J Gastric Cancer. 2021 Dec;21(4):439-456
https://doi.org/10.5230/jgc.2021.21.e40
pISSN 2093-582X·eISSN 2093-5641

Journal of Gastric Cancer

Original Article

Vitexin Inhibits Gastric Cancer Growth and Metastasis through HMGB1-mediated Inactivation of the PI3K/AKT/mTOR/HIF-1α Signaling Pathway

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Received: Jul 21, 2021
Revised: Nov 29, 2021
Accepted: Dec 14, 2021

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Author Contributions
Conceptualization: Z.P.; Data curation: Z.H.Z.; Formal analysis: W.T., W.J.; Writing - original draft: L.C.W.; Writing - review & editing: S.X.J.

Conflict of Interest
No potential conflict of interest relevant to this article was reported.

ABSTRACT

Purpose: Gastric cancer (GC) has high morbidity and mortality and is a serious threat to public health. The flavonoid compound vitexin is known to exhibit anti-tumor activity. In this study, we explored the therapeutic potential of vitexin in GC and its underlying mechanism.
Materials and Methods: The viability, migration, and invasion of GC cells were determined using MTT, scratch wound healing, and transwell assays, respectively. Target molecule expression was determined by western blotting. Tumor growth and liver metastasis were evaluated in vivo using nude mice. Protein expression in the tumor tissues was examined by immunohistochemistry.
Results: Vitexin inhibited GC cell viability, migration, invasion, and epithelial-mesenchymal transition (EMT) in a dose-dependent manner. Vitexin treatment led to the inactivation of phosphatidylinositol-3-kinase (PI3K)/AKT/hypoxia-inducible factor-1α (HIF-1α) pathway by repressing *HMGB1* expression. Vitexin-mediated inhibition in proliferation, migration, invasion and EMT of GC cells were counteracted by hyper-activation of PI3K/AKT/HIF-1α pathway or *HMGB1* overexpression. Finally, vitexin inhibited the xenograft tumor growth and liver metastasis in vivo by suppressing HMGB1 expression.
Conclusions: Vitexin inhibited the malignant progression of GC in vitro and in vivo by suppressing HMGB1-mediated activation of PI3K/Akt/HIF-1α signaling pathway. Thus, vitexin may serve as a promising therapeutic agent for the treatment of GC.

Keywords: Epithelial-Mesenchymal transition; Gastric cancer; Hypoxia; Phosphatidylinositols; Vitexin

INTRODUCTION

Gastric cancer (GC) is the fifth most common malignancy worldwide, with 989,600 new cases/year reported in 2008. Furthermore, GC is the third leading cause of cancer-related death worldwide, with 783,000 deaths reported in 2018 [1,2]. Notably, the survival rate of patients with GC in developed countries is poor [1]; however, in Korea, the 5-year survival rate of patients with GC, who typically obtain an early and comprehensive diagnosis, is nearly 90% [3,4]. The high mortality rate of GC in other developed countries is mainly caused by the high rate of recurrence

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and metastasis; the high 5-year survival rates for patients with GC without lymph node metastasis after surgery drop sharply for patients with metastatic nodes [5]. Although there has been recent improvement in the survival rate with postoperative and perioperative chemoradiotherapy, the regional and distant recurrences remain to be resolved due to lack of effective treatment [6].

Tumor metastasis is one of the major causes of GC recurrence [7]. Epithelial-mesenchymal transition (EMT) is an essential process that plays a pivotal role in GC metastasis, as well as normal development [8,9]. EMT in tumor progression allows a polarized epithelial cell to acquire a mesenchymal cell phenotype that facilitates the intravasation of tumor cells into blood or lymph vessels and the subsequent distant metastasis [10]. Phosphatidylinositol-3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) is one of the most essential intracellular signaling pathways involved in the regulation of EMT [11]. The hyper-activation of PI3K/AKT/mTOR signaling has been implicated in metastasis of multiple types of cancers including GC [12,13]. Inhibition of PI3K/AKT/mTOR signaling, mediated by various inhibitors, effectively reduces tumor metastasis [14,15], suggesting that PI3K/AKT/mTOR signaling can serve as a potential therapeutic target for GC.

Vitexin, a bioactive flavonoid component derived from many traditional Chinese herbal medicines, has recently drawn growing attention due to its wide range of biological and pharmacological activities, including anti-cancer effects [16]. Vitexin inhibits glioblastoma and non-small cell lung cancer progression via inactivation of the PI3K/AKT/mTOR signaling pathway [17,18]. However, the effect of vitexin and its detailed mechanisms in GC remain unclear. High-mobility group box 1 protein (HMGB1) is regarded as one of the essential activators of the PI3K/AKT/mTOR pathway and is known to facilitate cancer cell metastasis [19]. Hence, it is important to investigate whether vitexin affects GC metastasis by regulating the HMGB1-mediated PI3K/AKT pathway.

A hypoxic microenvironment is a common feature of solid tumors due to the compact and avascular histological structure of tumor tissues [20]. Studies have shown that a hypoxic microenvironment may facilitate tumor cell metastasis [21,22]. Hypoxia-induced expression of hypoxia-inducible factor-1α (HIF-1α) is a key promoter of EMT in various cancer cells [23,24]. Interestingly, HIF-1α has been well documented as a downstream target of PI3K/AKT signaling pathway [25]. He et al. [26] have reported that HMGB1 facilitates angiogenesis and metastasis of breast cancer cells by modulating HIF-1α expression through the PI3K/AKT/mTOR pathway. Additionally, *HMGB1* exerts a cardio-protective effect by enhancing HIF-1α expression in ischemic hearts by modulation of the PI3K/AKT pathway [27]. Furthermore, vitexin has been shown to act as an antagonist of HIF-1α to attenuate hypoxia-ischemia neonatal brain injury [28].

These findings led us to investigate whether vitexin may inactivate PI3K/AKT/mTOR/HIF-1α pathway via HMGB1, which may delay GC progression. We investigated the suppressive effect of vitexin on GC cell proliferation, migration, invasion, and EMT in vitro and xenograft tumor growth and liver metastasis in vivo, and also clarified the underlying molecular mechanisms.

MATERIALS AND METHODS

Cell lines and reagents

KRAS wild and mutant GC cell lines were used in this study. Human gastric adenocarcinoma cell line AGS with *KRAS* mutation (CRL-1739) and the normal human gastric epithelial cell

https://doi.org/10.5230/jgc.2021.21.e40

line GES-1 were obtained from the American Type Culture Collection (Manassas, VA, USA). GC cell line SGC-7901 with wild type *KRAS* was obtained from the National Infrastructure of Cell Line Resource (Beijing, China). All cells were cultured in RPMI 1640 supplemented with 10% fetal bovine solution (GIBCO, Carlsbad, CA, USA) at 37°C with 5% CO_2 supplement.

Vitexin (Selleck Chemicals, Houston, TX, USA) was dissolved in dimethyl sulfoxide (Sigma, St. Louis, MO, USA) to prepare the stock solution (100 mM). Before experiments, the stock solution was diluted with phosphate-buffered saline (PBS) to the desired concentration.

Cell transfection

HMGB1 overexpression plasmid and pcDNA 3.1 vector were obtained from GenePharma (Shanghai, China). GC cells were transfected with the 2 plasmids using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. GC cells were subjected to subsequent experiments 48 hours after the transfection.

Scratch wound healing assay

For the scratch wound healing assay, treated GC cells were first cultured to confluence. Next, the cell monolayer was scratched with a pipet tip to create a "scratch wound." The scratched cells and debris were removed by washing with pre-warmed PBS. Cells were maintained in a serum-free medium. Images were taken at 0 and 24 hours after scratch with a light microscope. The results were quantitatively analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Transwell invasion assay

The transwell chamber was pre-coated with 70 μL Matrigel Matrix (356234; Corning Inc., Corning, NY, USA) at 37°C for 1 hour. Cells at a density of 5×10^4 suspended in 200 μL of serum-free media were seeded in the upper chamber. Five hundred microliters of complete medium with 10% fetal bovine solution was added to the lower chamber. After incubation for 24 hours at 37°C, the cells in the lower chamber were fixed and stained with 0.2% crystal violet, and the number of invasive cells were counted.

MTT assay

GC cells were seeded at a density of 2,000 cells per well into 96-well plates. At the indicated time points, MTT reagent (20 μL, 5 mg/mL; Abcam, Cambridge, UK) was added to each well. After incubation for 4 hours at 37°C, 150 μL of dimethyl sulfoxide was added to dissolve the formazan crystals. Absorbance was assessed at 492 nm using a microplate reader. The assays were performed in triplicate.

Western blotting

Total protein was extracted from tumor tissues or cells using RIPA Lysis Buffer (Beyotime, Shanghai, China). For detection of HMGB1, the cytosolic and nuclear extracts were obtained using the NE-PER nuclear and cytoplasmic extraction kit (Piece, Rockford, IL, USA) following the manufacturer's instructions. Equal amount of protein (10 μg) was loaded onto 10% SDS-PAGE and then transferred to nitrocellulose membranes (0.2 μm; 1620112; Bio-Rad, Hercules, CA, USA). The membranes were blocked with 5% non-fat milk. Next, the membranes were probed with primary antibodies against E-cadherin (1:2,000; R&D, Minneapolis, MN, USA), HIF-1α (1:1,000, Abcam), HMGB1 (1:1000, CST, Trask Lane Danvers, MA, USA), MMP2 (1:1,000; CST), MMP9 (1:1,000; CST), mTOR (1:1,000; CST), N-cadherin (1:1,000; Abcam), PI3K (1:1,000; Abcam), p-PI3K (1:1,000; Abcam), p-AKT

(1:1,000; CST), AKT (1:2,000, CST), p-mTOR (1:1,000; CST), and β-actin (1:1,000; CST), followed by incubation with the secondary antibody (1:10,000; LI-COR Biosciences, Lincoln, NE, USA). The protein band signals were captured and analyzed using an Odyssey CLx Imaging System (LI-COR Biosciences). β-actin was used as an endogenous reference.

In vivo experiments

All experimental protocols were approved and conducted under the supervision of the Institutional Animal Care and Use Committee of Xi'an Jiaotong University. Athymic nude mice (6-week-old females) were provided by the Laboratory Animal Center of the Institute of Genetics (Beijing, China). The mice were maintained in a specific pathogen free facility at 26°C with 12/12 hours light/dark cycles. The mice were assigned into the following groups (n=5 per group): control (0.1% DMSO), low dose (intraperitoneal, 1 mg/kg vitexin, once a day for 4 weeks), high dose (intraperitoneal, 2 mg/kg vitexin, once a day for 4 weeks), high dose + pcDNA 3.1 (AGS cells stably transfected with pcDNA 3.1), and high dose + *HMGB1* (AGS cells stably transfected with *HMGB1* overexpression plasmid).

To generate xenograft tumors from GC cells, 1×10^6 AGS cells were suspended in 50 μL PBS and injected into one side of the posterior flank of nude mice subcutaneously. Tumor volume was determined weekly, by measuring the length, width, and thickness of tumors with a caliper. Four weeks post-injection, the xenografted tumors were collected and weighed. To evaluate GC metastasis in vivo, 1×10^7 AGS cells suspended in 200 μL PBS suspension were injected into the tail vein of nude mice [29,30]. Four weeks later, liver tissues were isolated and fixed in 4% paraformaldehyde for routine hematoxylin and eosin staining. The liver tissues were imaged and macroscopic nodules in the tissues were quantified.

Immunohistochemistry

The xenograft tumor tissues were fixed in 10% formalin for 24 hours and embedded in paraffin. Tissue blocks were cut into 5 μm-thick sections using Leica RM2155 Microtome. The sections were dewaxed in xylene and rehydrated in an ethanol gradient. For the antigen retrieval step, sections were immersed in 0.01% sodium citrate buffer and heated till the boiling point using a microwave oven. Sections were blocked with 5% normal goat serum for 1 hour and incubated with primary antibodies against E-cadherin (1:200; R&D), HMGB1 (1:100; CST), Ki67 (1:200; CST), and N-cadherin (1:100; Abcam) overnight at 4°C. Subsequently, sections were developed using the biotin-streptavidin HRP detection system (ZSGB-BIO, Beijing, China) followed by 3,3′-diaminobenzidine staining. The sections were imaged using a Zeiss imaging system (Zeiss, Oberkochen, Germany).

Statistical analyses

All data from at least 3 biological replicates were expressed as mean±standard deviation. Data were analyzed using Student's t-test or analysis of variance using GraphPad Prism software (GraphPad Software, San Diego, CA, USA). Statistical significance was set at $P<0.05$.

RESULTS

Vitexin suppresses GC cell viability in a dose-dependent manner

To determine the effect of vitexin on GC cell viability, GC cells were treated with different concentrations of vitexin (0, 10, 20, 40, 80, and 160 μM) for 24 hours and 48 hours. The MTT assay demonstrated that exposure to vitexin for 24 hours and 48 hours led to a significant

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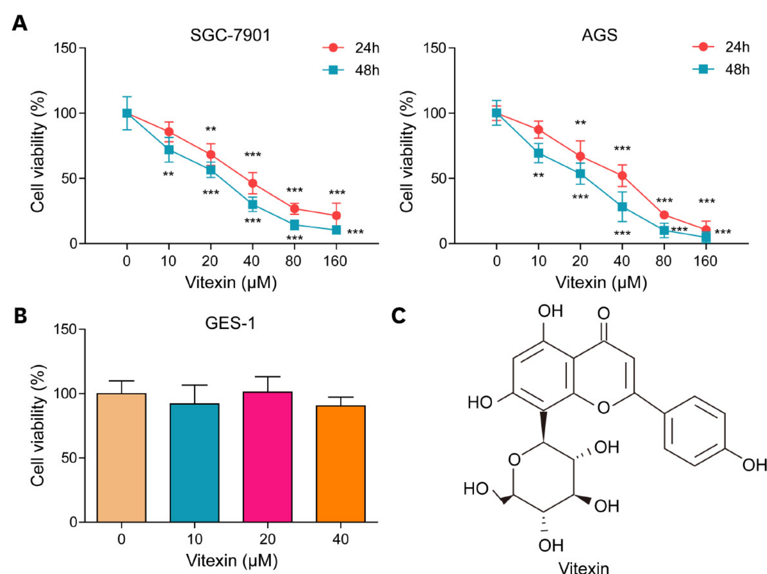

Fig. 1. Vitexin suppresses gastric cancer cell viability in a dose-dependent manner. (A) The viability of SGC-7901 and AGS cells treated with different concentrations (0, 10, 20, 40, 80, 160 μM) of vitexin for 24 hours and 48 hours was determined using an MTT assay. (B) The viability of GES-1 cells treated with different dosages of vitexin (0, 10, 20, 40 μM) for 48 hours was determined using an MTT assay. (C) Chemical structure of vitexin.
**P<0.01; ***P<0.001.

dose- and time-dependent decline in GC cell viability (**Fig. 1A**). However, treatment with vitexin for 48 hours did not affect the viability of the normal human gastric epithelial cell line GES-1 (**Fig. 1B**). The chemical structure of vitexin is shown in **Fig. 1C**.

Vitexin suppresses the migration, invasion, and EMT of GC cells

As migration and invasion are 2 distinct features of cancerous cells, we investigated whether vitexin could affect the migration and invasion of GC cells. Scratch wound healing assays indicated that vitexin treatment remarkably reduced the migration rate of SGC-7901 and AGS cells in a dose dependent manner (**Fig. 2A**). Similarly, transwell invasion assays showed that an increase in vitexin concentration was coupled with a decline in the number of invasive cells (**Fig. 2B**). The protein levels of EMT markers closely related with the migratory and invasive behaviors were also assessed. In both GC cell lines, treatment with vitexin significantly increased E-cadherin expression, but decreased N-cadherin, MMP9 and MMP2 levels in a dose-dependent manner (**Fig. 2C**). These observations indicated that the migration, invasion, and EMT of GC cells was attenuated by vitexin treatment.

Vitexin inhibits the activation of PI3K/AKT/mTOR/HIF-1α pathway

In order to explore the underlying mechanism of the inhibitory effect of vitexin on EMT, we investigated the PI3K/AKT/mTOR/HIF-1α pathway. After exposure to vitexin for 48 hours, no change was detected in the total protein levels of PI3K, AKT, and mTOR (**Fig. 3A**). However, treatment with vitexin resulted in a decline in the phosphorylation of PI3K, AKT, and mTOR in a dose-dependent fashion (**Fig. 3A**). HIF-1α, a downstream effector of the PI3K/AKT/mTOR pathway, was also downregulated by vitexin treatment (**Fig. 3A**).

Next, the kinetics of vitexin-mediated inhibition of PI3K/AKT/mTOR/HIF-1α pathway was assessed. Decreased levels of p-PI3K, p-AKT, p-mTOR, and HIF-1α were first observed at 6 hours after treatment with 40 μM vitexin (**Fig. 3B**), and continued to decline in a time-dependent fashion up to 48 hours. These results suggested that the PI3K/AKT/mTOR/HIF-1α pathway may be involved in vitexin-mediated inhibition in EMT of GC cells.

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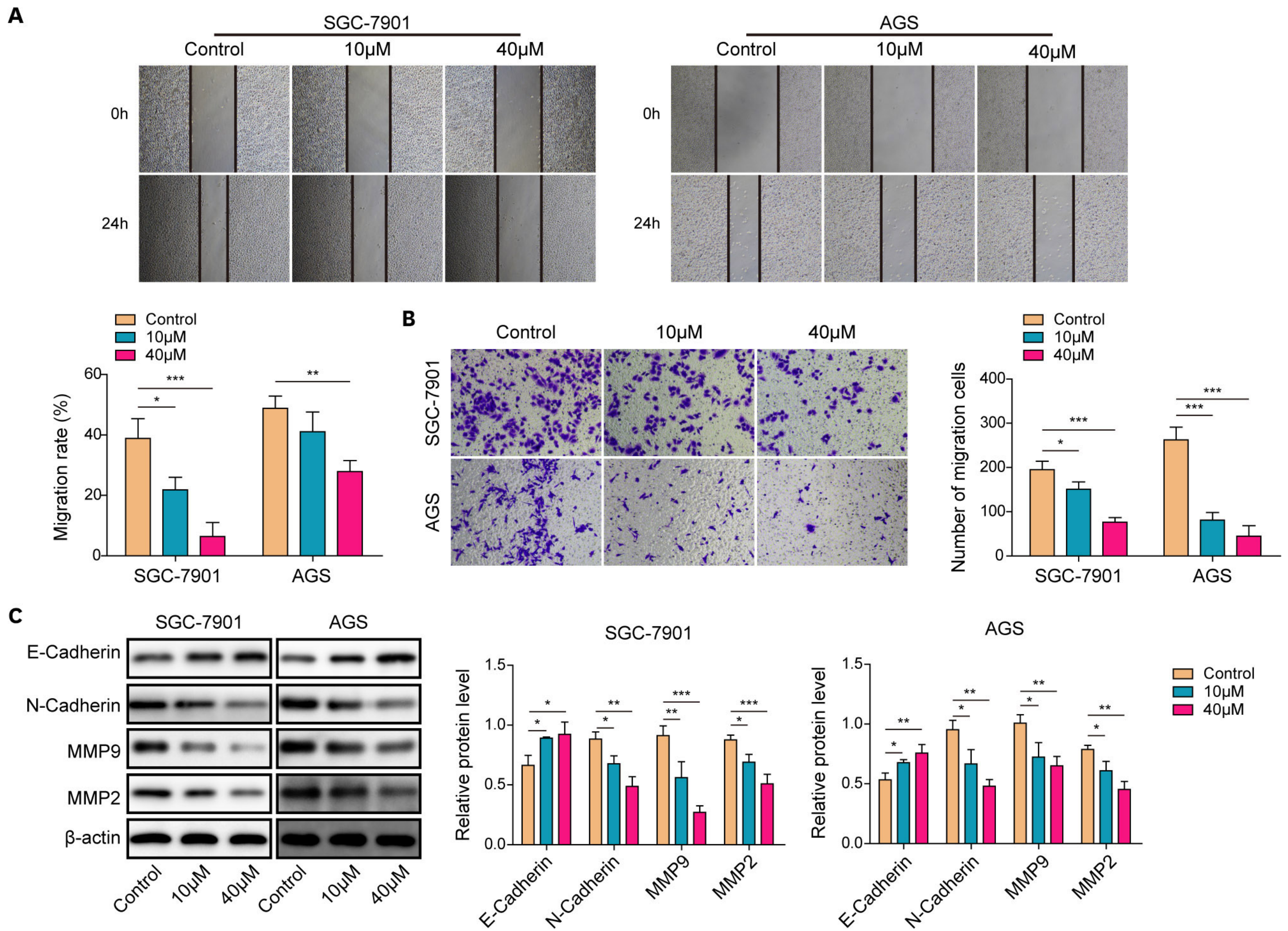

Fig. 2. Vitexin inhibits the migration, invasion, and epithelialmesenchymal transition of GC cells. SGC-7901 and AGS cells were treated with 10 μM and 40 μM vitexin for 48 hours. (A) Effect of vitexin on GC cell migration was measured using a scratch wound healing assay. (B) Effect of vitexin on GC cell invasion was measured using a transwell invasion assay. (C) Protein levels of E-cadherin, N-cadherin, MMP9, and MMP2 in response to vitexin treatment were detected through western blotting.
*P<0.05; **P<0.01; ***P<0.001.

Hyper-activation of PI3K/AKT/mTOR/HIF-1α pathway abolished the anti-cancer effects of vitexin

To further assess the role of PI3K/AKT/mTOR/HIF-1α pathway in the inhibitory effect of vitexin on the malignant phenotypes of GC cells, we included an AKT agonist, SC79, along with the vitexin treatment. As assessed by MTT assay, the decreased cell viability in vitexin-treated SGC-7901 and AGS cells was alleviated with the addition of SC79 (**Fig. 4A**). Treatment with SC79 significantly increased the migration rate of SGC-7901 and AGS cells exposed to vitexin (**Fig. 4B**). Similarly, SC79 treatment dramatically increased the number of invasive GC cells in the presence of vitexin (**Fig. 4C**). Western blotting analysis confirmed that the decrease in p-AKT and p-mTOR levels observed in vitexin-treated GC cells was recovered after addition of SC79. Furthermore, the vitexin-mediated downregulation of HIF-1α, N-cadherin, MMP9, and MMP2 and upregulation of E-cadherin were partly reversed by SC79 treatment (**Fig. 4D**). Thus, forceful activation of PI3K/AKT/mTOR/HIF-1α pathway reversed the anti-tumor effects of vitexin on GC cells.

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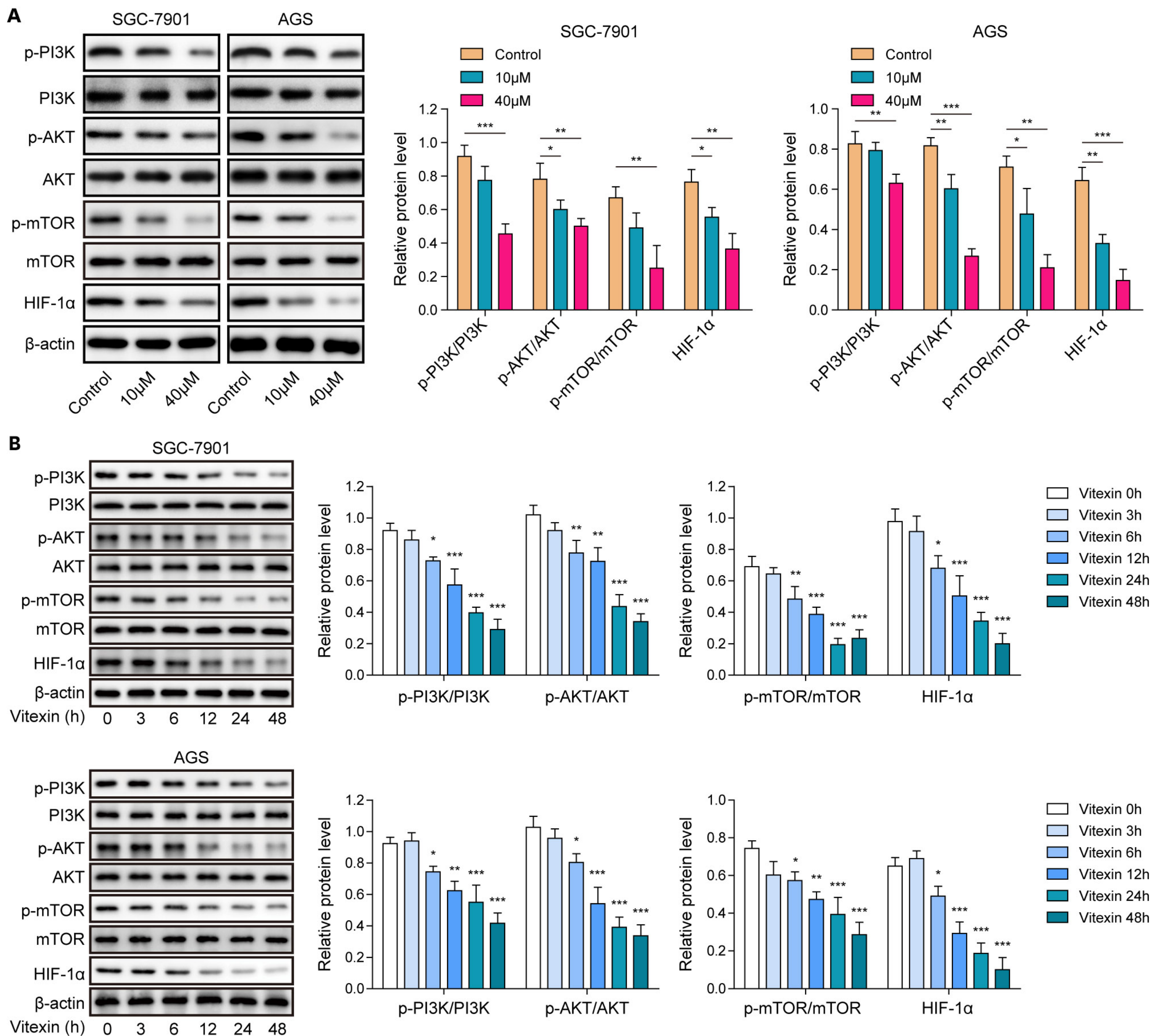

Fig. 3. Vitexin inhibits the activation of PI3K/AKT/mTOR/HIF-1α pathway in GC cells. (A) SGC-7901 and AGS cells were incubated with 10 μM and 40 μM vitexin for 48 hours. The protein levels of PI3K, p-PI3K, AKT, p-AKT, mTOR, p-mTOR, and HIF-1α were detected through western blotting. B. SGC-7901 and AGS cells were treated with 40 μM vitexin for 0, 3, 6, 12, 24, and 48 hours. Protein levels of PI3K, p-PI3K, AKT, p-AKT, mTOR, p-mTOR and HIF-1α were assessed through western blotting.
PI3K = phosphatidylinositol-3-kinase; mTOR = mammalian target of rapamycin; HIF-1α = hypoxia-inducible factor-1α; GC = gastric cancer.
*P<0.05; **P<0.01; ***P<0.001.

HMGB1 participates in the inhibition of vitexin in GC cell growth, migration, invasion, and EMT

To determine whether *HMGB1* was responsible for the inhibitory effect of vitexin on GC cell malignant properties, we examined the protein expression of HMGB1 in GC cells in response to various concentrations of vitexin. As determined by western blotting, in both SGC-7901 and AGS cells, vitexin strikingly decreased HMGB1 protein levels in a dose-dependent manner (**Fig. 5A**).

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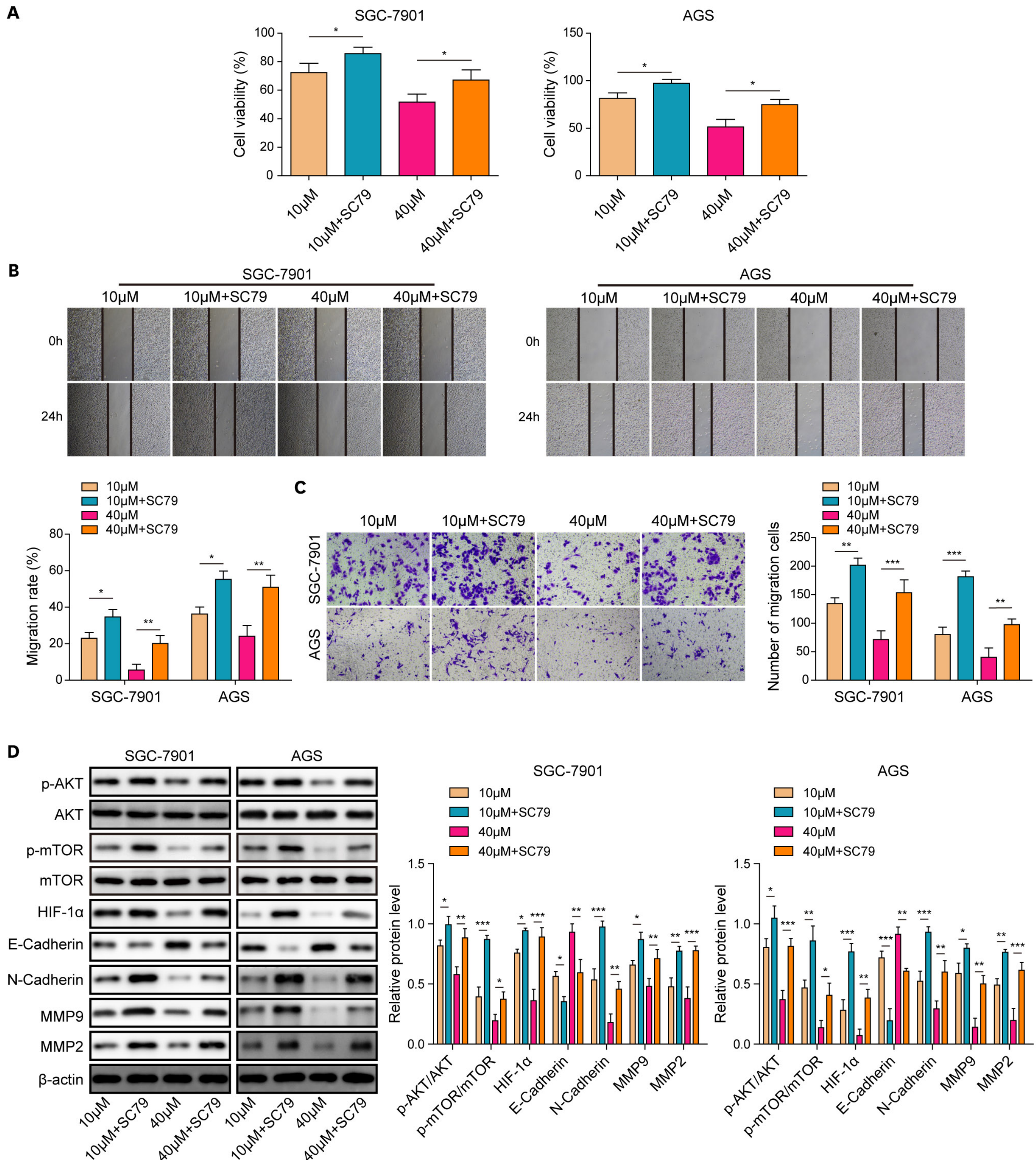

Fig. 4. Hyper-activation of the PI3K/AKT/mTOR/HIF-1α pathway reversed the inhibitory effects of vitexin on GC cell viability, migration, invasion, and EMT. SGC-7901 and AGS cells were treated with 10 μM and 40 μM vitexin, or a combination of SC79 (AKT agonist) for 48 hours. (A) Viability of GC cells was determined using an MTT assay. (B) Migration of GC cells was determined using the scratch wound healing assay. (C) Invasion of GC cells was measured using a transwell invasion assay. (D) Protein levels of p-AKT, AKT, mTOR, p-mTOR, E-cadherin, N-cadherin, MMP9, and MMP2 in GC cells with various treatments were detected through western blotting.

PI3K = phosphatidylinositol-3-kinase; mTOR = mammalian target of rapamycin; HIF-1α = hypoxia-inducible factor-1α; GC = gastric cancer; EMT = epithelial-mesenchymal transition.

*$P<0.05$; **$P<0.01$; ***$P<0.001$.

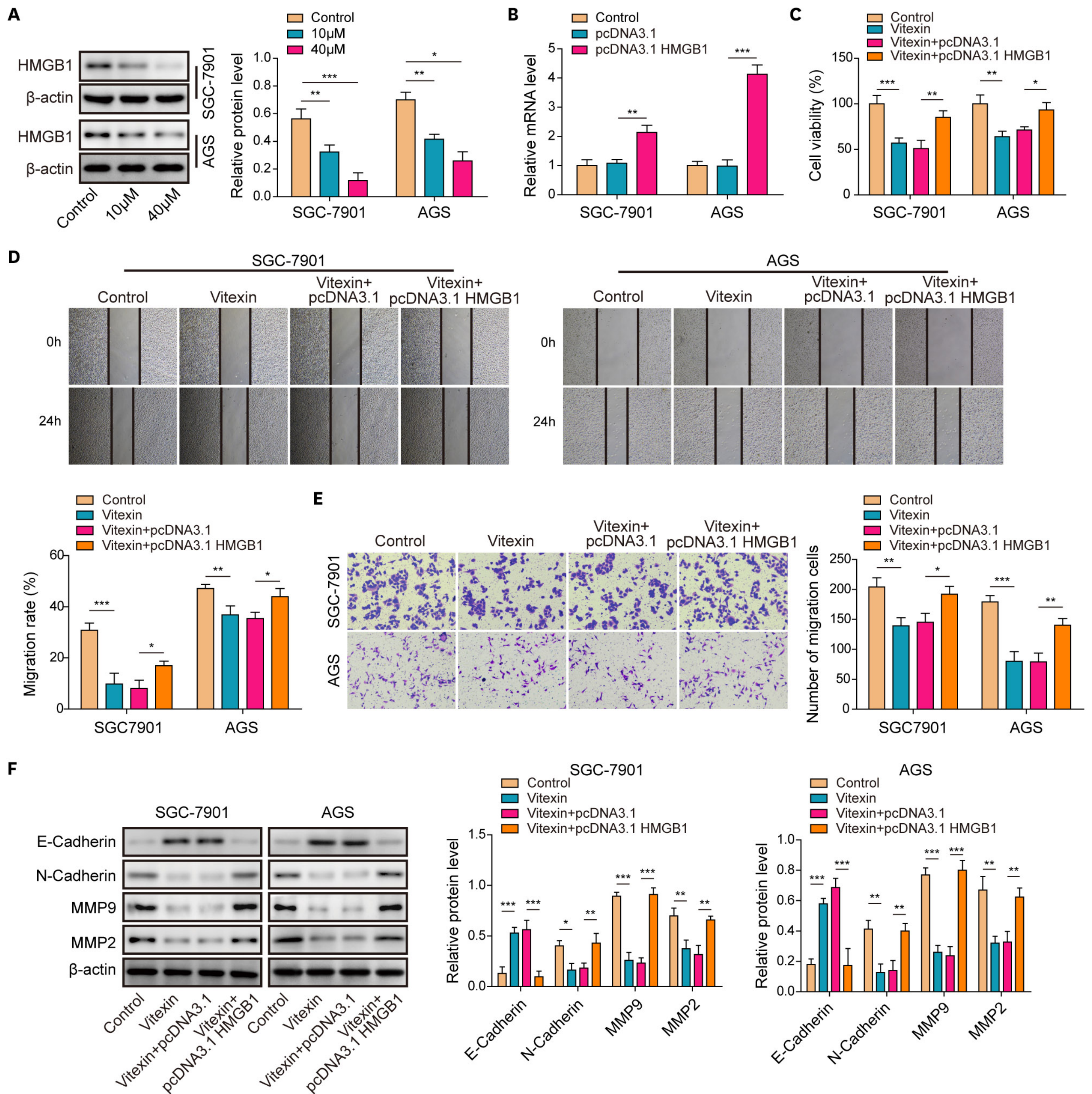

Fig. 5. HMGB1 inhibition is involved in the anti-cancerous effect of vitexin. (A) HMGB1 protein level in GC cells exposed to 10 μM and 40 μM vitexin for 48 hours was detected by western blotting. (B) The mRNA expression of *HMGB1* in GC cells after transfection with pcDNA3.1 or *HMGB1* overexpression plasmid for 48 hours was examined through qPCR. SGC-7901 and AGS cells were treated with 40 μM vitexin, or a combination with pcDNA3.1/*HMGB1* overexpression plasmid transfection for 48 hours. (C) Viability of GC cells was determined using an MTT assay. (D) Migration was determined using a scratch wound healing assay. (E) The invasive ability of GC cells was measured using a transwell invasion assay. (F) Protein levels of E-cadherin, N-cadherin, MMP9, and MMP2 were measured through western blotting.
GC = gastric cancer; HMGB1 = high-mobility group box 1 protein; qPCR = quantitative polymerase chain reaction.
*P<0.05; **P<0.01; ***P<0.001.

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GC cells were transfected with an overexpression plasmid of *HMGB1*, resulting in a significant increase in *HMGB1* mRNA expression in the cells (**Fig. 5B**). Vitexin-mediated suppression in GC cell viability (**Fig. 5C**), migration (**Fig. 5D**), and invasion (**Fig. 5E**) were partly rescued by the overexpression of *HMGB1*. Likewise, the increased expression of E-cadherin, but decreased expression of N-cadherin, MMP9, and MMP2 in vitexin-treated GC cells were counteracted by the overexpression of *HMGB1* (**Fig. 5F**). These results suggested that vitexin inhibited GC cell growth, migration, invasion, and EMT by downregulating *HMGB1*.

Vitexin restrained the activation of PI3K/AKT/mTOR/HIF-1α pathway through *HMGB1*

Next, we sought to investigate the involvement of *HMGB1* in vitexin-mediated inactivation of PI3K/AKT/mTOR/HIF-1α pathway. Western blotting of nuclear and cytosolic fractions of SGC-7901 and AGS cells indicated that treatment with vitexin promoted the translocation of HMGB1 from the cytoplasm to the nucleus. The cytoplasmic level of HMGB1 was increased after overexpression of *HMGB1* (**Fig. 6A**). Furthermore, vitexin-mediated downregulation of

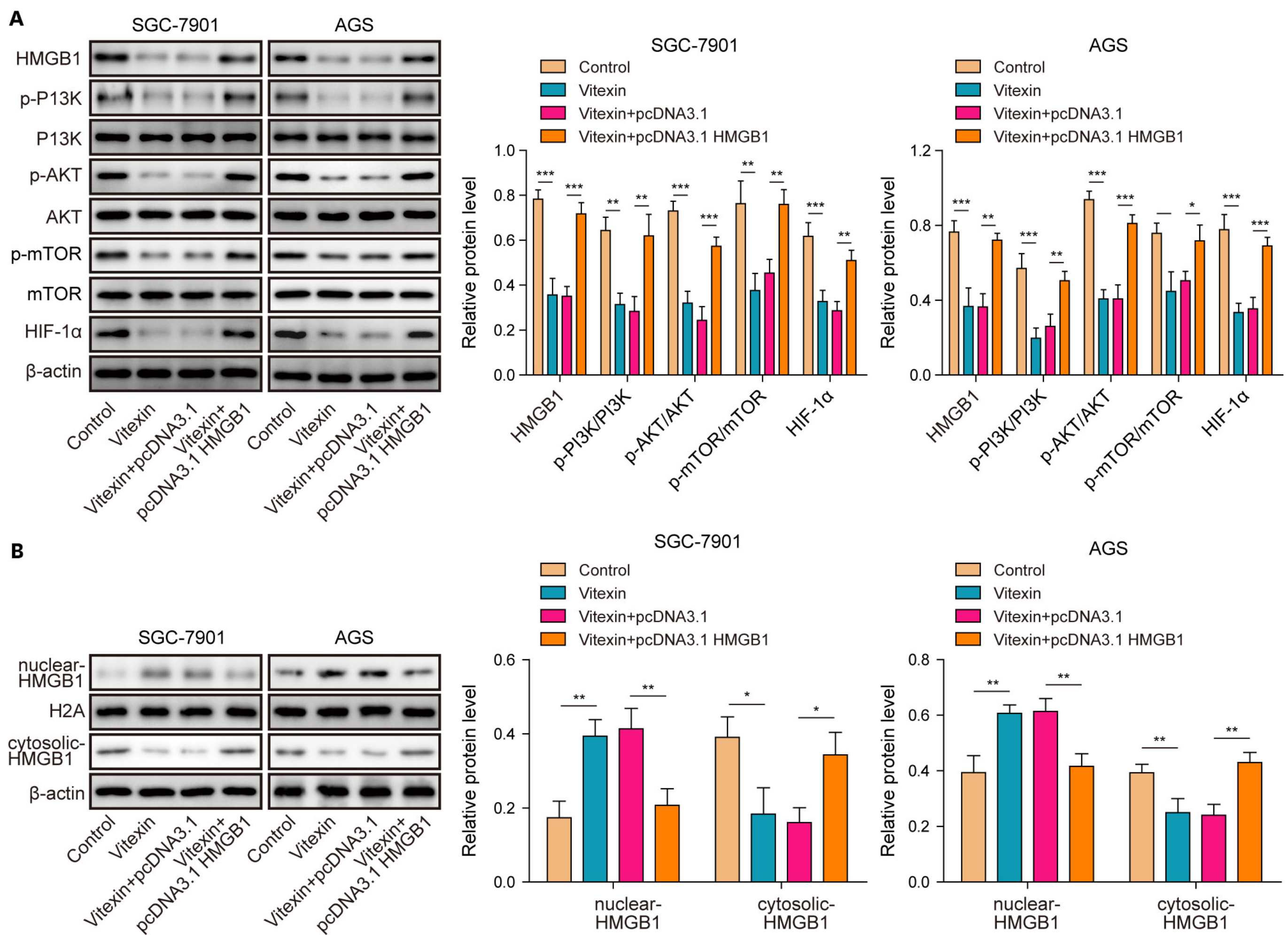

Fig. 6. Vitexin restrains the activation of PI3K/AKT/mTOR/HIF-1α pathway through HMGB1. SGC-7901 and AGS cells were incubated with 40 μM vitexin, or a combination with pcDNA3.1/*HMGB1* overexpression plasmid transfection for 48 hours. The protein levels of cytoplasmic/nuclear HMGB1 (A), PI3K, p-PI3K, AKT, p-AKT, mTOR, p-mTOR and HIF-1α (B) in SGC-7901 and AGS cells were detected through western blotting.
HMGB1 = high-mobility group box 1 protein; PI3K = phosphatidylinositol-3-kinase; mTOR = mammalian target of rapamycin; HIF-1α = hypoxia-inducible factor-1α.
*P<0.05; **P<0.01; ***P<0.001.

p-PI3K, p-AKT, p-mTOR, and HIF-1α in GC cells was recovered by overexpression of *HMGB1*; however, there was no significant change in total PI3K, AKT, and mTOR levels among the different groups (**Fig. 6B**). These results indicated that vitexin repressed the activation of PI3K/AKT/mTOR/HIF-1α pathway in GC cells by inhibiting cytosolic HMGB1 expression.

Vitexin inhibits xenograft tumor growth and liver metastasis in vivo

To determine the effect of vitexin on in vivo solid tumor growth, we established a xenograft mouse model. The tumor volume and weight were inhibited in a dose-dependent manner by the administration of 1 mg/kg and 2 mg/kg vitexin (**Fig. 7A-C**). In addition, the protein levels of HMGB1, p-PI3K, p-AKT, p-mTOR, and HIF-1α in the xenograft tumor tissues were decreased by vitexin in a dose-dependent manner (**Fig. 7D**). Immunohistological staining further showed that the expression levels of Ki67, HMGB1 and N-cadherin was decreased, while the expression level of E-cadherin was increased in the vitexin treatment groups (**Fig. 7E**).

The metastatic potential of GC cells was also evaluated using a nude mouse model in vivo. As shown in **Fig. 7F**, the number of metastatic nodules in liver tissues was reduced by treatment with 1 mg/kg and 2 mg/kg vitexin. The histological examination of liver tissues indicated that the area of metastatic tumor cells significantly decreased in mice treated with vitexin (**Fig. 7G**). Thus, vitexin exerted an inhibitory effect on in vivo GC tumor growth and liver metastasis.

Overexpression of *HMGB1* attenuated the anti-cancer activity of vitexin in vivo

To verify the involvement of *HMGB1* in the anti-cancer activity of vitexin in vivo, AGS cells stably transfected with pcDNA 3.1 or *HMGB1* plasmid were subcutaneously injected into the nude mice. Overexpression of *HMGB1* reversed the 2 mg/kg vitexin-induced decrease in tumor size and weight 28 days after the injection (**Fig. 8A-C**). Western blotting results indicated that the reduced levels of HMGB1, p-PI3K, p-AKT, p-mTOR and HIF-1α in 2 mg/kg vitexin-treated tumor tissues were partly reversed by *HMGB1* overexpression (**Fig. 8D**). Additionally, treatment with 2 mg/kg vitexin resulted in a decrease in Ki67, HMGB1, and N-cadherin expression and an increase in E-cadherin expression; these changes could be prevented by *HMGB1* overexpression (**Fig. 8E**). Overexpression of *HMGB1* also counteracted the inhibitory effect of 2 mg/kg vitexin on liver metastasis (**Fig. 8F and G**). These results suggested that vitexin suppressed tumor growth and liver metastasis in vivo by inhibiting *HMGB1* expression.

DISCUSSION

GC is one of the most life-threatening forms of cancers and is a severe threat to public health [31]. The high recurrence rate observed in patients with GC after surgical treatment is the leading cause of short survival in these patients [32]. Metastasis is a major contributor in GC recurrence and EMT plays an important role during metastasis [7]. Vitexin is considered a therapeutic candidate for GC because of its inhibition of EMT in multiple types of cancers [17,33] and also suppression of HIF-1α expression, which plays an important role in EMT-mediated metastasis in cancer [28]. In the present study, vitexin suppressed the viability, migration, invasion, and EMT of GC cells. Mechanistically, vitexin restrained *HMGB1* expression and the activation of the downstream PI3K/AKT/mTOR/HIF-1α signaling pathway. More importantly, vitexin significantly inhibited tumor growth and liver metastasis in vivo in a dose-dependent manner. Thus, this study may provide a theoretical foundation for the feasibility of vitexin as a potential therapeutic agent in the treatment of GC.

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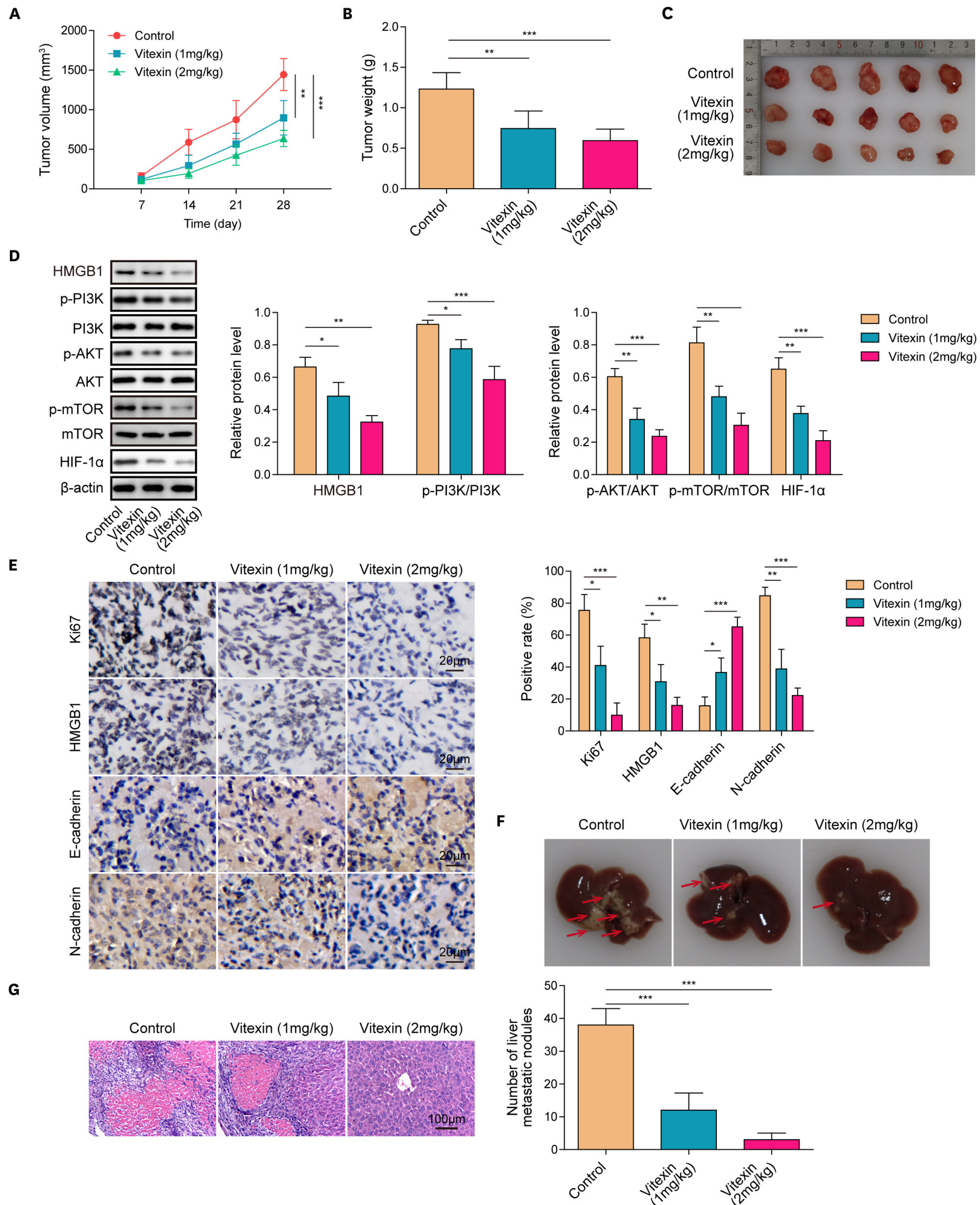

Fig. 7. Vitexin inhibits tumor growth and liver metastasis in vivo. To generate tumor xenografts, 1×10^6 AGS cells were subcutaneously injected into one side of the posterior flank of nude mice. The mice in low-dose and high-dose groups were intraperitoneally injected with 1 mg/kg and 2 mg/kg vitexin, respectively. The tumor tissues were collected 28 days after the injection. (A) Growth curves of the xenografted tumors were plotted. (B) Weight of xenografted tumors. (C) Representative photographs of GC xenografted tumors. (D) Protein levels of HMGB1, PI3K, p-PI3K, AKT, p-AKT, mTOR, p-mTOR and HIF-1α in tumor tissues were detected through western blotting. (E) The expression of Ki67, HMGB1, E-Cadherin, and N-Cadherin in tumor tissues was examined through immunohistochemistry staining. (F) To evaluate GC metastasis in vivo, 1×10^7 AGS cells were injected into the tail vein of nude mice. Four weeks later, the liver tissues were imaged. Macroscopic nodules in the liver tissues were quantified. (G) Liver metastasis was determined by hematoxylin and eosin staining.
GC = gastric cancer; HMGB1 = high-mobility group box 1 protein; PI3K = phosphatidylinositol-3-kinase; mTOR = mammalian target of rapamycin; HIF-1α = hypoxia-inducible factor-1α.
*P<0.05; **P<0.01; ***P<0.001.

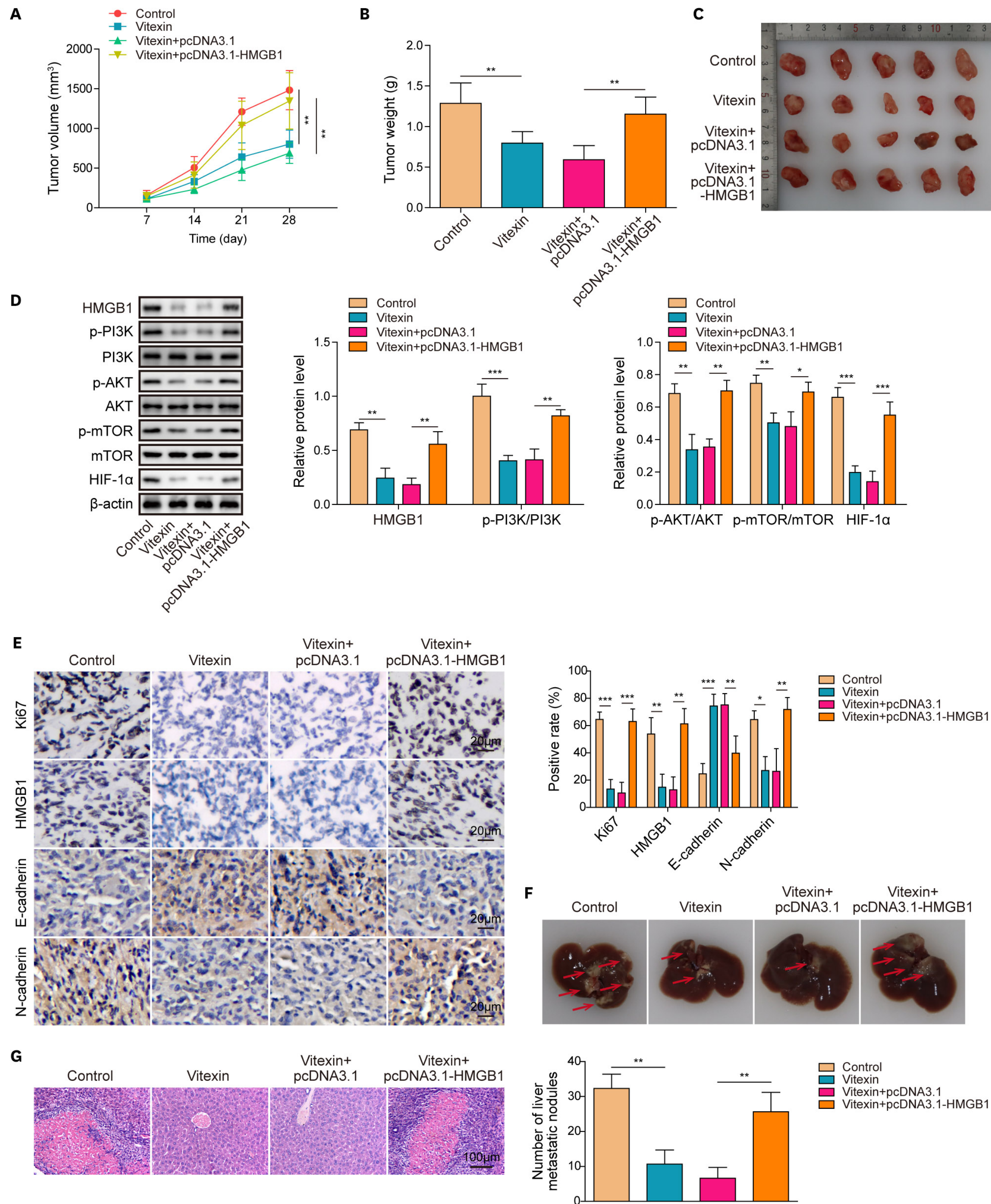

Fig. 8. Overexpression of *HMGB1* counteracts the anti-cancer activity of vitexin in vivo. AGS cells (1×10^{6}) stably transfected with pcDNA3.1/*HMGB1* overexpression plasmid were subcutaneously injected into nude mice. The mice in vitexin groups were intraperitoneally injected with 2 mg/kg vitexin. The tumor tissues were collected 28 days after the injection. (A) Growth curves of xenografted tumors were plotted. (B) Weight of xenografted tumors. (C) Representative photographs of GC xenografted tumors. (D) Western blotting was used to assess the protein levels of HMGB1, PI3K, p-PI3K, AKT, p-AKT, mTOR, p-mTOR, and HIF-1α in tumor tissues. (E) Immunohistochemistry staining was conducted to determine the expression of Ki67, HMGB1, E-cadherin, and N-cadherin in tumor tissues. (F) AGS cells (1×10^{6}) stably transfected with pcDNA3.1/*HMGB1* overexpression plasmid were injected into the tail vein of nude mice to evaluate GC metastasis in vivo. Four weeks later, the liver tissues were imaged. Macroscopic nodules in the liver tissues were quantified. (G) Liver metastasis was observed through hematoxylin and eosin staining.
GC = gastric cancer; HMGB1 = high-mobility group box 1 protein; PI3K = phosphatidylinositol-3-kinase; mTOR = mammalian target of rapamycin; HIF-1α = hypoxia-inducible factor-1α.
*P<0.05; **P<0.01; ***P<0.001.

The inhibitory effect of vitexin in cancer progression has been documented in various types of cancers including glioblastoma, lung cancer, and leukemia [17,18,34]. Vitexin exerts anti-tumor effects by the promotion of apoptosis and autophagy and the suppression of proliferation and migration through several signaling pathways, including increased caspase-3, caspase-9, and Hsp90 expression, inhibition of HIF-1α and Bcl-2 expression, as well as binding to transferrin receptor [16]. Additionally, mitogen-activated protein kinase, protein kinase C, PI3K/AKT, and β-catenin pathways are the molecular targets for the anti-cancer activity of vitexin [16]. However, the effect of vitexin on GC has rarely been studied.

In the present study, we used 2 different GC cell lines (SGC-7901 and AGS) and one normal non-transformed gastric cell line (GES-1) to evaluate the anti-tumor potential of vitexin. Vitexin exhibited a significant inhibitory effect on the viability of the 2 GC cell lines, but did not affect the growth of GES-1 cells. The 2 GC cell lines used in the study differ in terms of their genetic background and origination. SGC-7901 cells are derived from the metastatic lymph node of an East-Asian female patient [35]. In contrast, AGS cells were obtained from the resected stomach adenocarcinoma of an untreated Caucasian female [36]. Additionally, AGS cells carry a *KRAS* mutation, while this mutation is absent in SGC-7901 cells. Its inhibitory effect on these 2 different GC cell lines indicated that vitexin may be effective in patients with GC with different stages of development and diverse genetic background and genomic characteristics.

This is the first study to report the effect of vitexin on GC cell migration, invasion, and EMT. In this study, the concentrations of vitexin were chosen based on previous studies focusing on other types of cells [17,28]. We demonstrated that vitexin inhibited the migration, invasion, and EMT of both SGC-7901 and AGS cells in a dose-dependent fashion. In several other studies, the anti-cancer effects of vitexin were mainly attributed to its effect on cell proliferation and apoptosis [18,33,37,38]. The findings of this study help broaden our understanding of the anti-tumor mechanism of vitexin. Based on the results from GES-1 cells, vitexin did not exhibit obvious toxicity to normal tissues, further establishing its potential as an ideal therapeutic drug.

The rapid growth of tumor cells may result in hypoxia even with an abundant supply of oxygen, and the insufficient vascularization in solid tumors further amplifies the hypoxia response within tumor cells [39]. In response to hypoxia, cancer cells exhibit modified expression of many genes regulated by HIF-1α, which is the major component of hypoxia signaling pathways [40]. Most of the HIF-1α-mediated changes are observed in the expression of genes associated with proliferation, EMT, and metastasis [41], which allows cancer cells to survive in the hypoxic environment. Consistent with these studies, our results indicated that vitexin inhibited HIF-1α expression in GC cells in a dose-dependent manner. The activation of tyrosine kinases, such as IGFR and EGFR, stimulates the activation of PI3K/AKT/mTOR pathway, which may result in increased HIF-1α expression [42]. The PI3K/AKT/mTOR pathway is essential for the regulation of cell survival and apoptosis [43], and abnormal activation of this signaling pathway is commonly observed in cancer, including GC [44]. Hence, the present study investigated the effect of vitexin on PI3K/AKT/mTOR signaling pathway in GC. Our data suggested that vitexin treatment significantly restrained the activation of the PI3K/AKT/mTOR signaling pathway. Hyper-activation of the PI3K/AKT/mTOR pathway reversed the anti-cancerous effects of vitexin on GC cells through suppression of HIF-1α expression. These findings revealed that vitexin delayed the progression of GC cells by inactivating the PI3K/AKT/mTOR/HIF-1α pathway.

The role of vitexin in HMGB1 regulation in GC cells is another fundamental discovery of this study. HMGB1, a highly conserved nuclear protein, acts as a pivotal regulator of apoptosis and cell viability [45]. A previous study has shown that HMGB1 expression is strongly correlated with higher stage of GC [46], and inhibition of HMGB1 expression inhibits the malignant development of GC [47]. Previous studies have reported that HMGB1 is a contributor to EMT. Upregulation of HMGB1 promotes EMT and angiogenesis during liver cancer progression [48]. Ma et al. [49] have previously revealed that HMGB1 aggravates the development of silicosis by facilitating EMT. Hence, we also evaluated the involvement of HMGB1 in the inhibition of vitexin during EMT of GC cells. This is the first study to report that vitexin downregulates HMGB1 expression in GC cells, and the overexpression of *HMGB1* could counteract the inhibitory effects of vitexin on growth, migration, invasion, and EMT of GC cells. More importantly, vitexin promoted the nuclear translocation of HMGB1, thereby suppressing the activation of PI3K/AKT/mTOR pathway. Thus, cytoplasmic HMGB1 was responsible for the activation of the PI3K/AKT/mTOR pathway. However, the mechanism by which vitexin may downregulate HMGB1 in GC cells remains unclear. There are several possible underlying mechanisms for this effect. Firstly, vitexin may directly bind to the HMGB1 protein and promote its degradation. Secondly, vitexin may inhibit *HMGB1* expression by upregulating a series of miRNAs that target *HMGB1*. These speculations will be tested in our future studies.

This study investigated the connection between HMGB1 and PI3K/AKT/mTOR/HIF-1α pathway in GC cells. In a previous study, HMGB1 was demonstrated to be a modulator of HIF-1α via PI3K/AKT/mTOR pathway during breast cancer progression [26]. Recent studies have also indicated that HMGB1 modulates the PI3K/AKT/mTOR signaling pathway in myocardial ischemia/reperfusion injury and LPS-induced acute lung injury [50,51]. Yao et al. [27] have documented that HMGB1 upregulates HIF-1α via the PI3K/AKT pathway, attenuating acute myocardial ischemia/reperfusion injury in rats. In the present study, overexpression of *HMGB1* promoted the expression of p-PI3K, p-AKT, and p-mTOR in vitexin-treated GC cells, suggesting the activation of the PI3K/AKT/mTOR pathway. Vitexin inhibited cytoplasmic translocation of HMGB1 and subsequent activation of PI3K/AKT/mTOR pathway in GC cells. Therefore, vitexin repressed the development of GC cells by inhibiting HMGB1-mediated activation of PI3K/AKT/mTOR/HIF-1α pathway.

In summary, our findings demonstrated that vitexin suppressed the in vitro and in vivo growth and metastasis of GC cells. Additionally, vitexin effectively suppressed the activation of PI3K/AKT/mTOR pathway by inhibiting HMGB1, which led to a decrease in HIF-1α expression. Thus, based on the findings of this study, vitexin may be considered as a novel effective agent for the treatment of GC.

ACKNOWLEDGMENTS

We would like to give our sincere gratitude to the reviewers for their constructive comments.

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