Ginsenoside 20(S)-Rh2 exerts anti-cancer activity through the Akt/GSK3β signaling pathway in human cervical cancer cells

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Abstract. Ginsenoside 20(S)-Rh2 (GRh2) is a bioactive compound derived from ginseng that is believed to maintain health in traditional Chinese medicine. Emerging evidence has suggested that GRh2 exhibits anti-cancer activity. The present study hypothesized that GRh2 has an anti-cancer function in human cervical cancer cells. An MTS assay demonstrated that GRh2 attenuated proliferation of HeLa cells in a dose- and time-dependent manner. In addition, GRh2 inhibited migration and invasion, as determined by wound healing and transwell invasion assays, respectively. Furthermore, GRh2 treatment reduced expression of mesenchymal markers N-cadherin and vimentin as well as epithelial mesenchymal transition transcriptional factor zinc finger E-box-binding homeobox 1 and snail1, and increased the protein expression levels of epithelial marker E-cadherin. In addition, the results revealed that GRh2 prevented activation of the protein kinase B (Akt)/glycogen synthase kinase (GSK)3β signaling pathway in HeLa cells. In conclusion, the results suggested that GRh2 inhibits cervical cancer cell proliferation by targeting the Akt pathway, and prevents cervical cancer cell migration and invasion by suppressing the Akt/GSK3β regulated EMT process, and therefore, GRh2 may have the potential to be a novel anti-cancer agent for cervical cancer.

Introduction

Cervical cancer is one of the most common malignancies in woman worldwide, which occurs and develops on the surface of the cervix (1). A total of >510,000 patients are diagnosed with cervical cancer, and >288,000 patients die from the disease each year worldwide (1). The primary cause of cervical cancer is infection with human papillomavirus (HPV) (2). Although vaccines to high-risk human HPV (hrHPV) strains may prevent infection with hrHPV, cervical cancer remains a primary cause of cancer-associated mortality, particularly in under-developed countries (3,4).

Tumor metastasis is the primary cause of cancer-associated death. Numerous studies have suggested that epithelial mesenchymal transition (EMT) contributes to migration, invasion and metastasis of cancer (5,6). During the EMT process, epithelial cells undergo a cell morphology alteration from the polarized epithelial phenotype to mesenchymal phenotype, loss of cell-cell junction and epithelial markers including E-cadherin, acquirement of mesenchymal markers N-cadherin, vimentin and febronectin, and cytoskeleton rearrangement, which leads to gain of mesenchymal traits including cell migration and invasion (7,8). The EMT process is regulated by several transcription factors including zinc finger E-box-binding homeobox 1 (Zeb1), snail1, snail2 (Slug) and Twist, which repress the expression of E-cadherin and other cell adhesion and cytokeratin genes, and increase expression of mesenchymal markers vimentin, fibronectin and N-cadherin (9). Several signaling pathways are activated during EMT, including nuclear factor (NF)-κB, glycogen synthase kinase (GSK)3β, Notch, protein kinase B (Akt) and mitogen activated protein kinase (MAPK), which have been demonstrated to promote metastasis of lung, breast, ovarian and prostate cancers (10-13). In addition, EMT is involved in anti-cancer drug resistance (14). Therefore, EMT is a target of cancer therapy, particularly in metastasized cancer types.

Currently, chemotherapy and radiotherapy are the principal therapeutic strategies for cervical cancer. However, these two therapeutic approaches have severe side effects including headache, abnormal respiration, cirrhosis and cardiac dysfunction. There is therefore increasing interest in the study of safe and effective anti-cancer compounds from natural plants (15). Ginseng is considered as a panacea and is frequently used in traditional Chinese medicine (16,17). Ginsenosides are one of the primary bioactive ingredients in ginseng, which have various pharmacological effects including anti-obesity, anti-diabetic, regulation of blood pressure, anti-aging, immune regulation and anti-tumor functions (18-22). Ginsenosides are classified into three types, including protopanaxatriol-type, oleanolic acid-type and protopanaxadiol-type ginsenosides (23). Ginsenoside 20(S)-Rh2 (GRh2) belongs to the protopanaxadiol-type ginsenoside group. Several studies have reported that GRh2 exhibits anticancer activities in

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glioblastoma, skin squamous cell carcinoma, pancreatic cancer, prostatic cancer and leukemia (24-28). A previous study used GRh2 to inhibit cancer cell proliferation and induce transformation to normal cells in patients with cervical cancer (29). However, the effect of GRh2 on cervical cancer, the EMT process and its underlying mechanism remains unclear.

In the present study, it was demonstrated that GRh2 effectively inhibited proliferation, migration, invasion and EMT by targeting the Akt/GSK3β signaling pathway in human cervical cancer cells. Therefore, the results suggested that GRh2 may have the potential to be a novel anti-cancer agent for cervical cancer.

Materials and methods

Reagents and antibodies. Commercial GRh2 was obtained from Sichuan Weike Biotechnology Co., Ltd. (Chengdu, China). GRh2 was prepared in a stock of 100 mg/ml and applied to cultured HeLa cells at 10, 25 and 50 μM. Antibodies for N-cadherin (cat. no. 13116), E-cadherin (cat. no. 3195), vimentin (cat. no. 5741), Zeb1 (cat. no. 3396), snail1 (cat. no. 3879), phosphorylated (p)-Akt (cat. no. 4060), Akt (cat. no. 4691), p-GSK3β (cat. no. 9327), GSK3β (cat. no. 5676) and β-actin (cat. no. 4970) were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Cell culture. Human cervical carcinoma HeLa cells were purchased from Shanghai Institute of Pharmaceutical Industry (Shanghai, China). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% streptomycin and penicillin at 37˚C in an environment containing 5% CO_2. DMEM and FBS were purchased from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA).

Cell proliferation assay. Cell proliferation was detected by 3-(4, 5-dimethylthiazol-2-yl)-5 3-carboxymethonyphenol)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. $1x10^3$/ml HeLa were seeded into each well of a 96-well microplate. Then, cells were incubated with media containing normal saline (control) or different concentrations of GRh2 (10, 25 and 50 μM) respectively. Cells were incubated for 6, 24, 48, 72, 96 and 120 h and then incubated with 10 μl MTS reagent (Promega Corporation, Madison, WI, USA) at 37˚C for 4 h according to the manufacturer's protocol. The absorbance was measured at a wavelength of 490 nm using a microplate reader. Proliferation inhibition rates were determined following treatment for 120 h. % proliferation inhibition rate=(1-drug group/control group) x100.

Transwell invasion assays. For the matrigel assay, $5x10^4$ HeLa cells at the logarithmic growth phase were supplemented with 100 μl serum-free DMEM and seeded into the upper chamber of a transwell plate with 8-mm pore size (BD Biosciences, Franklin Lakes, NJ, USA). The upper side of the filter member of the chamber was coated with Matrigel (BD Biosciences) that was diluted with DMEM media (1:3). A total of 600 μl DMEM media supplemented with 1% FBS was added in the lower chamber, and GRh2 at a concentration of 0 μM (control) or 10, 25 and 50 μM was added into the upper chamber. Following incubation for 24 h at 37˚C, the cells in the upper membrane were discarded and cells on the lower membrane were fixed using 4% paraformaldehyde for 30 min and stained with crystal violet (Beyotime Institute of Biotechnology, Haimen, China) for 10 min at room temperature. Next, five random fields were captured with a phase contrast video microscope (ELWD 0.3; Nikon Corporation, Tokyo, Japan; magnification, x40) and then the cells were counted. Each experiment was performed in triplicate.

Wound healing assay. For wound healing assay, $3x10^5$ HeLa cells at the logarithmic growth phase were seeded into 6-well plates. At 80% confluence, cells were incubated with GRh2 at a concentration of 0 μM (control), or 10, 25 and 50 μM, and the monolayer was disrupted with a cell scraper and captured at 0 and 48 h with a phase contrast video microscope (model ELWD 0.3; Nikon Corporation, Tokyo, Japan; magnification, x100). Each experiment was performed in triplicate. The percentage of wound closure between the wound edges at different time points was measured with Image-Pro Plus version 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA). % wound closure=(1-distance at 0 h/distance at 48 h) x100.

Protein extraction and western blotting. For detection of N-cadherin, E-cadherin, vimentin, zeb1 and snail1 expression, HeLa cells were treated with GRh2 at a concentration of 0 μM (control), or 25, and 50 μM for 48 h at 37˚C in an environment containing 5% CO_2. To investigate the activity of the Akt and GSK3β signaling pathway, HeLa cells were treated with GRh2 at the concentration of 0 μM (control) or 25 and 50 μM for 12 h at 37˚C in an environment containing 5% CO_2. Cells were lysed using a radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology) supplemented with complete EDTA-free protease inhibitor cocktail tablets (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol. Total protein concentrations were determined with the bicinchoninic acid protein assay kit (Applygen Technologies Inc., Beijing, China). A total of 40 μg protein in each sample was loaded onto 8% SDS-PAGE gels, and transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Following blocking with 5% nonfat milk for 30 min at room temperature, the membranes were incubated with primary antibodies including N-cadherin (1:1,000 dilution), E-cadherin (1:1,000 dilution), vimentin (1:1,000 dilution), Zeb1 (1:1,000 dilution), snail1 (1:1,000 dilution), phosphorylated (p)-Akt (1:2,000 dilution), Akt (1:1,000 dilution), p-GSK3β (1:1,000 dilution), GSK3β (1:1,000 dilution) and β-actin (1:1,000 dilution) at 4˚C overnight. The membranes were next incubated with horseradish peroxidase-conjugated anti-rabbit IgG (cat. no. A0545; 1:5,000 dilution; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 1 h at room temperature. Signals were visualized using enhanced chemiluminescence reagent (EMD Millipore, Billerica, USA) and protein expression levels normalized to β-actin were calculated by using ImageJ software (version 1.48; National Institutes of Health, Bethesda, MD, USA). Detection of the protein of interest was repeated three times. The quantification of the western blots presented in the figures are representative of three independent experiments.

Statistical analysis. All tests were performed in triplicate and the experiments were repeated three times independently.

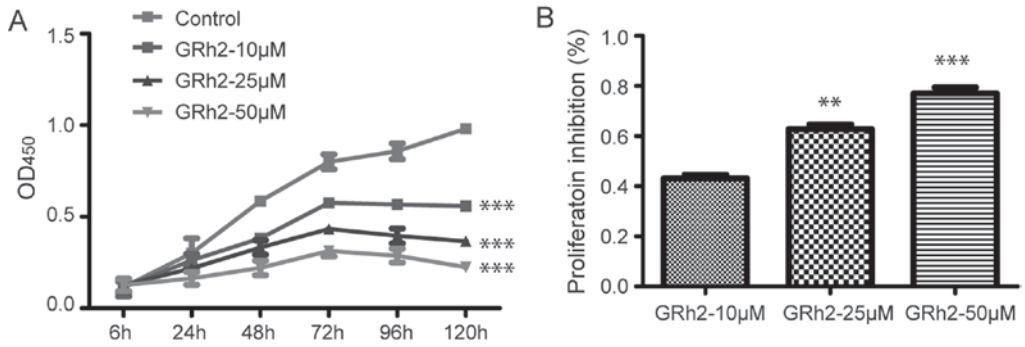

Figure 1. Effect of GRh2 on the proliferation of human cervical cancer cells. HeLa cells were treated with cell culture media containing GRh2 at 10, 20 and 50 μM, or normal saline as a control. Cell proliferation was analyzed following treatment for 6, 24, 48, 72, 96 and 120 h. (A) The absorbance at a wavelength of 450 nm was measured. (B) Following treatment with GRh2 for 120 h, the proliferation inhibition rate was determined. The results are representative of three independent experiments. All data represent the mean ± standard deviation. **P<0.01 and ***P<0.001 vs. control. GRh2, Ginsenoside 20(S)-Rh2; OD, optical density.

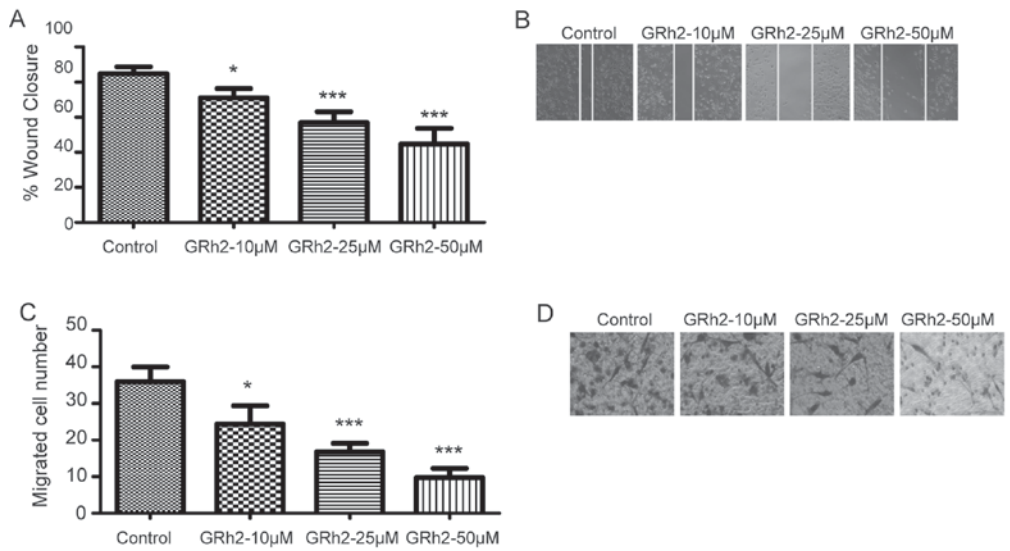

Figure 2. Effect of GRh2 on migration and invasion of human cervical cancer cells. HeLa cells were treated with GRh2 at concentrations of 0 μM (control group), or 10, 20 and 50 μM. (A) A wound healing assay was performed and the percentage of scratch wound healing (n=9) was calculated following treatment for 48 h. (B) Representative images of wound healing. (C) A transwell invasion assay was performed and the migrated cell number (n=3) was calculated following treatment for 24 h. (D) Representative images of transwell invasion. All data represent the mean ± standard deviation. *P<0.05 and ***P<0.001 vs. control group. GRh2, Ginsenoside 20(S)-Rh2.

Data was expressed as mean ± standard deviation. A one-way analysis of variance followed by Tukey's post hoc test was conducted to assess differences among multiple groups. All statistical calculations were carried out using SPSS software, version 19.0 (IBM Corp., Armonk, NY, USA) and P<0.05 was considered to indicate a statistically significant difference.

Results

GRh2 inhibits cell viability and proliferation of cervical cancer. To evaluate the function of GRh2 on cervical cancer cells, HeLa cells were exposed to various concentrations of GRh2 and the cell proliferation was assessed. Compared with the control group, GRh2 treatments inhibited HeLa cell proliferation in a dose- and time-dependent manner (P<0.001; Fig. 1A). Compared with the control group, proliferation inhibition rates were 40, 60 and 80% at GRh2 concentrations of 10, 25 and 50 μM, respectively, at 120 h (Fig. 1B). These results suggested that GRh2 inhibited the proliferation of cervical cancer cells.

GRh2 inhibits cell migration and invasion of cervical cancer. To ascertain the role of GRh2 in cervical cancer cell migration, wound healing and transwell invasion assays were performed in Hela cells treated with GRh2 at concentrations of 0 μM (control), or 10, 25 and 50 μM. The migration abilities were inhibited in HeLa cells treated with GRh2 in a dose-dependent manner (P<0.05 vs. control; Fig. 2A and B). Similar inhibitory effects were observed when detecting the invasion ability of HeLa cells by a Matrigel-coated transwell invasion assay (P<0.05 vs. control; Fig. 2C and D). These results suggested that GRh2 inhibited cell migration and invasion of cervical cancer.

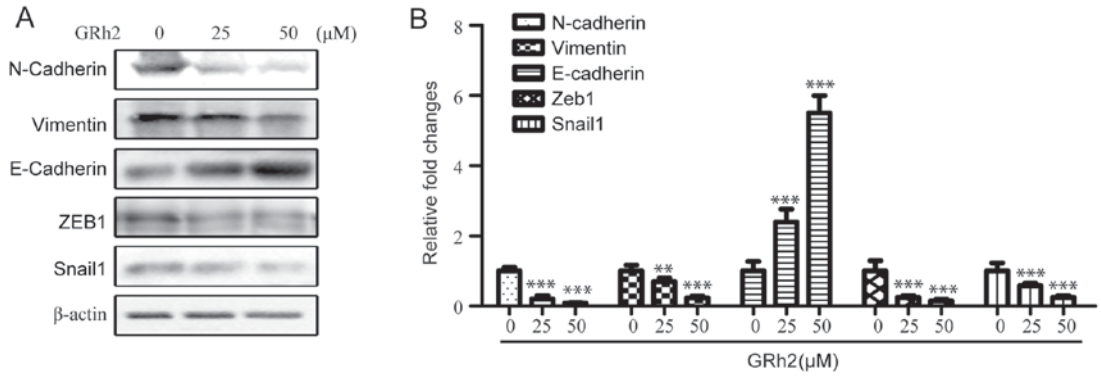

Figure 3. Effect of GRh2 on EMT in human cervical cancer cells. HeLa cells were treated with GRh2 at concentrations of 0 μM (control group), or 25 and 50 μM for 24 h. (A) Western blotting was carried out to detect the protein expression level of EMT markers N-cadherin, vimentin and E-cadherin as well as EMT transcription factors Zeb1 and snail1. (B) The results of the western blotting were quantified. The results are representative of three independent experiments. All data represent the mean ± standard deviation. **P<0.01 and ***P<0.001 vs. control group. GRh2, Ginsenoside 20(S)-Rh2; EMT, epithelial mesenchymal transition; Zeb1, zinc finger E-box-binding homeobox 1.

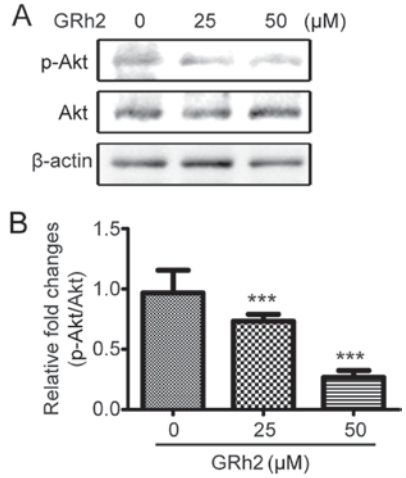

Figure 4. Effect of GRh2 on the Akt signaling pathway in human cervical cancer cells. HeLa cells were treated with GRh2 at concentrations of 0 μM (control group), or 25 and 50 μM for 10 min. (A) Basal protein and phosphorylation levels of Akt were detected by western blotting. (B) The results of the western blotting were quantified. The results are representative of three independent experiments. All data represent the mean ± standard deviation. ***P<0.001 vs. control group. GRh2, Ginsenoside 20(S)-Rh2; Akt, protein kinase B; p, phosphorylated.

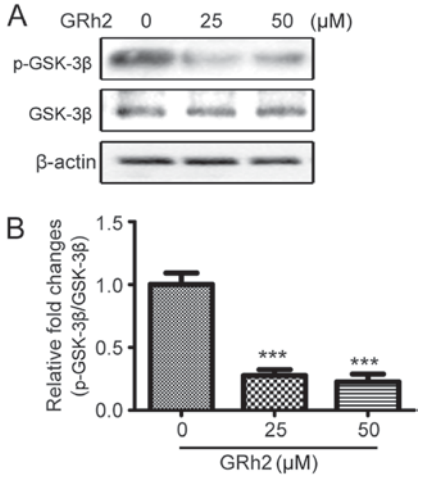

Figure 5. Effect of GRh2 on the GSK3β signaling pathway in human cervical cancer cells. HeLa cells were treated with GRh2 at concentrations of 0 μM (control group), or 25 and 50 μM for 10 min. (A) Basal protein and phosphorylation levels of GSK3β were detected by western blotting. (B) The results of the western blotting were quantified. The data represent the mean ± standard deviation of three independent experiments. ***P<0.001 vs. control group. GRh2, Ginsenoside 20(S)-Rh2; GSK3β, glycogen synthase kinase 3β, p, phosphorylated.

GRh2 inhibits epithelial to mesenchymal transition of cervical cancer. To investigate whether GRh2 inhibits the EMT process, EMT markers were detected in HeLa cells treated with different concentrations of GRh2 (0, 25 and 50 μM) for 48 h. As expected, GRh2 treatment decreased the expression levels of N-cadherin and vimentin, and increased the protein expression levels of epithelial marker E-cadherin (Fig. 3A and B). The effects of GRh2 on the expression levels of EMT control transcription factors, Zeb1 and snail1, were also determined. The results revealed a downregulation in the expression levels of Zeb1 and snail1 in the GRh2-treated groups (Fig. 3A and B). These data suggested that GRh2 effectively inhibited EMT in a dose-dependent manner in cervical cancer cells.

GRh2 inhibits the Akt signaling pathway in cervical cancer cells. The effect of GRh2 on the Akt signaling pathway was investigated. HeLa cells were treated with different concentrations of GRh2 (0, 10, 25 and 50 μM) for 12 h. The phosphorylation and protein expression levels of Akt were then detected. GRh2 treatment significantly inhibited the phosphorylation of Akt compared with cells treated with 0 μM GRh2 (Fig. 4A and B), which suggested that GRh2 inhibited the activation of the Akt pathway.

GRh2 inhibits the GSK3β signaling pathway in cervical cancer cells. GSK3β is a downstream pathway of Akt. It has been reported that the Akt/GSK3β pathway regulates EMT (30). Therefore, the effect of GRh2 on the GSK3β pathway was investigated. HeLa cells were treated with different concentrations of GRh2 (0, 10, 25 and 50 μM) for 12 h. The activation of GSK3β was then detected. GRh2 treatment significantly suppressed the phosphorylation of GSK3β compared with cells treated with 0 μM GRh2 (Fig. 5A and B), which suggested that GRh2 inhibited the activation of the Akt/GSK3β pathway.

Discussion

Currently, cancer is one of the most common diseases that leads to mortality in humans (31). Chemotherapy and radiotherapy are the primary clinical therapeutic approaches. However, a limitation of these treatment strategies is toxicity to normal cells in addition to the tumor cells. The identification of safe and efficacious anticancer drugs is of primary concern (32). Natural products are the primary source for the development of anti-cancer agents (33). The anticancer activities of Panax ginseng have been demonstrated by several studies (34,35). Ginsenosides, a group of bioactive compounds of Panax ginseng, exhibit anticancer activities (36,37). GRh2 has been demonstrated to inhibit cancer cell proliferation, migration and invasion, and prevent tumor growth and metastasis (24-28). However, only a small number of studies have explored the effects of GRh2 in human cervical cancer.

In the present study, the effect of GRh2 on cell proliferation of human cervical cancer cells was investigated. The results revealed that GRh2 impaired HeLa cell proliferation in a dose- and time-dependent manner. These findings are in accordance with previous reports that demonstrated that ginseng Rh2 prevents cell proliferation in colon, lung, liver, and gastric cancers, in addition to leukemia and glioblastoma (38-42). In addition, it was observed that GRh2 inhibited the activation of the Akt pathway in HeLa cells. Akt mediates various biological processes, including glucose metabolism, cell survival, proliferation and differentiation (43,44). Collectively, the results of the present study, and previous studies, suggest that GRh2 may inhibit human cervical cancer cell proliferation via impairment of the Akt signaling pathway.

Furthermore, the present study revealed that GRh2 decreased the migration and invasion of human cervical cancer cells, which concurred with previous reports that Rh2 inhibits cell migration and invasion in C2C12 skeletal muscle cells (45,46). EMT is an important cellular process that promotes tumor progression and metastasis (47). To ascertain the underlying molecular mechanism of GRh2 inhibition of cervical cancer migration and invasion, the present study investigated the effects of GRh2 on EMT. The results demonstrated that Rh2 treatment increased the expression levels of epithelial marker E-cadherin and decreased the expression levels of mesenchymal N-cadherin and vimentin, in addition to EMT-control transcription factors Zeb1 and snail1. In addition, GRh2 treatment impaired the activation of Akt and the phosphorylation of GSK3β. Aberrant activation of the Akt signaling pathway was observed during tumor proliferation and metastasis as well as during the progression of EMT (45,48,49). GSK3β is activated when it is dephosphorylated. The inhibition of Akt pathway can activateGSK3β by preventing its phosphorylation (50,51). The activation of GSK3β induces the degradation of Snail. As a transcriptional factor, Snail inhibits the expression of epithelial marker E-cadherin and activates the transcription of mesenchymal N-cadherin and vimentin (47,52-54). Thus, degradation of Snail increases the expression of epithelial marker E-cadherin and decreases the expression of mesenchymal N-cadherin and vimentin. Accordingly, the results of the present study demonstrated that GRh2 inhibited AKT pathway, dephosphorylated and activated GSK3β and inhibited the expression of Snail, which lead to upregulation of E-cadherin and downregulation of N-cadherin and vimentin. Taken together with the data from the present study and previous observations, it is suggested that GRh2 inhibited the expression of Snail and prevented EMT by inhibiting AKT and activating GSK3β.

In conclusion, the present study demonstrated that GRh2 inhibits cell proliferation of cervical cancer by suppressing the Akt signaling pathway. In addition, the data suggested that GRh2 prevents cell migration, invasion and EMT via inhibition of the Akt/GSK3β signaling pathway. Therefore, these findings suggested that GRh2 may be a potential treatment strategy for cervical cancer treatment.

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