt/ β -catenin pathway remains unknown. β -catenin is a main member of the Wnt signaling pathway. In general, Wnt/ β -catenin

signals promote tumor spread and development by activating downstream genes β -catenin, cyclin D and proto-oncogenes. In this study, GLA reduced the protein levels of β -catenin in SGC-7901 and MGC-803 cells under hypoxic conditions in a concentration-dependent manner, and thereby inhibited cell growth and EMT in gastric cancer. Notably, Wnt3a reversed the inhibitory effect of GLA on β -catenin expression, and further reversed the inhibitory effect of GLA on gastric cancer cell growth and EMT. These results indicated that Wnt/ β -catenin signals were involved in the regulatory effects of GLA in gastric cancer cells.

In summary, our study showed that GLA inhibited hypoxia-induced cell growth and EMT by inhibiting the Wnt/ β -catenin signaling pathway, thus demonstrating antitumor activity in gastric cancer cell lines. Therefore, GLA may be a potentially attractive bioactive phytochemical for the treatment of gastric cancer. Our findings provide a theoretical basis for GLA as a potential clinical drug for gastric cancer.

Competing interests

The authors declare that they have no competing interests, and all authors should confirm its accuracy.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

Yan Wang conceived and designed the experiments, Jian Shi analyzed and interpreted the results of the experiments, Liya Gong performed the experiments.

Informed consent

Written informed consent was obtained from a legally authorized representative(s) for anonymized patient information to be published in this article.

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