Original Article

Tumor Biology

Tumor Biology
May 2017: 1–10

DOI: 10.1177/1010428317692225
journals.sagepub.com/home/tub
SAGE

Ginsenoside 20(S)-Rg3 suppresses ovarian cancer migration via hypoxia-inducible factor 1 alpha and nuclear factor-kappa B signals

Ting Liu[1,2]*, Le Zhao[3]*, Huilian Hou[4], Lu Ding[5], Wei Chen[6] and Xu Li[3]

Abstract

Hypoxia-inducible factor 1 is believed to play a prominent role in the survival and developing progress of cancers. As a result, inhibiting α subunit of hypoxia-inducible factor 1 represents an attractive strategy against tumor. Although hypoxia-inducible factor 1α is a hypoxia-regulated subunit, increasing evidence indicates that hypoxia-inducible factor 1α could stable expression under normoxic conditions, regulated by non-hypoxia-mediated mechanisms. However, there are few strategies to target hypoxia-inducible factor 1α under normoxic conditions. Here, we report that ginsenoside 20(S)-Rg3, one of the main active ingredients in red ginseng, restrains hypoxia-inducible factor 1α expression under normal oxygen levels in human ovarian cancer cell lines, SKOV3 and 3AO, which leads to potently inhibits migration of ovarian cancer in vitro and in vivo. 20(S)-Rg3 could decrease the expression of hypoxia-inducible factor 1α by upregulation of prolyl hydroxylase domain protein 1 to promoting hypoxia-inducible factor 1α ubiquitin–proteasome degradation under normal oxygen levels. Furthermore, 20(S)-Rg3 could attenuate the expression of nuclear factor-κ B, which may be another possible mechanism for 20(S)-Rg3 to block ovarian cancer migration. Taken together, our study suggests that 20(S)-Rg3 is a strong inhibitor of hypoxia-inducible factor 1α, which may provide a novel agent for future treatments for ovarian cancer.

Keywords

20(S)-Rg3, hypoxia-inducible factor 1α, nuclear factor-κ B, migration, ovarian cancer

Date received: 28 April 2016; accepted: 17 August 2016

Introduction

Although great advances have been made in therapeutic management, ovarian cancer remains the most lethal gynecologic malignancy. Numerous patients with ovarian cancer already harbor tumor invasion and metastasis upon initial diagnosis, which leads to poor prognosis. It has been proved that invasion and metastasis are the major causes for high mortality of ovarian cancer. Therefore, devising novel therapeutic strategies against the invasion and metastasis of ovarian cancer is very important for improving patient outcomes in the clinical treatment.

Hypoxia-inducible factor 1 (HIF-1) is a vitally important transcription factor for intratumoral hypoxia, which can make a significant contribution toward tumor invasion and metastasis.[1] HIF-1 consists of a hypoxia-regulated α subunit and a stable β subunit. Importantly, more and more new medicines targeting HIF-1α have been discovered to

[1]Department of Radiology, the First Affiliated hospital of Xi'an Jiaotong University, Xi'an, China
[2]Department of Obstetrics & Gynecology, the First Affiliated hospital of Xi'an Jiaotong University, Xi'an, China
[3]Center for Translational Medicine, First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, China
[4]Department of Pathology, First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, China
[5]Department of Anesthesiology, First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, China
[6]Center for Laboratory Medicine, the First Affiliated Hospital School of Medicine, Xi'an Jiaotong University, Xi'an, China

*These authors contributed equally to this work.

Corresponding author:
Xu Li, Center for Translational Medicine, First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, 710061, China.
Email: lixu56@mail.xjtu.edu.cn

block tumor progression. Zhang et al.[2] reported that digoxin suppressed tumor growth by decreasing HIF-1α synthesis. Zagzag et al.[3] demonstrated that geldanamycin could prevent migration of glioma cells through interfering with the induction of HIF-1α. Sluis showed that the gene encoding COMM domain–containing 1 (COMMD1) disrupted HIF-1α/β dimerization and repressed tumor cell invasion.[4] There are two prolyl residues (Pro402 and 564) in human HIF-1α which are called the oxygen degradation domain (ODD). Generally, HIF-1α is rapidly degraded under well-oxygenated conditions. The ODD could be post-translationally hydroxylated by prolyl hydroxylase domain (PHD) proteins (PHD1-3). Then, HIF-1α can be recognized by von Hippel–Lindau protein (VHL), an E3 ubiquitin ligase complex, to form a VHL-HIF complex. The formation of the complex leads to degradation by the 26S proteasome. However, evidences are accumulating to suggest that various non-hypoxia-mediated mechanisms might also contribute to HIF-1α stable expression. Tumor necrosis factor alpha (TNF-α) has been proved to regulate chondrocyte HIF-1α protein expression under normoxic conditions.[5] ODD mutations caused HIF-1α protein expression in invasive breast cancer independent of hypoxia.[6] However, there are few strategies to target HIF-1α in tumors under normoxic conditions.

Red ginseng has been widely used in Asian countries as a traditional medicine. Ginsenoside 20(S)-Rg3 is one of the main active ingredients in red ginseng. Because of the different orientations of the C-20 hydroxyl, there are two stereoisomers of Rg3-20(S) and 20(R) forms. It is reported that 20(S)-Rg3 may have more effective antitumor activity than 20(R)-Rg3 due to its orientation of the C-20 hydroxyl, making it an more efficient regulator of ion channels.[7] It has been shown that 20(S)-Rg3 owns a wide spectrum of biological activities including anticancer, anti-inflammatory, and anti-angiogensis.[7–10] Recently, 20(S)-Rg3 was proved to be capable of promoting senescence and apoptosis in gallbladder cancer, ovarian cancer, and leukemia cells.[10–12] It has also been demonstrated that 20(S)-Rg3 could suppress cells' migration and invasion in lung cancer and colorectal cancer.[13,14] Interestingly, our previous results have shown that 20(S)-Rg3 could inhibit the Warburg effect in ovarian cancer cells.[15] All of these above suggest that 20(S)-Rg3, an active and safe nature medicine, has a potential anticancer activity.

Our previous studies have shown that 20(S)-Rg3 could inhibit hypoxia-induced epithelial–mesenchymal transition in ovarian cancer cells by reducing the expression of HIF-1α under hypoxia conditions.[14] Significantly, there are various non-hypoxia-mediated mechanisms that might also lead to HIF-1α stable expression and tumor progression under normoxic conditions.[5] However, there has not been any evidence to show whether 20(S)-Rg3 may regulate HIF-1α expression under normal oxygen levels before. Here, we found the inhibitory effect of the 20(S)-Rg3 on migration of ovarian cancer cells. Mechanism dissection revealed that 20(S)-Rg3 could decrease HIF-1α expression via upregulation of PHD1 expression under normal oxygen levels, besides 20(S)-Rg3 could also inhibit nuclear factor-kappa B (NF-κb) expression. These findings suggest that 20(S)-Rg3 could serve as a novel strategy in treating ovarian cancer.

Materials and methods

Chemicals

Ginsenoside 20(S)-Rg3 is a reference compound (purity ⩾ 99%) purchased from Tasly Pharmaceutical Company (Tianjin, China). 20(S)-Rg3 was dissolved in dimethyl sulfoxide (DMSO) in a 4 mg/mL stock solution and stored at −20°C after filtering by 0.2 mm membrane. The final concentrations of DMSO in the culture medium were less than 0.05%. It was diluted by cell culture media to final concentrations (80 μg/mL for SKOV3, 160 μg/mL for 3AO) for the in vitro study, and diluted by phosphate-buffered saline (PBS) to 5 mg/kg for the in vivo study. The concentrations of the 20(S)-Rg3 were designed according to our previous study.[14] For transwell migration assay, western blot, and real-time polymerase chain reaction (RT-PCR), cells were treated with 20(S)-Rg3 for 24 h. For wound-healing assay, cells were treated with 20(S)-Rg3 for 36 h. For transient transfection, cells were treated by 20(S)-Rg3 for 24 h followed by transient transfection treatment with small interfering RNA (siRNA) for 24 h.

MG132 (Cayman Chemical, Ann Arbor, MI, USA) was dissolved in DMSO in a 25 mg/mL stock solution and stored at −20°C. The final concentrations of DMSO in the culture medium were less than 0.05%. The cells were treated with 20 μM of MG132 for 30 min before treatment with 20(S)-Rg3.

Cell culture

Human ovarian cancer cell lines, SKOV3 and 3AO, were purchased from the Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China) and the Shandong Academy of Medical Sciences (Jinan, China). Cells were grown in RPMI-1640 (Gibco, Grand Island, NY, USA) supplemented with 10% newborn bovine serum (NBS; Sijiqing, Hangzhou, China) and penicillin/streptomycin (100 U/mL of penicillin, 100 μg/mL of streptomycin). These cell lines were cultured at 37°C in a humidified incubator containing 21% O_2 and 5% CO_2. Cells with a passage number less than 20 were used in all experiments.

siRNA transient transfection

PHD1 siRNA (siPHD1) and negative control siRNA were designed and synthesized by GenePharma Company (Shanghai, China). The siPHD1 sequence was as follows: 5′-GCAUCACCUGUAUCUAUUATT-3′. This siPHD1 is

a very effective siRNA for silencing PHD1 which is chosen according to our previous research. Scrambled siRNA was used as negative control (5′-UUCUCCGAACGUG UCACGUTT-3′). Cells were cultivated in six-well plates (Corning Inc., Corning, NY, USA) at a density of 5×10^4 cells/well. siPHD1 or control siRNA was transfected to cells at 40%–50% confluence. Then, 15 μL siRNA transfection reagent (Roche, Indianapolis, IN, USA) mixed with 10 nM siRNA was used to treatment for each well according to the manufacturer's instructions. Total RNA and protein were extracted after incubation with siRNA for 48 and 72 h, respectively. RT-PCR and western blot were used to detect the effect of the siRNA.

Wound-healing assay

SKOV3 and 3AO cells were cultivated in six-well plates (Corning Inc., Corning, NY, USA) at a density of 3×10^5 cells/well 24 h before treatment. When the cell density reached more than 90% confluence, the NBS-containing medium was removed, and cells were serum starved for 24 h. Cells were washed with NBS-free media. A line was scratched using the end of a 200 mL pipette tip (time 0 h) and cells were washed with NBS-free media to remove the loose cells. Cells were treated with or without 20(S)-Rg3 for 36 h. Images of migrating cells were sequentially taken after 36 h. More than three images were captured for each wound. The experiments were repeated at least three times.

Transwell migration assay

About 1×10^5 cells/well were seeded in the upper transwell chambers (8 mm pore size, 24-well; Millipore Co., Bedford, MA, USA) in serum-free medium, and RPMI-1640 with 20% NBS as chemotactic factor was added to the bottom chambers. After treatment for 24 h at 37°C under 5% CO_2, the top cells which were not migrated were removed from the upper chamber using a cotton swab. The bottom cells which were migrated through the pores onto the lower side of the filter were fixed with 5% glutaric dialdehyde at 4°C for 30 min, and the cells were washed with PBS (PBS, pH 7.2–7.4: NaCl 137 mmol/L, KCl 2.7 mmol/L, Na_2HPO_4 10 mmol/L, KH_2PO_4 2 mmol/L). Then, the cells were stained with Giemsa at room temperature for 30 min. The number of migrant cells in five fields under 200× magnification was counted, and the mean values for five fields were determined. The results were repeated three times.

Western blot

Western blot assay was used to analyze proteins. First, the SKOV3 or 3AO cells were washed with ice-cold PBS for three times and cracked in lysis buffer, radio immunoprecipitation assay (RIPA; Biyuntian, Shanghai, China):protease inhibitor (PI; Roche, Basel, Switzerland): phenylmethanesulfonyl fluoride (PMSF; DingGuo, Shanghai, China) = 50:2:1, on ice for 30 min. Then, the cells were centrifuged at 12,000 r/min for 30 min to collect the supernatant. The Quick Start Bradford Protein Assay Kit (Bio-Rad Inc., Hercules, CA, USA) was used to quantify the level of total cell protein for each sample. The samples were diluted in 5× sodium dodecyl sulfate (SDS) loading buffer (1:50 volume of sample; Biyuntian, Shanghai, China) and boiled for 5 min. An equivalent amount of proteins (no more than 30 μL) from each sample was electrophoresed by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for about 40 min and then transferred onto nitrocellulose membranes (0.45 μm, Pall Life Science, Port Washington, NY, USA) using a wet transmembrane device (Amersham Pharmacia Biotech., Piscataway, NJ, USA). After that, the membranes were blocked with 5% non-fat milk (milk was mixed in 1× Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBST): pH 7.6) for about 1 h at room temperature and then incubated overnight at 4°C with the primary antibody–rabbit antibody to E-cadherin (Bioworld Technology, Louis Park, MN, USA) 1:500, rabbit antibody to vimentin (Cell Signaling Technology, Beverly, MA, USA) 1:2000, rabbit antibody to β-actin (Cell Signaling Technology, Beverly, MA, USA) 1:1000, mouse antibody to HIF-1α (Abcam Inc., Cambridge, MA, USA) 1:500, sheep antibody to PHD1 (R&D Systems Inc., Minneapolis, MN, USA) 1:2000, rabbit antibody to VHL (Cell Signaling Technology) 1:1000, mouse antibody to NF-κB (Cell Signaling Technology) 1:10000. All the primary antibody was diluted with 5% non-fat milk. The membranes were washed in 1× TBST (5 min, six times) and then incubated for 1 h at room temperature with the appropriate secondary antibody—horse radish peroxidase (HRP)–conjugated goat anti-mouse/rabbit IgG 1:2000, rabbit anti-sheep IgG 1:1000. All the secondary antibody was diluted with 5% non-fat milk. After washing with 1× TBST (5 min, six times), the membranes were developed using ECL reagent (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and were analyzed by electrochemiluminescence (ECL) detection systems (Bio-Rad Inc.).

RT-PCR

For RT-PCR, the cells were washed with ice-cold PBS for three times and were crushed in 0.5 mL lysis buffer (RNAfast 200; Fastagen Biotech Co., Ltd., Shanghai, China) according to the manufacturer's recommendations. RNA concentration and purity were measured by an ultraviolet (UV) spectrophotometer (Bio-Rad Inc.). Equal concentrations of RNA samples (2.0 mg) were then reverse transcribed to complementary DNA (cDNA) by the RT-PCR system (Applied Biosystems Inc., Foster, CA, USA) using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer's instructions. The cDNA

reaction was performed 5 min at 25°C, followed by 60 min at 42°C and 5 min at 70°C and stored at −20°C. Specific cDNAs were then amplified by RT-PCR system (Bio-Rad Inc.). RT-PCR was performed using the SYBR Green Master Mix (Takara Biotechnology Co. Ltd., Dalian, China) in a 25 μL reaction volume, which contained 12.5 μL of SYBR Green master PCR mix, 0.5 μL of each of forward and reverse primers (20 sμM), 1 μL of diluted cDNA template, and appropriate amounts of sterile distilled water. RT-PCR was performed using the following primers (Shenggong Biotechnological Ltd., Shanghai, China): HIF-1α-F, 5′-ATCCATGTGACCATGAGGAAATG-3′; HIF -1α-R, 5′-TCGGCTAGTTAGGGTACACTTC-3′; PHD1-F, 5′-AGGCTGTCGAAG CATTGGTG-3′; PHD1-R, 5′-GG GATTGTCAACGTGCCTTAC-3′; PHD2-F, 5′-A AGC CCAGTTTGCTGACATT-3′; PHD2-F, 5′-TTACCGACC GAATCTGAAGG-3′; PHD3-F, 5′AGCCCATTTTTGC CAGACTCC-3′; PHD3-R, 5′-GATTTCAGAGCA CGGT CAGTC-3′;VHL-F,5′-GCAGGCGTCGAAGAGTACG-3′; VHL-R, 5′-CGG ACTGCGATTGCAGAAGA-3′; β-actin-F, 5′- TCCCTGGAGAAGAGCTACGA-3′; β-actin-R, 5′-AG CACTGTGTTGGCGTACAG-3′. *β-actin* gene was used to normalize the expression levels in subsequent quantitative analyses. The PCR program consisted of an initial melting step of 30 s at 95°C, followed by 40 cycles of 5 s at 95°C, 31 s at 58−60°C. Relative gene expression was analyzed using the $2^{-\Delta\Delta CT}$ method. Each sample was performed in triplicate.

Animal study

Female, nude mice (6- to 8-week-old) from Animal Center of Xi'an Jiaotong University Medicine School were fed in specific pathogen free (SPF) barrier facilities and were maintained under 12 h dark/light cycle at 21°C. SKOV3 cells were harvested and mixed with PBS, and then the cells were inoculated (1×10^7 SKOV3) into the abdominal cavity of each mice. Mice were randomly divided into two groups (control and 20(S)-Rg3, n = 8/group) and were given tail vein injections of 5 mg/kg 20(S)-Rg3 or the equal volume of PBS every other day for 30 days. The change of rats' body weight and ascites was observed. Following the experiment, the mice were killed and the tumors were excised. Histopathological analysis for tumors was carried out using hematoxylin and eosin (H&E) staining and immunohistochemistry. All the contents of animal experiment we have reported here are in accordance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. The care and use of experimental animals were approved by the ethical committee of the First Affiliated Hospital.

Histological analysis with H&E staining

All the tissue samples from control and treated groups were fixed in 4% formaldehyde overnight at 4°C. Then, they were dehydrated in a graded series of ethanol, embedded in paraffin, sectioned at 2- to 3-μm thickness and stuck onto glass slides. After that, glass slides were stained using H&E for microscopic observation.

Immunohistochemistry

Paraffinized ovarian cancer tissue blocks were sectioned, deparaffinized, and hydrated in descending alcohol dilutions. The primary antibody was an anti-HIF-1α antibody (Abcam Inc., Cambridge, MA, USA). The 3-μm-thick sections were deparaffinized and boiled in 0.01 M citrate buffer (pH 6.0) for 30 min in a microwave oven. The sections were then treated with 0.3% hydrogen peroxide and incubated with 10% normal rabbit serum in PBS for 10 min to block non-specific binding. The sections were then incubated with the primary antibody (1:200) for 2 h at room temperature. Negative control specimens were incubated in PBS. The sections were then washed in TBS. After that the sections were incubated for 30 min at RT with HRP-labeled secondary antibody (MaxVision HRP-Polymer anti-Mouse/Rabbit IHC Kit, 1:200, Maixin Inc., Fuzhou, Fujian, China) and color development with diaminobenzidine (DAB) (Abcam Inc.). Positive and negative immunohistochemistry controls were routinely employed. The results of immunohistochemistry were evaluated by two independent observers unaware of the fate of the tissue site.

Statistical analysis

The Student's *t*-test was used to analyze the results of transwell migration assay and RT-PCR. All statistical analyses were performed using SPSS software (Chicago, IL, USA). Differences were considered significant (**) at $p < 0.01$ for all comparisons.

Results

20(S)-Rg3 inhibits migratory ability of ovarian cancer cells

Our previous study showed that 20(S)-Rg3 could inhibit cell growth in a dose-dependent manner, and we also tested the half maximal inhibitory concentration (IC_{50}) for SKOV3 and 3AO cells, respectively. In this study, 80 and 160 mg/mL 20(S)-Rg3 were chosen to treat SKOV3 and 3AO cells, respectively. In our previous studies, migratory ability of ovarian cancer cell lines SKOV3 and 3AO was enhanced greatly under hypoxia conditions. 20(S)-Rg3 could restrain migration of SKOV3 and 3AO cells under hypoxia conditions obviously.[14] In this study, we applied wound-healing assay and transwell assay to test migratory ability of SKOV3 and 3AO cells with the treatment of 20(S)-Rg3 under normoxic conditions. Wound-healing assay showed that in comparison with control cells, the

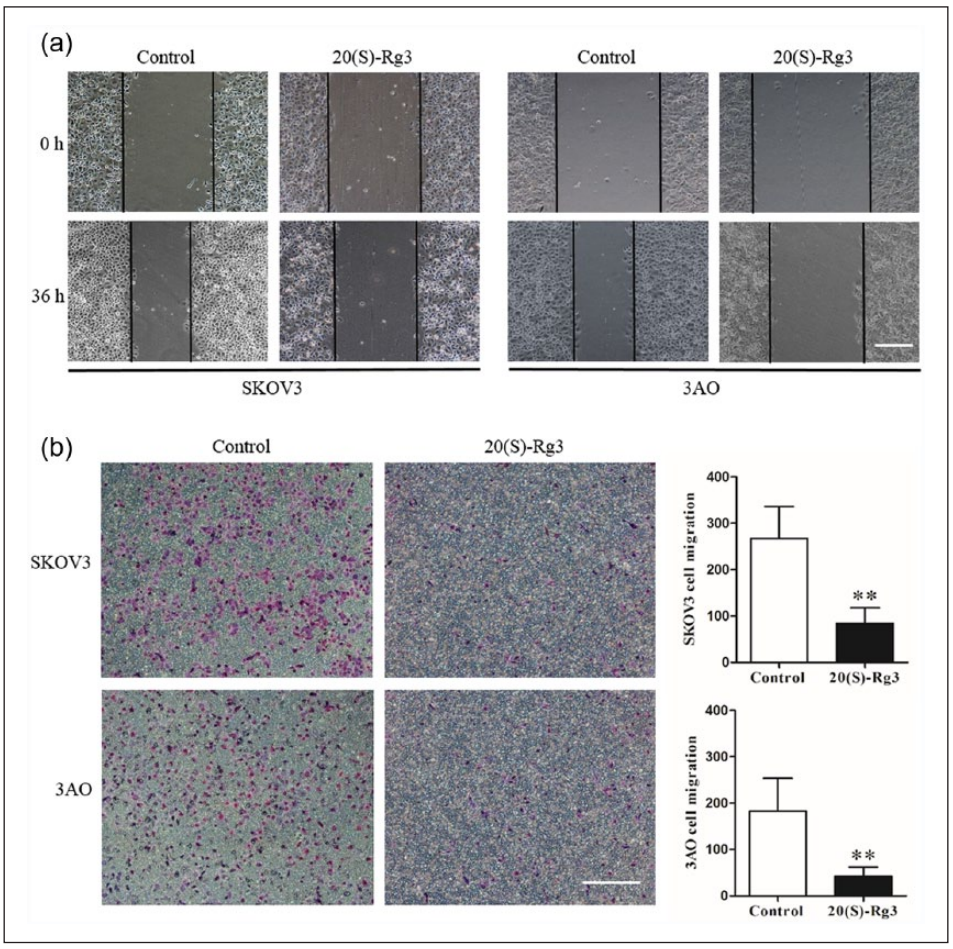

Figure 1. 20(S)-Rg3 inhibited migration of SKOV3 and 3AO ovarian cancer cells. (a) Cell mobility detected by wound-healing assay. Cells were cultured in six-well plates for 24 h. Wounds were created by 200 µL pipette tips, and cell migration rate was calculated at 0 and 24 h of culturing. Representative examples of images are shown (scale bars: 50 µm). (b) Cellular migration was assessed by a transwell system. Cells were seeded into Millicell. After 24 h of incubation, migratory cells were fixed and stained with crystal violet and were counted under 20× magnified lens. Representative photomicrographs of are shown on the left (scale bar: 50 µm). Quantitation of migratory cells was performed with five randomly selected microscopic fields. The ruler (the white line) in the figure is 50 µm. All of the treatments in this figure were carried out in triplicate, and the results of transwell migration assay are displayed as the mean values ± SD. $^{**}p < 0.01$, *t*-test.

mobility of SKOV3 and 3AO cells was suppressed with the treatment of 20(S)-Rg3 for 24 h (Figure 1(a)). Similar results were also obtained when we replaced wound-healing assay with transwell assay (Figure 1(b)). Together, results from Figure 1(a) and (b) demonstrated that 20(S)-Rg3 notably impaired the migration ability of SKOV3 and 3AO cells.

20(S)-Rg3 reduces migration of ovarian cancer cells through suppressing HIF-1α protein expression

Given that stabilization of HIF-1α protein is responsible for progression and metastasis in many types of human cancers,[16] we detected the HIF-1α level of SKOV3 and 3AO cells after 20(S)-Rg3 treatment. We found that there was no change of HIF-1α messenger RNA (mRNA) level in the presence of 20(S)-Rg3 in cells (Figure 2(a)); however, HIF-1α protein expression decreased significantly in both SKOV3 and 3AO cells treated with 20(S)-Rg3 (Figure 2(b)). These data indicate that HIF-1α protein level can be post-transcriptionally regulated by 20(S)-Rg3 in both SKOV3 and 3AO cells.

20(S)-Rg3 elevates PHD1 expression to facilitate HIF-1α degradation via the ubiquitin/proteasome pathway

20(S)-Rg3 could inhibit HIF-1α protein level but have no influence on its mRNA level, which suggested that 20(S)-Rg3 regulated HIF-1α expression via a post-transcriptional mechanism. Ubiquitin-mediated proteasomal degradation

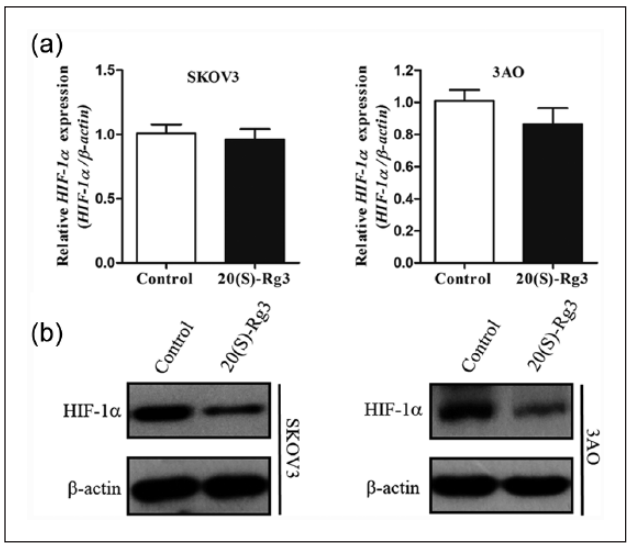

Figure 2. 20(S)-Rg3 suppressed HIF-1α protein expression. Total RNA or protein of cells exposed to 20(S)-Rg3 for 24 h and control group was extracted. (a) The relative mRNA levels of HIF-1α were analyzed by real-time PCR. (b) The relative protein levels of HIF-1α were monitored by western blot and normalized to β-actin.

is one of the commonest way to the regulate HIF-1α protein level, so we analyzed whether the 26S proteasome-specific PI MG132 could prevent the effect of 20(S)-Rg3 on HIF-1α expression. As expected, we found that when treated with MG132, HIF-1α protein of SKOV3 and 3AO cells was not degraded even in the presence of 20(S)-Rg3 (Figure 3(a)). This result supports that 20(S)-Rg3 could decrease HIF-1α protein level by promoting ubiquitin/proteasome degradation pathway.

PHDs and VHL play critical role in HIF-1α ubiquitin/proteasome-dependent protein degradation. In order to verify whether 20(S)-Rg3 can regulate HIF-1α ubiquitin/proteasome-dependent protein degradation via PHDs or VHL, we detected the mRNA level of PHDs and VHL in cells after treatment of 20(S)-Rg3 (Figure 3(b)). We found PHD1 and VHL mRNA were upregulated in both SKOV3 and 3AO cells treated with 20(S)-Rg3, but there was almost no effect of 20(S)-Rg3 on the mRNA level of PHD2 and PHD3. To further confirm the data in mRNA level, we applied western blot to detect the protein level of PHD1 and VHL in SKOV3 cells treated with 20(S)-Rg3, and we found that 20(S)-Rg3 could increase the expression of PHD1, but not the expression of VHL (Figure 4(a)), which indicated that 20(S)-Rg3 may enhance HIF-1α ubiquitin/proteasome degradation via increased PHD1 expression.

To further confirm these conclusions that 20(S)-Rg3-induced HIF-1α degradation was mediated by PHD1, we applied the interruption approach via knocking down PHD1 with siRNA of PHD1 and found that knocking down PHD1 could partially reverse 20(S)-Rg3-induced HIF-1α degradation (Figure 4(b)). Together, these results suggest that 20(S)-Rg3 could decrease the expression of HIF-1α dependent on ubiquitin/proteasome degradation pathway via induced expression of PHD1 in ovarian cancer cells.

20(S)-Rg3 suppresses migration of ovarian cancer cells by inhibiting NF-κB

NF-κB plays an important role in cancer cell invasion and migration.[17] Hence, it has been reported that 20(S)-Rg3 could prevent colon cancer cells migration by reducing NF-κB.[18] We also wanted to know whether NF-κB was involved in the process that 20(S)-Rg3 decreased migration of ovarian cancer cells. Interestingly, the results showed that NF-κB expression was impaired notably by 20(S)-Rg3 in SKOV3 cells (Figure 5), which implied that, in ovarian cancer, inhibition of NF-κB was also a possible mechanism for 20(S)-Rg3 to block ovarian cancer migration.

20(S)-Rg3 represses HIF-1α expression in vivo

We had reported that, after continuous monitoring of the ovarian cancer metastasis of intraperitoneal xenograft model, the metastasis of 20(S)-Rg3 group reduced significantly. To demonstrate the HIF-1α results above in vivo animal models, we then xenografted SKOV3 cells into abdominal cavity of 6- to 8-week-old female nude mice. Eight mice were then treated with 20(S)-Rg3, and the other eight mice were treated with vehicle solvent. The subcutaneous tumors were fixated for H&E staining and immunohistochemistry analysis of HIF-1α. We found that results from immunohistochemical (IHC) staining of HIF-1α also matched well with those data in vitro co-culture studies (Figure 6(a)).

It was interesting that we found that the boundary of hepatic and muscle tissues was damaged by ovarian neoplastic tissue obviously in control group, which was not to seen in 20(S)-Rg3 group (Figure 6(b) and (c)). That may give evidence that 20(S)-Rg3 prevent ovarian cancer metastasis in vivo.

Discussion

Ovarian cancer, with a 5-year survival rate of less than 30%, is the most deadly cancer of gynecological tumor.[19] Strong invasiveness and migration accelerate the progression of ovarian cancer. Consequently, suppression of cancer cells invasion and migration is a key strategy for therapy of ovarian carcinoma. Increasing evidence shows that HIF-1α could promote tumor progression by improving cancer cells' invasion and migration. Therefore, HIF-1α is a significant target for therapy of carcinoma. More and more new medicines which were considered as HIF-1α inhibitors were discovered to block tumor progression.

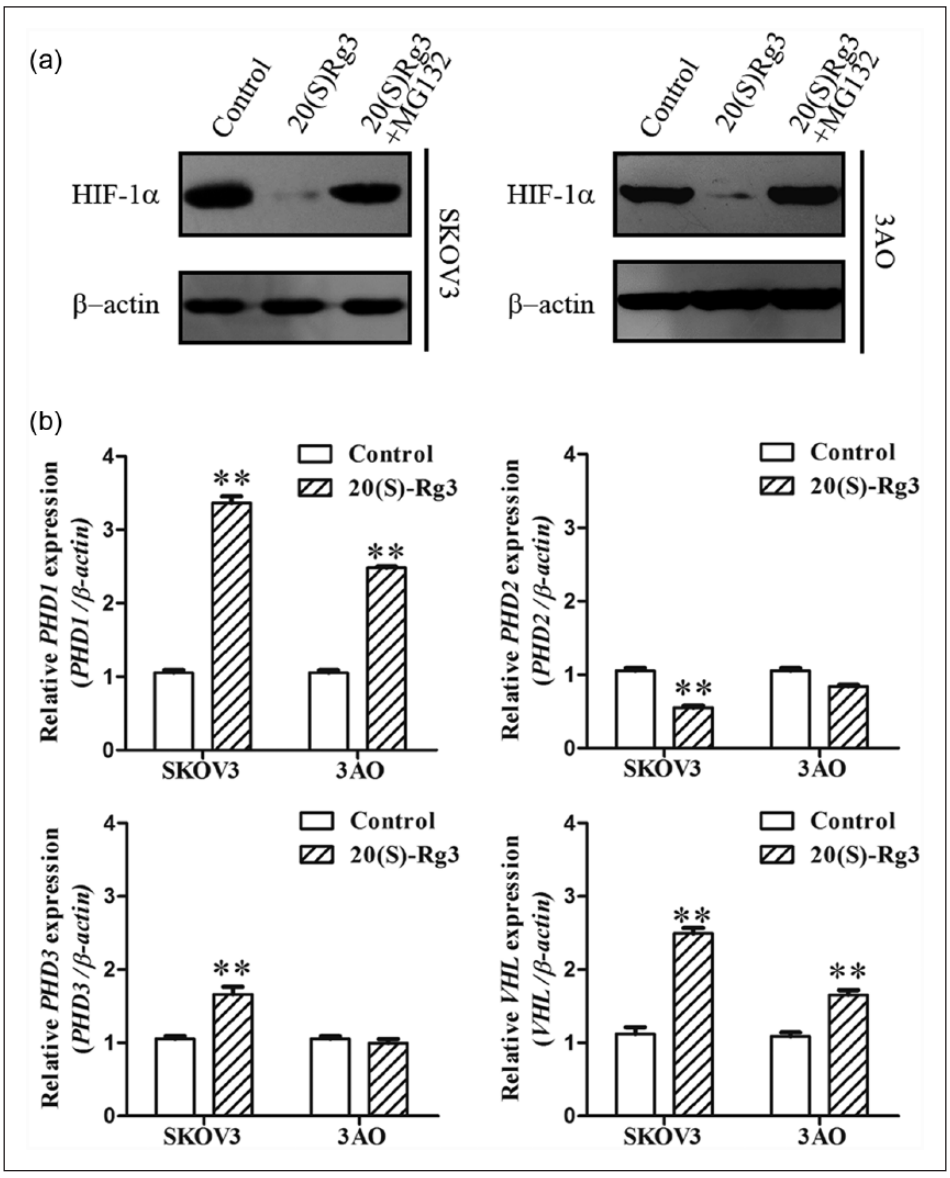

Figure 3. 20(S)-Rg3 increased HIF-1α degradation through ubiquitin–proteasome pathway. (a) MG132 suppressed the inhibition effect of 20(S)-Rg3 on HIF-1α protein level. Total protein from cells exposed to control, 20(S)-Rg3, or 20(S)-Rg3 plus MG132 (cells pretreated with 20-μM MG132 for 30 min followed by exposure to hypoxia and 20(S)-Rg3 for 24 h), respectively, for 24 h was assessed by western blot and normalized to β-actin. (b) Cells exposed to control and 20(S)-Rg3, respectively, were assessed by real-time PCR for transcription of PHD1, PHD2, PHD3, and VHL. After 20(S)-Rg3 treatment, the transcription of PHD1 and VHL genes was increased in both SKOV3 and 3AO cells.

Herein, we present ginsenoside 20(S)-Rg3 which could block ovarian cancer migration by inhibiting HIF-1α expression.

Ginsenoside 20(S)-Rg3 has been proved to have notable anticancer activities, such as accelerating cancer cells senescence and apopotosis,[10,11] repressing cancer growth,[20] and inhibiting cancer invasion and metastasis.[13,14] The mechanism of 20(S)-Rg3 suppressing tumor is very complicated. It was reported that 20(S)-Rg3 repressed cancer cell proliferation through mitotic inhibition, DNA replication, and growth factor signaling rebuilding.[21] Some evidences demonstrated that 20(S)-Rg3 mediated some tumor-associated signaling pathways, such as PI3K/Akt,[10,12] AMPK,[22] p53,[11] and androgen receptor signaling pathway,[23] which induced cells' apoptosis and senescence. In addition, other results indicated that 20(S)-Rg3 repressing Warburg effect suppressed epithelial–mesenchymal transition to inhibit cancer metastasis[13,14] and enhanced radiosensitization by reducing vascular endothelial growth factor.[24] However, the possible mechanism is still not clear. In our study, we found 20(S)-Rg3 could block ovarian cancer invasion and migration by suppressing HIF-1α protein

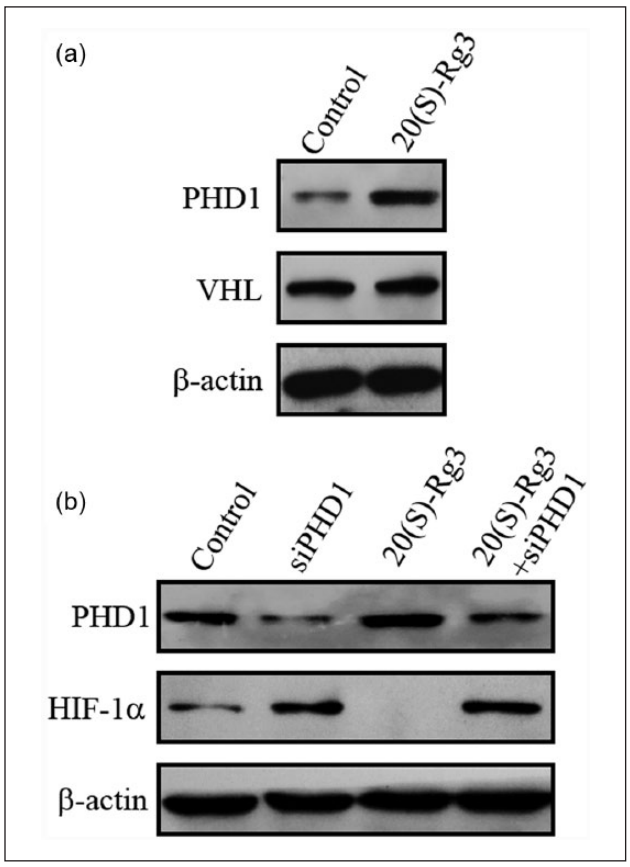

Figure 4. Deficiency of PHD1 counteracted inhibitory effect of 20(S)-Rg3 on HIF-1α protein level. (a) Cells exposed to control and 20(S)-Rg3, respectively, were assessed by western blot for expression of PHD1 and VHL. (b) Effect of PHD1 silence on 20(S)-Rg3-suppressed HIF-1α. SKOV3 cells were transiently transfected for 24 h with siPHD1, and the protein level of PHD1 and HIF-1α was then detected using western blot. All experiments were repeated at least three times.

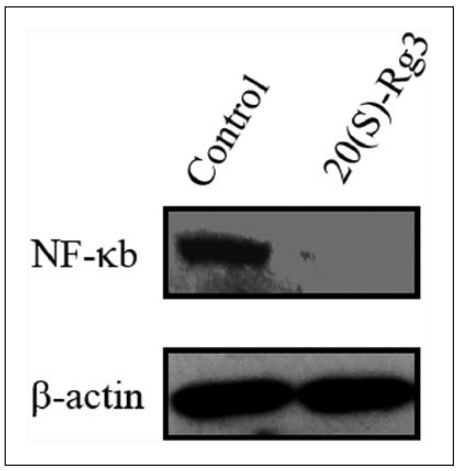

Figure 5. 20(S)-Rg3 inhibited NF-κB protein expression. Total protein of cells exposed to 20(S)-Rg3 for 24 h and control group was extracted. The relative protein levels of NF-κB were analyzed by western blot.

expression with no effect on mRNA level, and we confirmed 20(S)-Rg3 promoted proteasomal degradation of HIF-1α using 26S proteasome-specific PI MG132. Chen et al.[25] also discovered that Rg3 facilitates HIF-1α protein degradation and shortens the half-life of HIF-1α protein significantly in human esophageal carcinoma cell line Eca-109, which was consistent with our result. 20(S)-Rg3 was proved to prevent ovarian cancer growth and intraperitoneal dissemination in nude mice in our previous study (data not shown) and weakened HIF-1α level in vivo. These discovery support 20(S)-Rg3 may become a new HIF-1α inhibitor for tumor therapy.

In our study, we found that the mechanism of 20(S)-Rg3 inhibiting HIF-1α was relevant to proteasomal degradation of HIF-1α. It is known that the level of the HIF-1α protein is modified by PHDs and VHL, which could send HIF-1α to proteasomal degradation under normoxia. Impaired activity of PHDs and VHL, even under normoxia, causes accumulation of HIF-1α and activation of HIF-1α target gene expression. Some therapeutic agents have been proved to inhibit HIF-1α by enhancing its protein degradation. Wogonin, a plant-derived flavone, was shown to heighten HIF-1α prolyl hydroxylation to reduce HIF-1α expression.[26] LW6 was demonstrated to increase VHL to promoting proteasomal degradation of HIF-1α.[27] Apicidin was proved to accelerate degradation of HIF-1α via upregulation of PHD2.[28] Our study revealed that 20(S)-Rg3 elevated PHD1 expression to progress proteasomal degradation of HIF-1α under well-oxygenated conditions.

Increased evidence showed that PHD1 acts as a tumor suppressor, like PHD2 and PHD3. PHD1 could prevent colon carcinoma and breast cancer growth[29,30] and regulated colorectal cancer chemoresistance.[31] Our data showed that PHD1 played a key role in regulation of HIF-1α. Furthermore, we speculated that PHD1 also mediated ovarian cancer invasion and migration. It is very likely that PHD1 could become a new target for ovarian cancer therapy. Our previous study found that under hypoxia, 20(S)-Rg3 increases not only PHD1 expression but also VHL level to reduce HIF-1α protein. Whereas, we found that under well-oxygenated conditions, 20(S)-Rg3 activated only PHD1 but not VHL to accelerate HIF-1α ubiquitin–proteasome degradation. These results suggested that the mechanisms of the effect for 20(S)-Rg3 under normoxia or hypoxia were different. But why the situations under normoxia or hypoxia were different require further study. It is essential to clarify the mechanism for 20(S)-Rg3 working under normoxia conditions. Because HIF-1α shows significant upregulation in ovarian cancer under well-oxygenated conditions, it means that inhibition of HIF-1α under normoxia conditions is very important for ovarian cancer therapy. In addition, our data showed that 20(S)-Rg3 can reduce NF-κB expression, which may be another possible mechanism for 20(S)-Rg3 decreasing HIF-1α. It was

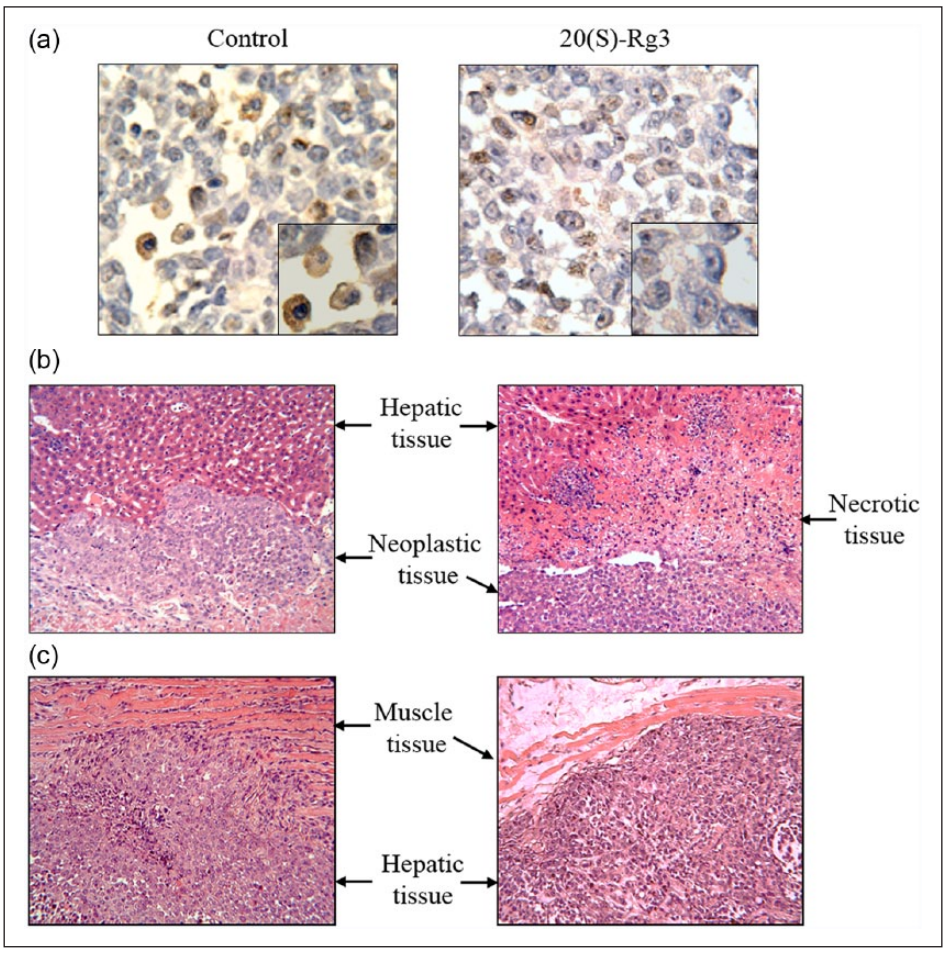

Figure 6. 20(S)-Rg3 decreased HIF-1α protein level in nude mice. Mice injected intraperitoneal with SKOV3 cells underwent 20(S)-Rg3 treatment for 30 days. (a) Immunohistochemical staining of HIF-1α expression in tumor samples (original magnification, 200×; insets, 400×) 20(S)-Rg3 weakened HIF-1α in vivo. (b and c) H&E staining for glass slides of hepatic and muscle tissue (100×). Hepatic tissue, muscle tissue, neoplastic tissue, and necrotic tissue were shown. Ovarian neoplastic tissue invaded the boundary of hepatic and muscle tissue obviously in control group, which was not to seen in 20(S)-Rg3 group.

reported that 20(S)-Rg3 could inhibit NF-κB in variety of tumors[18,32] and which is also a possible pathway to suppressing cancer growth and progression.

Collectively, we revealed that natural medicine ginsenoside 20(S)-Rg3 hindered ovarian cancer invasion and migration through decreasing HIF-1α protein level under normoxia. We found the mechanisms involved in upregulation of PHD1 by 20(S)-Rg3 which caused HIF-1α ubiquitin–proteasome degradation. Although the specific mechanism is still unclear, our data would suggest that 20(S)-Rg3 could became a new drug for ovarian cancer therapy.

Acknowledgements

The authors thank Ms Jing Li, Wen Zhai, and Mei Xue for technical assistance. Ting Liu and Le Zhao contributed equally to this work.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical approval

The study was conducted according to the guidelines of the institutional review boards at the First Affiliated Hospital of Xi'an Jiaotong University; we have obtained internal review board approval and/or patients' informed consent for this study.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by the National Natural Science Foundation of China (no. 30973429) and Foundation of First Affiliated Hospital of Xi'an Jiaotong University (no. YK201509).

References

1. Osman NA, Abd El-Rehim DM and Kamal IM. Defective Beclin-1 and elevated hypoxia-inducible factor (HIF)-1α expression are closely linked to tumorigenesis, differentiation, and progression of hepatocellular carcinoma. *Tumour Biol* 2015; 36(6): 4293–4299.

2. Zhang H, Qian DZ, Tan YS, et al. Digoxin and other cardiac glycosides inhibit HIF-1alpha synthesis and block tumor growth. *Proc Natl Acad Sci U S A* 2008; 105(50): 19579–19586.
3. Zagzag D, Nomura M, Friedlander DR, et al. Geldanamycin inhibits migration of glioma cells in vitro: a potential role for hypoxia-inducible factor (HIF-1alpha) in glioma cell invasion. *J Cell Physiol* 2003; 196(2): 394–402.
4. van de Sluis B, Mao X, Zhai Y, et al. COMMD1 disrupts HIF-1alpha/beta dimerization and inhibits human tumor cell invasion. *J Clin Invest* 2010; 120(6): 2119–2130.
5. Coimbra IB, Jimenez SA, Hawkins DF, et al. Hypoxia inducible factor-1 alpha expression in human normal and osteoarthritic chondrocytes. *Osteoarthritis Cartilage* 2004; 12(4): 336–345.
6. Vleugel MM, Greijer AE, van der Wall E, et al. Mutation analysis of the HIF-1alpha oxygen-dependent degradation domain in invasive breast cancer. *Cancer Genet Cytogenet* 2005; 163(2): 168–172.
7. Nag SA, Qin JJ, Wang W, et al. Ginsenosides as anticancer agents: in vitro and in vivo activities, structure-activity relationships, and molecular mechanisms of Action. *Front Pharmacol* 2012; 3: 25.
8. Wang L, Yang X, Yu X, et al. Evaluation of antibacterial and anti-inflammatory activities of less polar ginsenosides produced from polar ginsenosides by heat-transformation. *J Agric Food Chem* 2013; 61(50): 12274–12282.
9. Yue PY, Wong DY, Wu PK, et al. The angiosuppressive effects of 20(R)- ginsenoside Rg3. *Biochem Pharmacol* 2006; 72(4): 437–445.
10. Wang JH, Nao JF, Zhang M, et al. 20(s)-ginsenoside Rg3 promotes apoptosis in human ovarian cancer HO-8910 cells through PI3K/Akt and XIAP pathways. *Tumour Biol* 2014; 35(12): 11985–11994.
11. Zhang F, Li M, Wu X, et al. 20(S)-ginsenoside Rg3 promotes senescence and apoptosis in gallbladder cancer cells via the p53 pathway. *Drug Des Devel Ther* 2015; 9: 3969–3987.
12. Qiu XM, Bai X, Jiang HF, et al. 20-(s)-ginsenoside Rg3 induces apoptotic cell death in human leukemic U937 and HL-60 cells through PI3K/Akt pathways. *Anticancer Drugs* 2014; 25(9): 1072–1080.
13. Kim YJ, Choi WI, Jeon BN, et al. Stereospecific effects of ginsenoside 20-Rg3 inhibits TGF-β1-induced epithelial-mesenchymal transition and suppresses lung cancer migration, invasion and anoikis resistance. *Toxicology* 2014; 322: 23–33.
14. Liu T, Zhao L, Zhang Y, et al. Ginsenoside 20(S)-Rg3 targets HIF-1alpha to block hypoxia-induced epithelial-mesenchymal transition in ovarian cancer cells. *PloS ONE* 2014; 9(9): e103887.
15. Li J, Liu T, Zhao L, et al. Ginsenoside 20(S)Rg3 inhibits the Warburg effect through STAT3 pathways in ovarian cancer cells. *Int J Oncol* 2015; 46(2): 775–781.
16. Yang MH, Wu MZ, Chiou SH, et al. Direct regulation of TWIST by HIF-1alpha promotes metastasis. *Nat Cell Biol* 2008; 10(3): 295–305.
17. Dolcet X, Llobet D, Pallares J, et al. NF-κB in development and progression of human cancer. *Virchows Arch* 2005; 446(5): 475–482.
18. Junmin S, Hongxiang L, Zhen L, et al. Ginsenoside Rg3 inhibits colon cancer cell migration by suppressing nuclear factor kappa B activity. *J Tradit Chin Med* 2015; 35(4): 440–444.
19. Ruscito I, Gasparri ML, Marchetti C, et al. Cediranib in ovarian cancer: state of the art and future perspectives. *Tumour Biol* 2016; 37: 2833–2839.
20. Yang R, Chen D, Li M, et al. 20(s)-ginsenoside Rg3-loaded magnetic human serum albumin nanospheres applied to HeLa cervical cancer cells in vitro. *Biomed Mater Eng* 2014; 24(6): 1991–1998.
21. Lee SY, Kim GT, Roh SH, et al. Proteomic analysis of the anti-cancer effect of 20S-ginsenoside Rg3 in human colon cancer cell lines. *Biosci Biotechnol Biochem* 2009; 73(4): 811–816.
22. Yuan HD, Quan HY, Zhang Y, et al. 20(S)-Ginsenoside Rg3-induced apoptosis in HT-29 colon cancer cells is associated with AMPK signaling pathway. *Mol Med Rep* 2010; 3(5): 825–831.
23. Bae JS, Park HS, Park JW, et al. Red ginseng and 20(S)-Rg3 control testosterone-induced prostate hyperplasia by deregulating androgen receptor signaling. *J Nat Med* 2012; 66(3): 476–485.
24. Ge X, Zhen F, Yang B, et al. Ginsenoside Rg3 enhances radiosensitization of hypoxic oesophageal cancer cell lines through vascular endothelial growth factor and hypoxia inducible factor 1α. *J Int Med Res* 2014; 42(3): 628–640.
25. Chen QJ, Zhang MZ and Wang LX. Gensenoside Rg3 inhibits hypoxia-induced VEGF expression in human cancer cells. *Cell Physiol Biochem* 2010; 26(6): 849–858.
26. Song X, Yao J, Wang F, et al. Wogonin inhibits tumor angiogenesis via degradation of HIF-1α protein. *Toxicol Appl Pharmacol* 2013; 271(2): 144–155.
27. Lee K, Kang JE, Park SK, et al. LW6, a novel HIF-1 inhibitor, promotes proteasomal degradation of HIF-1alpha via upregulation of VHL in a colon cancer cell line. *Biochem Pharmacol* 2010; 80(7): 982–989.
28. Durczak M and Jagodzinski PP. Apicidin upregulates PHD2 prolyl hydroxylase gene expression in cervical cancer cells. *Anticancer Drugs* 2010; 21(6): 619–624.
29. Erez N, Milyavsky M, Eilam R, et al. Expression of prolyl-hydroxylase-1 (PHD1/EGLN2) suppresses hypoxia inducible factor-1alpha activation and inhibits tumor growth. *Cancer Res* 2003; 63(24): 8777–8783.
30. Zheng X, Zhai B, Koivunen P, et al. Prolyl hydroxylation by EglN2 destabilizes FOXO3a by blocking its interaction with the USP9x deubiquitinase. *Genes Dev* 2014; 28(13): 1429–1444.
31. Deschoemaeker S, Di Conza G, Lilla S, et al. PHD1 regulates p53-mediated colorectal cancer chemoresistance. *EMBO Mol Med* 2015; 7(10): 1350–1365.
32. Tian L, Shen D, Li X, et al. Ginsenoside Rg3 inhibits epithelial-mesenchymal transition (EMT) and invasion of lung cancer by down-regulating FUT4. *Oncotarget* 2015; 7: 1619–1632.