Geniposide inhibits non-small cell lung cancer cell migration and angiogenesis by regulating PPARγ/VEGF-A pathway

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Abstract

Geniposide, an iridoid glycoside derived from traditional Chinese herb, *Gardenia jasminoides* Ellis, exerts antitumor effect against distinct cancers. The role of geniposide in the migration and angiogenesis of non-small cell lung cancer (NSCLC) cell was investigated in this study. Cancer cells were incubated with various concentrations of geniposide, and proliferative ability was detected by Cell Counting Kit-8 (CCK-8) and 5-ethynyl-2′-deoxyuridine (EdU) staining. Wound healing and transwell were used to assess cell migration and invasion, respectively. Tube formation assay was performed to investigate angiogenesis. Geniposide reduced NSCLC cell proliferation, and suppressed NSCLC cell migration and invasion in a dosage-dependent manner. Angiogenesis of NSCLC was also inhibited by geniposide. Geniposide increased the protein expression of peroxisome proliferator-activated receptor gamma (PPARγ) and decreased vascular endothelial growth factor-A (VEGF-A) protein expression in NSCLC cells. Incubation with a PPARγ antagonist, GW9662, attenuated geniposide-induced up-regulation of PPARγ and down-regulation of VEGF-A. Over-expression of VEGF-A weakened geniposide-suppressed cell proliferation, migration, and angiogenesis of NSCLC. Geniposide exerted antitumor and anti-angiogenic actions on NSCLC through regulation of PPARγ/VEGF-A pathway.

Keywords: geniposide; migration; angiogenesis; non-small cell lung cancer; PPARγ; VEGF-A

Introduction

Lung cancer, a common cause of cancer-related mortality, is one of the most prevalent malignancies (Thawani *et al.*, 2020). Non-small cell lung cancer (NSCLC), with high morbidity and mortality, contributes to more than 80% of the diagnosed lung-cancer cases (Gridelli *et al.*, 2015). Therapeutic strategies, such as surgery, radiotherapy, chemotherapy, and molecular-targeted therapy, are commonly used in the treatment of early-stage NSCLC (Loong *et al.*, 2018). However, these strategies demonstrate less beneficial effects on patients having advanced-stage NSCLC because of high risk of recurrence (Kim *et al.*, 2013). The 5-year survival rate of patients with advanced-stage NSCLC is less than 10% (Garon *et al.*, 2019).

NSCLC cell migration and invasion have been demonstrated to be associated with the poor prognosis and lung-cancer mortality (Yang *et al.*, 2016). Moreover, angiogenesis is characterized by the abnormal proliferation and migration of vascular endothelial cells in response to the metabolic demand of tumor cells, and contributes to the metastasis and recurrence of NSCLC (D'Amico, 2004). Targeting angiogenesis of vascular endothelial cells is considered as a promising strategy

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for the treatment of NSCLC (Malapelle and Rossi, 2019). Vascular endothelial growth factor (VEGF) is the prime mediator of vascular endothelial cell proliferation and migration, contributing to the progression of NSCLC; inhibition of VEGF suppressed cancer growth and metastasis (Keedy and Sandler, 2007).

Geniposide is an iridoid glycoside derived from traditional Chinese herb, *Gardenia jasminoides* Ellis, and is widely known as an anti-inflammatory, antioxidant, and antitumor agent (Lichota and Gwozdzinski, 2018; Liu *et al.*, 2009, *et al.*2013). For example, geniposide inhibited the proliferation of diffused large B-cell lymphoma (Hu *et al.*, 2020), and suppressed gastric cancer cell migration and invasion (Ma and Ding, 2018), and promoted the apoptosis of oral squamous cell carcinoma cell (Cheng *et al.*, 2017). Geniposide also inhibited the secretion of VEGF in hepatocellular carcinoma, and suppressed the migration and angiogenesis of endothelial cells (Zhang *et al.*, 2020). However, there are few studies considering the role of geniposide in NSCLC, and the relevant mechanism is still unclear.

The effects of geniposide on NSCLC cell proliferation and metastasis as well as its role in angiogenesis were investigated in this study. The underling mechanism involved in geniposide-inhibited tumor migration and angiogenesis could provide a promising strategy for NSCLC.

Materials and Methods

Cell culture and treatment

Lung cancer cells (A549 and NCI-H1299; American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum and penicillin-streptomycin (Invitrogen). Cells were incubated with 100-, 200-, 300-, or 400-μM geniposide (Sigma-Aldrich, San Francisco, CA, USA) according to a previous study (Yu *et al.*, 2020), or co-incubated with 300-μM geniposide and 10-μM GW9662 (Sigma-Aldrich) for 24 h. Cells were transfected with plasmid cloning DNA (pcDNA) vector or pcDNA–VEGF-A (Genepharma, Suzhou, China) for 48 h before incubation with geniposide or GW9662.

Cell viability and proliferation assays

A549 and NCI-H1299 cells with indicated incubation and transfection were seeded in 96-well plates for 24, 48, or 72 h. Cells were then incubated with 10-μL cell counting kit-8 (CCK-8) reagent (Beyotime, Beijing, China) for another 2 h. Absorbance at 450 nm was detected by a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) according to the study conducted by Yu *et al.* (2020). For cell proliferation assay, cells were seeded in 48-well plates, and 200 μL of 5-ethynyl-2′-deoxyuridine (EdU; Beyotime) was added in each well and incubated for 2 h according to the study conducted by Chen *et al.* (2021). Cells were then fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, and incubated with Apollo Staining reaction liquid. Cells were measured under Olympus ZKX53 microscope (Olympus, Tokyo, Japan) followed by counterstaining with DAPI (Sigma-Aldrich).

Cell migration and invasion assays

A549 and NCI-H1299 cells with indicated incubation and transfection were seeded in 6-well plates for 24 h according to a previous study (Elkhider *et al.*, 2020). A 200-μL pipette tip was used to generate scratch in the middle of each well. The wound was observed under the microscope after 24 h, and the wound width was calculated by Image J (v.1.46; National Institutes of Health, Bethesda, MD, USA). For cell invasion assay, cells in serum-free medium were plated into the upper chambers of Transwell chambers (Corning Incorporated, Corning, NY, USA), and medium with 15% fetal bovine serum was planted into the lower chambers according to the study conducted by Elkhider *et al.* (2020). Cells in the upper chamber were removed after 24 h, and cells in the lower chamber were stained with crystal violet. Cells were observed under the stated microscope (Olympus).

Tube formation assay

Human umbilical vein endothelial cells (HUVECs) with conditioned medium (Invitrogen) were seeded in 24-well plates precoated with basement membrane matrix containing reduced growth factor (Invitrogen) according to a previous study (Wang *et al.*, 2021). The culture medium of A549 and NCI-H1299 cells with indicated incubation and transfection was added into each well, and incubated for 6 h. Cells were then washed and fixed with 4% paraformaldehyde. The figures of tube formation were observed under the stated microscope, and the number of tubes was calculated by ImageJ.

Western blot assay

A549 and NCI-H1299 cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (Beyotime), and the protein samples were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto nitrocellulose membranes. The

membranes were blocked in 5% bovine serum albumin and probed with specific antibodies: anti-peroxisome proliferator-activated receptor gamma (PPARγ, 1:2,500; Abcam), anti-VEGF-A (1:3,500; Abcam), and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:4,500; Abcam). The membranes were then washed and incubated with horseradish peroxidase-conjugated secondary antibody (1:5,000; Abcam) and tetramethylbenzidine. The immunoreactivities were visualized using enhanced chemiluminescence (Sigma-Aldrich).

Statistical analysis

The data with at least triple replicates were expressed as mean ± standard error of mean (SEM), and analyzed by Student's *t*-test or one-way analysis of variance (ANOVA) with Tukey's post-hoc test using the SPSS software. $P < 0.05$ was considered as statistically significant.

Results

Geniposide repressed NSCLC cell proliferation

In order to investigate the role of geniposide in the progression of NSCLC, NSCLC cell lines (A549 and NCI-H1299) were treated with different concentrations of geniposide. Incubation with geniposide reduced the viabilities of A549 and NCI-H1299 cells in a dosage-dependent manner (Figure 1A). The viabilities of A549 and NCI-H1299 cells were reduced by almost 50% followed

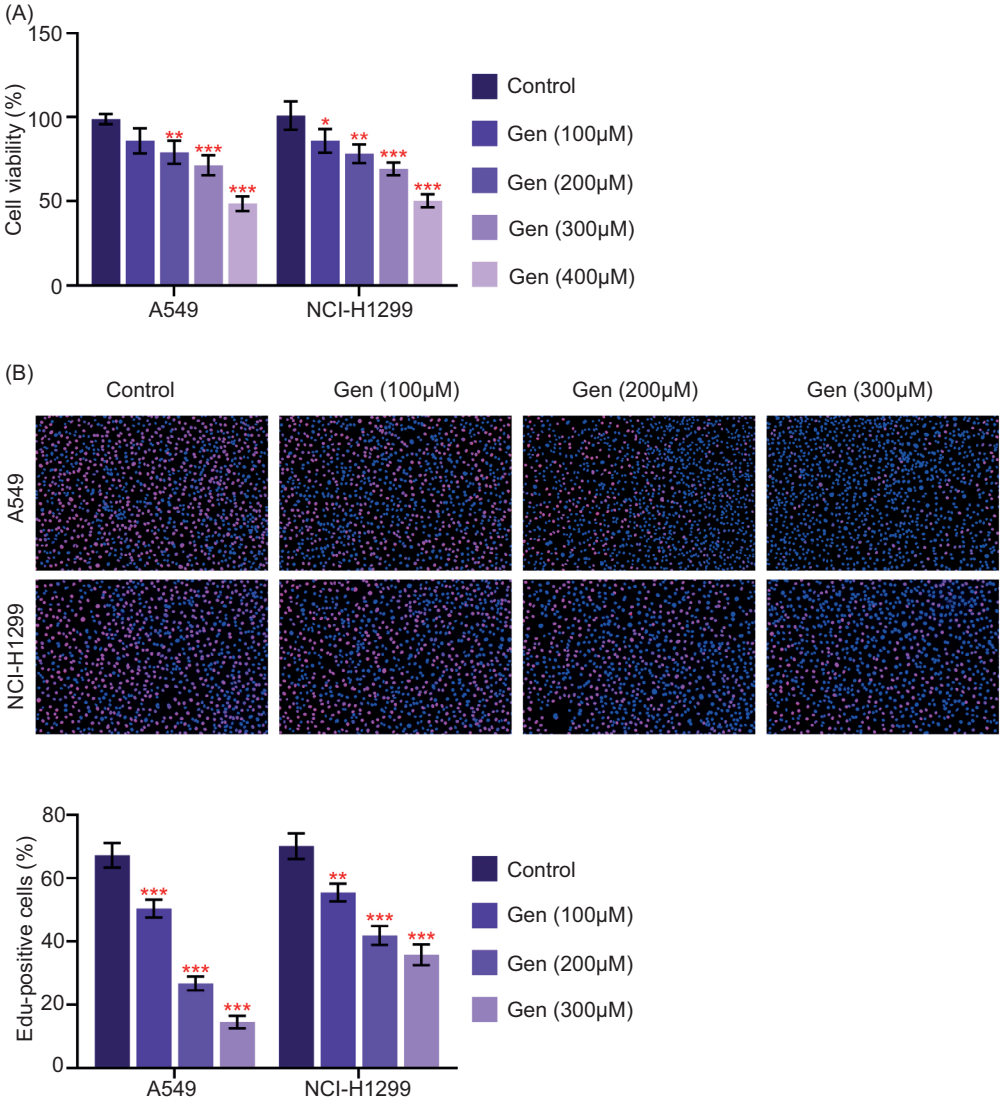

Figure 1. Geniposide repressed NSCLC cell proliferation. (A) Incubation with geniposide reduced the viability of A549 and NCI-H1299 cells in a dosage-dependent manner. (B) Incubation with geniposide reduced the number of EdU positive A549 and NCI-H1299 cells in a dosage-dependent manner. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

by 400-μM geniposide incubation (Figure 1A). Moreover, geniposide reduced the number of EdU positive A549 and NCI-H1299 cells in a dosage-dependent manner (Figure 1B), suggesting its antiproliferative effect against NSCLC.

Geniposide repressed NSCLC cell migration and invasion

Wound healing assay demonstrated that the wound width of A549 and NCI-H1299 cells was reduced by geniposide treatment (Figure 2A). The invasive number of A549 and NCI-H1299 cells was also reduced by geniposide treatment (Figure 2B), demonstrating the anti-migrative effect of geniposide against NSCLC.

Geniposide repressed angiogenesis of NSCLC

Human umbilical vein endothelial cells were treated with the supernatant of A549 and NCI-H1299 post-incubation with different concentrations of geniposide. The tube formation assay indicated that incubation with geniposide reduced the number of tubes (Figure 3), thus revealing its anti-angiogenic effect against NSCLC.

Geniposide reduced the expression of VEGF-A through activation of PPARγ

The protein expression of PPARγ was up-regulated, while VEGF-A was down-regulated in A549 and NCI-H1299 cells by geniposide treatment in a dosage-dependent manner (Figure 4A). A549 and NCI-H1299 cells were then co-treated with GW9662 and geniposide. Incubation with a potent antagonist of PPARγ, GW9662, attenuated geniposide-induced up-regulation of PPARγ and down-regulation of VEGF-A (Figure 4B), indicating that geniposide reduced VEGF-A in NSCLC through up-regulation of PPARγ.

Geniposide repressed NSCLC cell migration and angiogenesis through regulation of VEGF-A

In order to investigate the role of geniposide/VEGF-A in NSCLC, A549 cell was transfected with pcDNA–VEGF-A and incubated with 300 μM of geniposide. Over-expression of VEGF-A increased the viability of geniposide-induced A549 cell (Figure 5A). The suppressed A549 cell migration (Figure 5B) and invasion (Figures 5C and D) driven by geniposide were also

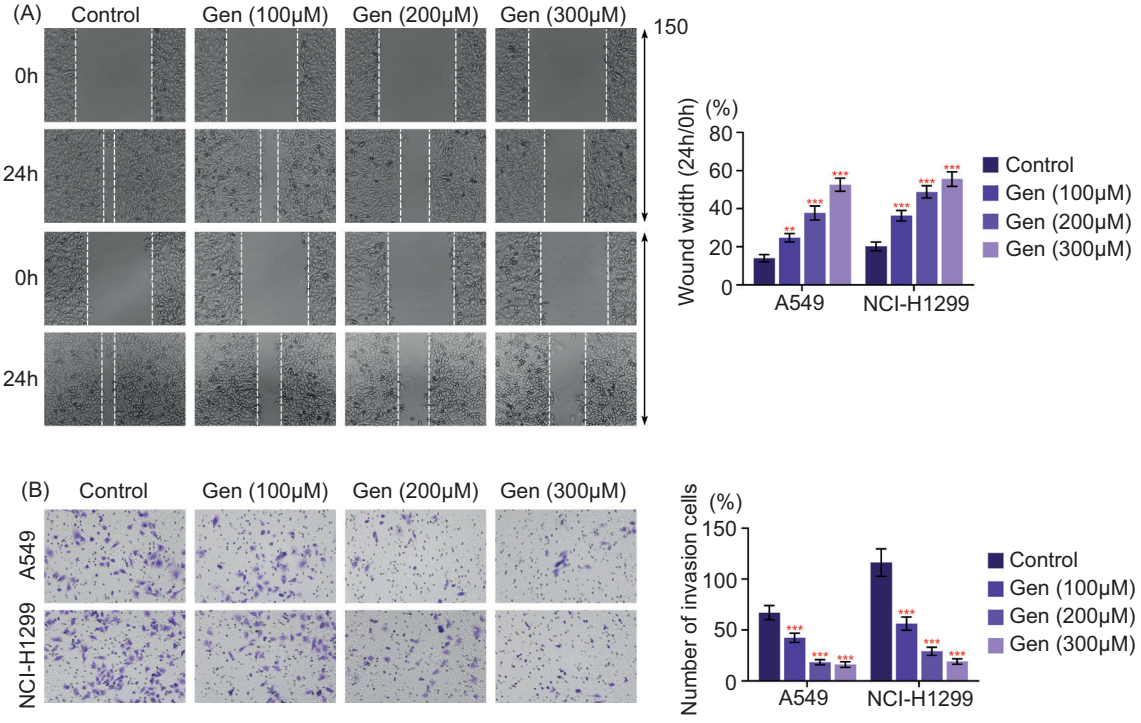

Figure 2. Geniposide repressed NSCLC cell migration and invasion. (A) Incubation with geniposide reduced wound width of A549 and NCI-H1299 cells in a dosage-dependent manner. (B) Incubation with geniposide reduced the number of invasive A549 and NCI-H1299 cells in a dosage-dependent manner. **$P < 0.01$, ***$P < 0.001$.

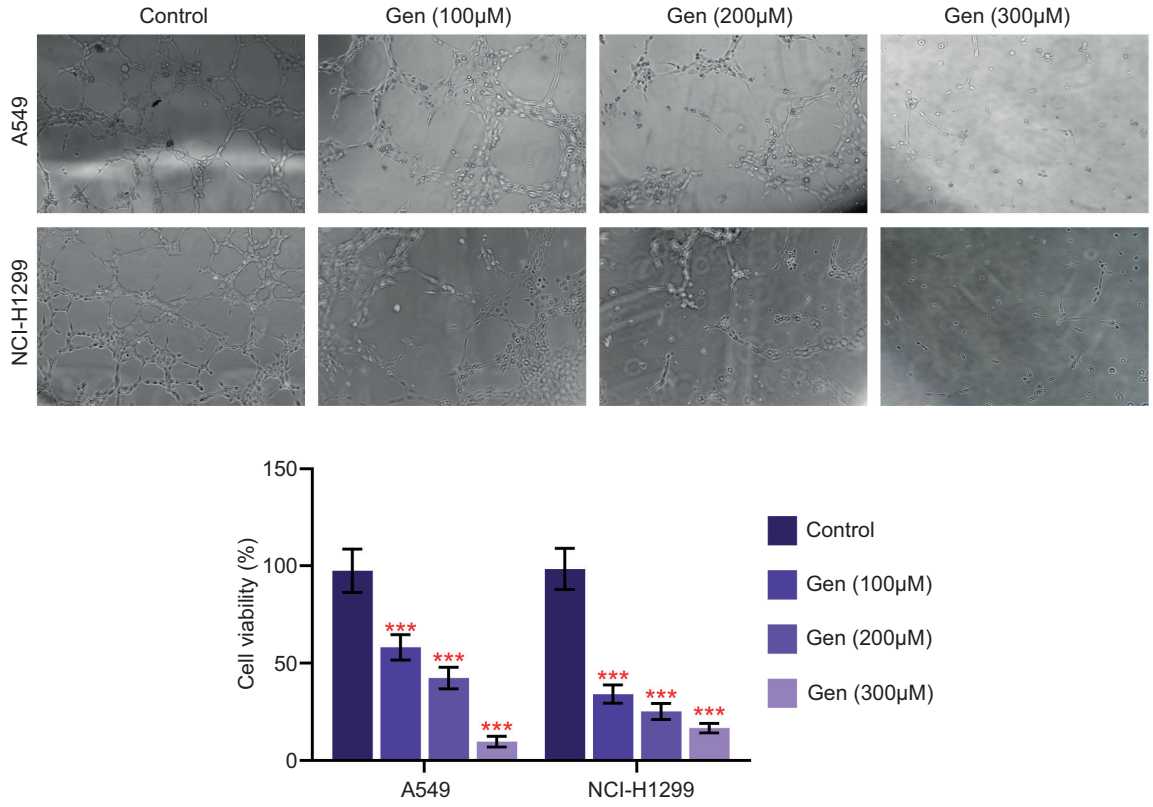

Figure 3. Geniposide repressed the angiogenesis of NSCLC. Incubation with geniposide reduced the number of tubes of HUVECs in a dosage-dependent manner. ***$P < 0.001$.

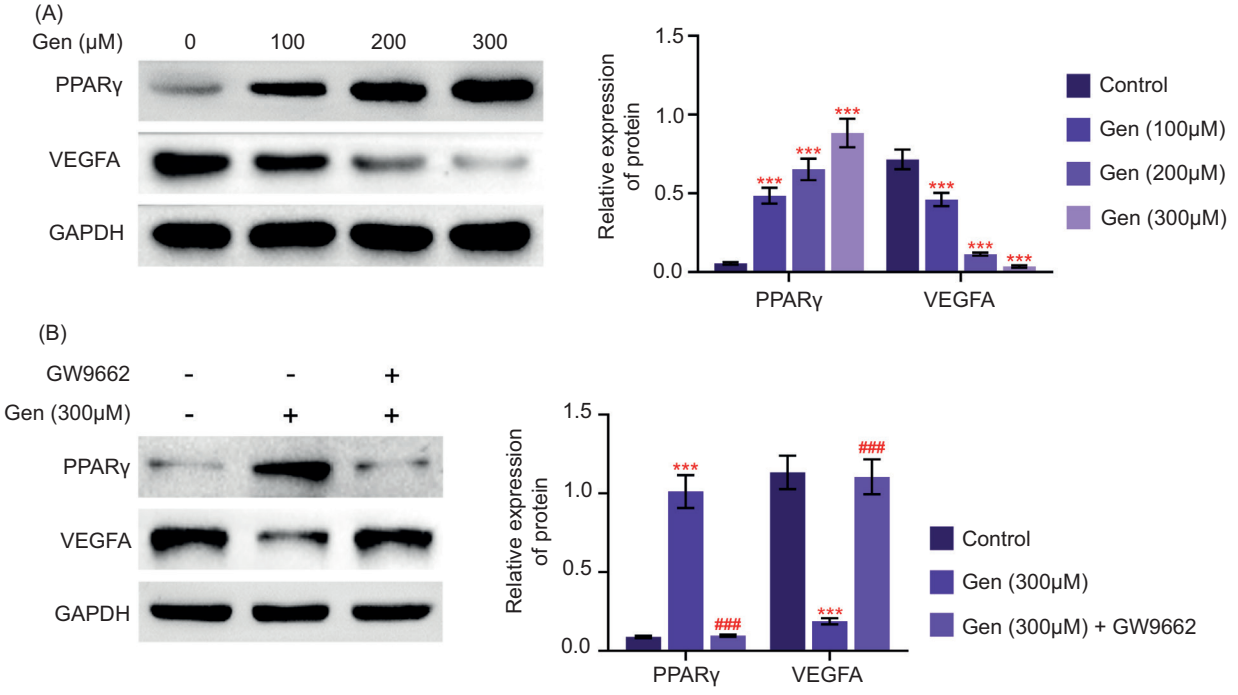

Figure 4. Geniposide reduced expression of VEGF-A through activation of PPARγ. (A) Incubation with geniposide reduced the protein expression of VEGF-A and enhanced PPARγ protein expression in A549 and NCI-H1299 cells in a dosage-dependent manner. (B) Incubation with a potent antagonist of PPARγ, GW9662, attenuated geniposide-induced up-regulation of PPARγ and down-regulation of VEGF-A in A549 and NCI-H1299 cells. *, ###$P < 0.001$.**

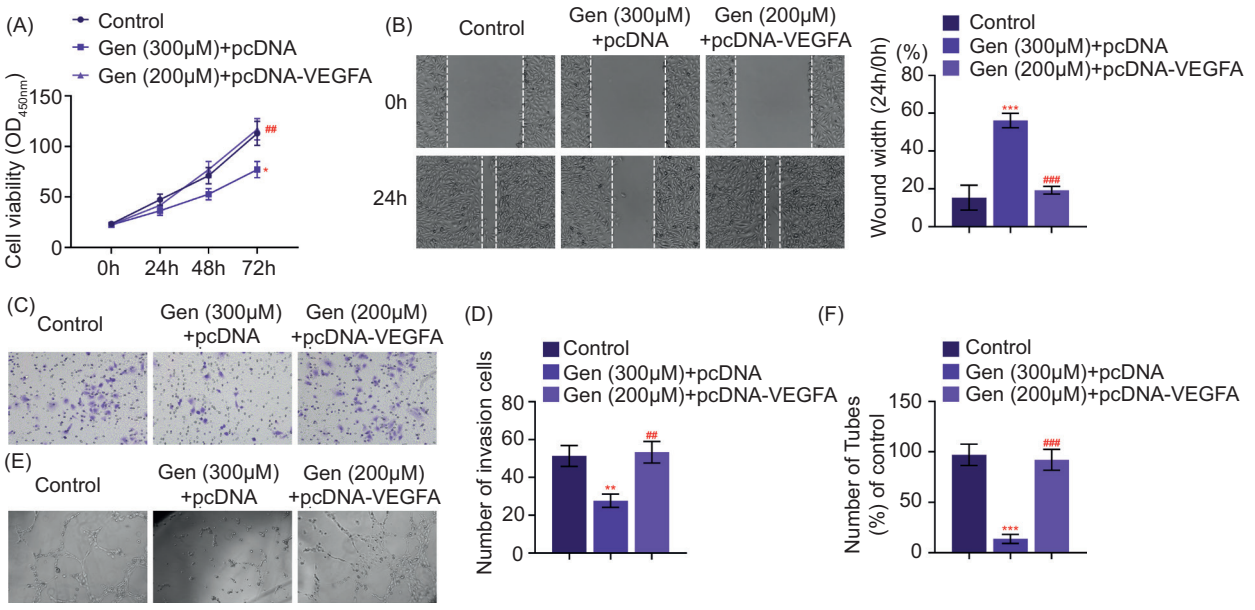

Figure 5. Geniposide repressed NSCLC cell migration and angiogenesis through regulation of VEGF-A. (A) Over-expression of VEGF-A increased the viability of geniposide-induced A549 cell. (B) Over-expression of VEGF-A increased the wound width of geniposide-induced A549 cell. (C) Over-expression of VEGF-A increased the number of invasive cells in geniposide-induced A549 cell. (D) The relative number of invasive cells in geniposide-induced A549 cell with transfection of pcDNA or pcDNA–VEGF-A. (E) Over-expression of VEGF-A increased the angiogenesis of geniposide-induced A549 cell. (F) The relative number of tubes in geniposide-induced A549 cell with transfection of pcDNA or pcDNA–VEGF-A. *$P < 0.05$, **,\#\#$P < 0.01$, *,\#\#\#$P < 0.001$.**

promoted by pcDNA–VEGF-A transfection. Over-expression attenuated geniposide-suppressed angiogenesis of A549 cell (Figures 5E and F), demonstrating that geniposide repressed NSCLC cell migration and angiogenesis through regulation of VEGF-A.

Discussion

This study found that geniposide repressed NSCLC cell proliferation and metastasis. Moreover, the angiogenesis of NSCLC was also repressed by geniposide. Traditional Chinese herbs are widely used as therapies or adjuvant therapies for the treatment of advanced NSCLC (Liu *et al.*, 2014; Zhang *et al.*, 2018). The derivative of geniposide, genipin, has been evidenced to promote the initiation of mitochondrial death cascade through regulation of p38MAPK signaling in NSCLC (Zhang *et al.*, 2015). Moreover, geniposide and genipin exerted cytotoxic effect on cancer cells, suppressing carcinogenesis and metastasis through regulation of cell cycle arrest, cell apoptosis, cell metastasis, invasion, and angiogenesis (Habtemariam and Lentini, 2018). Therefore, geniposide could be regarded as an anticancer agent for the prevention of NSCLC.

Genipin reduced the viability of NSCLC cell, H1299 (Zhang *et al.*, 2015). Results in this study also established that geniposide reduced the viability and suppressed the proliferation of A549 and H1299 cells in a dosage-dependent manner. Moreover, genipin induced G2/M arrest and promoted the apoptosis in H1299 cell through mitochondrial cellular execution pathway (Zhang *et al.*, 2015). Geniposide was also involved in mitochondrial-mediated apoptosis of PC12 cells (Guo *et al.*, 2009). Therefore, geniposide might exert pro-apoptotic effects against NSCLC through mitochondrial apoptotic cascade. Furthermore, NSCLC cell migration and invasion were suppressed by geniposide, suggesting that geniposide exerted anti-proliferative, pro-apoptotic and anti-invasive effects against NSCLC.

Angiogenesis and its related pathological process, metastasis, contribute to the release and activation of distinct factors to promote NSCLC cell proliferation and invasion (D'Amico, 2004). Geniposide has been depicted to suppress the angiogenesis of hepatocellular carcinoma through down-regulation of VEGF (Zhang *et al.*, 2020). Consistently, the results of this study evidenced the anti-angiogenic effect of geniposide against NSCLC, as demonstrated by the reduced number of tubes based on the tube formation assay. Moreover, the protein expression of VEGF, an important mediator in angiogenesis of NSCLC, was also reduced by geniposide. PPARγ functions as a tumor suppressor in NSCLC through regulation of tumor cell proliferation, differentiation, apoptosis, and

invasion (Han and Roman, 2010). VEGF was reported to be a downstream signaling of PPARγ in the regulation of cell apoptosis, proliferation, invasion, and angiogenesis (Han and Roman, 2010). Depletion of PPARγ promoted the invasiveness of lung cancer cells through activation of VEGF-A-mediated inhibition of cell apoptosis (Tian *et al.*, 2016). Antagonist of PPARγ reversed thiazolidinedione-induced up-regulation of VEGF in NSCLC cell (Yoshizaki *et al.*, 2010). Therefore, PPARγ/VEGF signaling was involved in NSCLC cell metastasis and angiogenesis. Geniposide has been reported to up-regulate PPARγ in lipopolysaccharide (LPS)-stimulated human renal tubular epithelial (HK-2) cells and cecal ligation in puncture-induced septic mice to improve renal injury in sepsis (Liu *et al.*, 2020). Moreover, VEGF-A was also predicted as a geniposide-related target (Jin *et al.*, 2021), as geniposide reduced the expression of VEGF-A to attenuate angiogenesis in rheumatoid arthritis (Wang *et al.*, 2021). Here, the protein expression of PPARγ was enhanced by geniposide in NSCLC, and antagonist of PPARγ reversed geniposide-induced up-regulation of PPARγ and down-regulation of VEGF-A, suggesting that geniposide reduced VEGF-A in NSCLC through up-regulation of PPARγ. Additionally, functional assays demonstrated that over-expression of VEGF-A attenuated geniposide-induced reduction of cell viability, migration, invasion, and angiogenesis in NSCLC, demonstrating that geniposide exerted anti-invasive and anti-angiogenic effects against NSCLC through PPARγ/VEGF signaling. Geniposide has been regarded as an agonist of glucagon-like peptide-1 receptor (Li, Li, Wu, & Zhou, 2019), and PPARγ is the downstream target of glucagon-like peptide-1 receptor(Onuma *et al.*, 2014). Therefore, geniposide might regulate PPARγ/VEGF signaling by targeting glucagon-like peptide-1 receptor.

Conclusion

In summary, this study indicated the *in vitro* antitumor effect of geniposide against NSCLC through inhibition of cell proliferation, migration, invasion, and angiogenesis. The underlying mechanism was associated with up-regulation of PPARγ and down-regulation of VEGF. Taken together, geniposide might function as an inhibitor of angiogenesis in NSCLC. However, its *in vivo* effect on tumor growth and angiogenesis of NSCLC should be investigated in the future research. The present research established that geniposide could be transformed to genipin by β-glucosidase in the bowel (Li *et al.*, 2019). Genipin induced NSCLC cell apoptosis (Yang *et al.*, 2013), and suppressed angiogenesis of hepatocellular carcinoma (Hong *et al.*, 2020). Therefore, geniposide might undergo biotransformation into genipin to exert antitumor and anti-angiogenic actions in NSCLC.

Competing Interests

The authors state that there Were no conflict of interest to disclose.

Author Contributions

Ming Jiang designed and conducted the experiments. Shiying Zheng analyzed and interpreted the data, prepared the manuscript with contributions from the co-author.

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