Effects of ginsenoside Rg3 on epigenetic modification in ovarian cancer cells

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Abstract. Epigenetic modifications are closely related to oncogene activation and tumor suppressor gene inactivation. The aim of this study was to determine the effects of ginsenoside Rg3 on epigenetic modification in ovarian cancer cells. Cell proliferation, metastasis, invasion and apoptosis were respectively determined using Cell Counting Kit-8 (CCK-8), wound healing, Transwell and flow cytometric assays. Methylation levels were determined using methylation specific PCR (MSP). Related-factor expression was detected by conducting real-time-qPCR (RT-qPCR) and western blotting. The results revealed that cell proliferation was inhibited by ginsenoside Rg3 (0, 25, 50, 100 and 200 μg/ml) in a time-dependent manner (12, 24 and 48 h). Ginsenoside Rg3 (50, 100 and 200 μg/ml) was selected to treat cells in various experiments. When ovarian cells were treated with ginsenoside Rg3, cell apoptosis was observed to be promoted, while cell metastasis and invasion were inhibited at 48 h. The results of the present study revealed that in the promoter regions of p53, p16 and hMLH1, the methylation levels decreased, while the mRNA and protein levels significantly increased. The activities of DNMTs and mRNA as well as protein levels of DNMT1, DNMT3a and DNMT3b were decreased by Rg3. The data also demonstrated that the mRNA and protein levels of acetyl-H3 K14/K9 and acetyl-H4 K12/K5/K16 were increased by Rg3. Hence, ginsenoside Rg3 inhibited ovarian cancer cell viability, migration and invasion as well as promoted cell apoptosis.

Introduction

Ovarian cancer, one of the most common malignant tumors of the female reproductive tract, has the third highest incidence rate and the highest mortality rate (3%) among other female malignant reproductive cancers (1). The 5-year survival rate of advanced stage disease (FIGOII-IV) accounts for 30-44% (2). However, although the 5-year survival rate is ~93% among patients who have early stage ovarian cancer (FIGO I), these patients account for <15% of all ovarian cancer patients (3). Therefore, an early diagnosis of ovarian cancer is critical in improving the prognosis of patients and reducing mortality.

Epigenetic modifications are closely related to oncogene activation, tumor suppressor gene inactivation, DNA damage repair defects and cancer stem cell differentiation, among which DNA methylation and histone acetylation are the two most widely used methods in diagnosing cancers (4). The study of epigenetic mechanisms of tumors is helpful during clinical diagnosis and preventive treatment of tumors (5). DNA methylation which is the transfer of methyl groups to the 5th carbon atom of cytosine producing 5-methylcytosine under the catalysis of DNA methyltransferases, is an important epigenetic mechanism. Abnormal hypermethylation of the DNA promoter region is a mechanism underlying the inactivation of tumor suppressor genes and it plays an important role in the development of a cancer (6,7). 5-Aza-2'-deoxycitydine (5-aza-dc) is presently recognized as a common demethylation drug and is commonly used in clinical treatment of hematological diseases and lung cancer. However, its high price and great side effects have generated research focusing on finding a demethylation substitute to 5-aza-dc. In addition to methylation, acetylation is another important post-transcriptional regulation of histones. In general, histone acetylation is related to the opening of chromatin and helps promote gene transcription (8). Histone acetylation has been revealed to be closely related to the proliferation, differentiation and progression of tumor cells (9). The regulation of histone acetylation is a reversible dynamic process that relies on both histone acetyltransferases (HATs) and histone deacetylases (HDACs) that regulate the conformation of the chromatin structure and promote or suppress gene transcription (10).

Ginsenosides, a type of steroid compound, mainly distributed in medical materials of the *Panax* species, are considered as active ingredients in ginseng and have long been used as a traditional Chinese medicine (11,12). As one of the most important ginsenoside monomers, ginsenoside Rg3 induces G2 phase cell cycle arrest, therefore inhibiting the synthesis of proteins and ATPs in the pre-mitotic phase, slowing down the

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proliferation and growth of cancer cells (13), and promoting tumor cell apoptosis (14), as well as inhibiting cancer cell infiltration and metastasis (15,16). The aforementioned functions have all been confirmed in ovarian cancer cells (17,18). However, the effect of ginsenoside Rg3 on epigenetic modification in ovarian cancer still remains unclear.

Therefore, the present study was conducted to investigate the role of ginsenoside Rg3 on epigenetic modification of ovarian cancer cells, providing a molecular basis for novel diagnosis and treatment strategies of ovarian cancer.

Materials and methods

Drugs. Ginsenoside Rg3 (CAS no. 14197-60-5) with a molecular weight of 785.02 kDa ($C_{42}H_{72}O_{13}$) was purchased from Chengdu Mansite Biotechnology Co., Ltd. (Cdmust; Chengdu, China) in July 2016. The HPLC purity of was ≥98%. It was diluted by culture media and prepared to be used in experiments. Cisplatin (BP809) was purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany).

Cell culture and treatment. Human normal ovarian epithelial cells HOSEpiC (cat. no. BNCC340096; Bena Culture Collection, Beijing, China) and human ovarian cancer SKOV3 cells (HTB-77) were purchased from the American Type Culture Collection, (ATCC; Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; high glucose) (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) containing 10% (v/v) fetal bovine serum (FBS) (Gemini Bio-Products, West Sacramento, CA, USA), 100 U/ml streptomycin and 100 μg/ml penicillin) (Biological Industries, Beit Haemek, Israel) in an incubator with 5% CO_2 at 37˚C. HOSEpiC cells were treated with different concentrations of cisplatin (0, 5, 10, 20, 40 and 80 μg/ml) and ginsenoside Rg3 (0, 100, 200, 400, 800 and 1,600 μg/ml). SKOV3 cells were treated with different concentrations of cisplatin (0, 2, 4, 8, 16 and 32 μg/ml) and ginsenoside Rg3 (0, 25, 50, 100, 200 and 500 μg/ml).

Cell viability assay. A Cell Counting Kit-8 (CCK-8; Beyotime Institute of Biotechnology, Haimen, China) assay was carried out to determine cell viabilities. Briefly, cells ($5x10^3$/well) treated with different concentrations of cisplatin and ginsenoside Rg3 were inoculated in 96-well plates. After having been incubated for 12, 24 and 48 h, the cells were stained with 20 μl staining reagent for 1 h. The optical density (OD) values at 450 nm were read using a MultiSkan 1500 microplate reader (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Cell apoptosis assay. Annexin V/propidium iodide (PI) double-staining assay (Roche Diagnostics, Basel, Switzerland) was performed to assess cell apoptosis rates. Briefly, SKOV3 cells were first treated with different concentrations of ginsenoside Rg3 (0, 50, 100 and 200 μg/ml) for 48 h and were then stained with 5 μl Annexin V and 5 μl PI for 5 min in the dark at 37˚C. The analysis was performed using BD CellQuest™ Pro Software (BD Biosciences, Franklin Lakes, NJ, USA) without delay.

Cell metastasis ability assay. Wound healing assay was applied to determine cell metastatic abilities. To be more specific, $1x10^5$ SKOV3 cells were treated with different concentrations of ginsenoside Rg3 (0, 50, 100 and 200 μg/ml). Next, the cells were inoculated in each well of 12-well plates and scratched gently to form a cell-free area and then cultured in an incubator for 12 and 24 h at 37˚C. Finally, the diameters of cell-free areas were assessed under an Olympus DSX100 optical microscope (Olympus Corp., Tokyo, Japan).

Cell invasion ability assay. Cell invasion abilities of ovarian cancer cells treated with ginsenoside Rg3 were assessed using 24-well Transwell chambers that contained 8-μm pore filters (Corning Inc., Corning, NY, USA). In brief, $5x10^4$ SKOV3 cells treated with different concentrations of ginsenoside Rg3 (0, 50, 100 and 200 μg/ml) were cultured in DMEM culture media in Matrigel GFR-coated (BD Biosciences) upper chambers of the Transwell. In addition, DMEM culture media containing 10% FBS was filled into the lower chambers. After having been incubated for 48 h, the bottom membrane was stained with 0.1% crystal violet for 30 min at 37˚C. The number of invasive cells was calculated using Olympus DSX100 optical microscope (Olympus Corp.) with a magnification of x200.

Methylation assay. The methylation degrees of tumor inhibitors p53, p16 and hMLH1 in SKOV3 ovarian cancer cells, which were treated with different concentrations of ginsenoside Rg3 (0, 50, 100 and 200 μg/ml), were assessed using methylation specific PCR (MSP). SKOV3 ($1.5x10^4$) cells were treated with ginsenoside Rg3 (0, 50, 100 and 200 μg/ml) for 48 h. The methylation was detected by EZ DNA Methylation-Startup kit (Zymo Research Corp., Irvine, CA, USA) for bisulfite conversion. Next, the converted DNA (30 ng) was subjected for MSP amplification. The promoter regions of p53, p16 and human mutL homolog-1 (hMLH1) were respectively identified using specific methylated (M) and unmethylated (U) allele-specific primers and observed with MethPrimer 2.0 (Chinese Academy of Medical Sciences, Beijing, China). The amplification process was conducted as follows: Pre-denaturation at 95˚C for 5 min; 35 (M) or 40 (U) cycles of denaturation at 95˚C for 40 sec, and annealing at 65˚C (M) or 56˚C (U) for 45 sec; and a final extension at 72˚C for 5 min.

Enzyme activity assays. The activity of DNMT was determined by EpiQuik DNMT Activity/Inhibition Assay Ultra Kit (Colorimetric) (Epigentec Group Inc., Farmingdale, NY, USA) in ovarian cancer cells treated with ginsenoside Rg3 (0, 50, 100 and 200 μg/ml) or 50 μg/ml 5-aza-dc (as a negative control). The samples were first co-incubated with cytimidine substrate and then with 5'-methyl-cytimidine antibody. The activity of HDAC was determined using Epigenase HDAC Activity/Inhibition Direct Assay Kit (Colorimetric) (Epigentec Group Inc.) in ovarian cancer cells treated with ginsenoside Rg3 (0, 50, 100 and 200 μg/ml) or 500 ng/ml HDAC inhibitor trichostatin A (TSA) (Selleck Chemicals, Houston, TX, USA) (as a negative control). The samples were co-incubated with acetylated substrate and then with photographic developer. The OD values at 450 nm were measured using a MultiSkan 1500 microplate reader (Thermo Fisher Scientific, Inc.).

Real-time quantitative polymerase chain reaction (RT-qPCR). The mRNA expression levels of methylation related factors

were detected by RT-qPCR in SKOV3 ovarian cancer cells treated with different concentrations of ginsenoside Rg3 (0, 50, 100 and 200 μg/ml) or 50 μg/ml 5-aza-dc (as a negative control). Total RNA was extracted from different cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), and reversely-transcribed to cDNA using Transcriptase (Takara Bio, Inc., Otsu, Japan). Next, the cDNA was amplified by LightCycler® Multiplex Masters on a LightCycler® 480 II System (both from Roche Diagnostics, Indianapolis, IN, USA). The thermocycling conditions were: Initial denaturation at 95˚C for 5 min, 35 cycles (a denaturation at 95˚C for 25 sec, annealing at 56˚C for 25 sec, an extension at 72˚C for 35 sec) and a final extension at 72˚C for 5 min. The primer sequences of p53, p16, hMLH1, DNMT1, DNMT3a and DNMT3b are listed in Table I.

Western blotting. The protein expression levels of methylation and acetylation-related factors were detected by performing western blotting in SKOV3 ovarian cancer cells treated with different concentrations of ginsenoside Rg3 (0, 50, 100 and 200 μg/ml), 50 μg/ml 5-aza-dc (as a negative control) or 500 ng/ml HDAC inhibitor TSA (as a positive control). Cells were lysed by RIPA lysis buffer (Wuhan Boster Biological Technology, Ltd., Wuhan, China) for 20 min and centrifuged on ice at 12,000 x g for 10 min. The supernatant with proteins was first quantified using a BCA protein assay kit (Pierce; Thermo Fisher Scientific, Inc.) and then subjected to 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), for protein separation. Next, the separated proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Thermo Fisher Scientific, Inc.). The membranes were blocked using 5% non-fat dry milk at 37˚C for 1 h, and were first probed with specific primary antibodies overnight at 4˚C and then with secondary antibody for 1 h at 37˚C. The immunoblots were visualized using enhanced chemiluminescense (ECL) detection reagents (Pierce; Thermo Fisher Scientific, Inc.) and analyzed by Bio-Rad ChemiDoc XRS densitometry with Image Lab™ Software version 6.0.1 (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The primary antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA): Rabbit anti-p53 (dilution 1:1,000; cat. no. 2527), p16 (dilution 1:1,000; cat. no. 80772), hMLH1 (dilution 1:1,000; cat. no. 4256), DNMT1 (dilution 1:1,000; cat. no. 5032), DNMT3a (dilution 1:1,000; cat. no. 32578), DNMT3b (dilution 1:1,000; cat. no. 57868), acetyl-H3 K14 (dilution 1:1,000; cat. no. 7627), acetyl-H3 K9 (dilution 1:1,000; cat. no. 9649), acetyl-H4 K12 (dilution 1:1,000; cat. no. 13944), acetyl-H4 K5 (dilution 1:1,000; cat. no. 8647), acetyl-H4 K16 (dilution 1:1,000; cat. no. 13534) and β-actin (dilution 1:1,000; cat. no. 4970). The secondary antibodies were anti-rabbit IgG and HRP-linked antibody (dilution 1:5,000; cat. no. 7074; Cell Signaling Technology, Inc.).

Statistical analysis. The statistical analysis was carried out using SPSS 19.0 (IBM Corp., Armonk, NY, USA). The data were obtained from at least three repeated experiments. The significance of difference was analysed by Dunnett's post hoc test. $P<0.05$ or $P<0.01$ was considered to indicate a statistically significant difference.

Table I. The primer sequences used in the present study.

Name	Type	Sequence (5'-3')
β-actin	F	GTGGACATCCGCAAAGAC
	R	GAAAGGGTGTAACGCAACT
p53	F	GCCCCTCCTCAGCATCTTAT
	R	AAAGCTGTTCCGTCCCAGTA
p16	F	CAGGTCATGATGATGGGCAG
	R	GATGGCCCAGCTCCTCAG
hMLH1	F	CTACTTCCAGCAACCCCAGA
	R	AGAACCTCATGTCCCTGCTC
DNMT1	F	CCGACTACATCAAAGGCAGC
	R	AGGTTGATGTCTGCGTGGTA
DNMT3a	F	GGGACCCCTACTACATCAGC
	R	CATTCTTGTCCCCAGCATCG
DNMT3b	F	GGCCACCTTCAATAAGCTCG
	R	GTTGCGTGTTGTTGGGTTTG

F, forward; R, reverse.

Results

Ginsenoside Rg3 inhibits cell proliferation and promotes apoptosis of ovarian cancer cells. The cell proliferation abilities using CCK-8 assay were detected in order to investigate the effect of ginsenoside Rg3 on SKOV3 ovarian cancer cell proliferation. First, the cytotoxic effects of classical anticancer drugs cisplatin (Fig. 1A) and Rg3 (Fig. 1B) were investigated in both normal HOSEpiC and ovarian cancer cells SKOV3. It was revealed that Rg3 had almost no cytotoxicity to HoSEpiC and SKOV3 cells with an IC_{50} of 1,000 and 400 μg/ml, respectively, compared to cisplatin (IC_{50} of HOSEpiC, 29 μg/ml; IC_{50} of SKOV3, 14 μg/ml) (Fig. 1C). Therefore, the concentrations of Rg3 used in subsequent experiments were significant to the study. The results of the present experiment revealed that cell proliferation abilities were inhibited by ginsenoside Rg3 treatment in dose-dependent (0, 25, 50, 100 and 200 μg/ml) and time-dependent (12, 24 and 48 h) manners. It was revealed that cell viability was significantly decreased when the cells were incubated with different concentrations of ginsenoside Rg3 for 48 h, compared to cells being incubated for 12 h ($P<0.01$, Fig. 1B). When the concentration of ginsenoside was 500 μg/ml (>200 μg/ml), the inhibition rate was revealed to decrease again. Thus, 50, 100 and 200 μg/ml ginsenoside Rg3 were selected to treat cells in subsequent experiments. The effect of ginsenoside Rg3 on cell apoptosis of SKOV3 ovarian cancer cells was then detected by carrying out Annexin V/PI assay. The cell apoptosis rates were revealed to be significantly promoted by ginsenoside Rg3 treatment (50, 100 and 200 μg/ml) ($P<0.01$, Fig. 1D and E).

Ginsenoside Rg3 inhibits cell invasion and metastatic abilities of ovarian cancer cells. The effect of ginsenoside Rg3 on cell invasion and metastatic abilities of SKOV3 ovarian

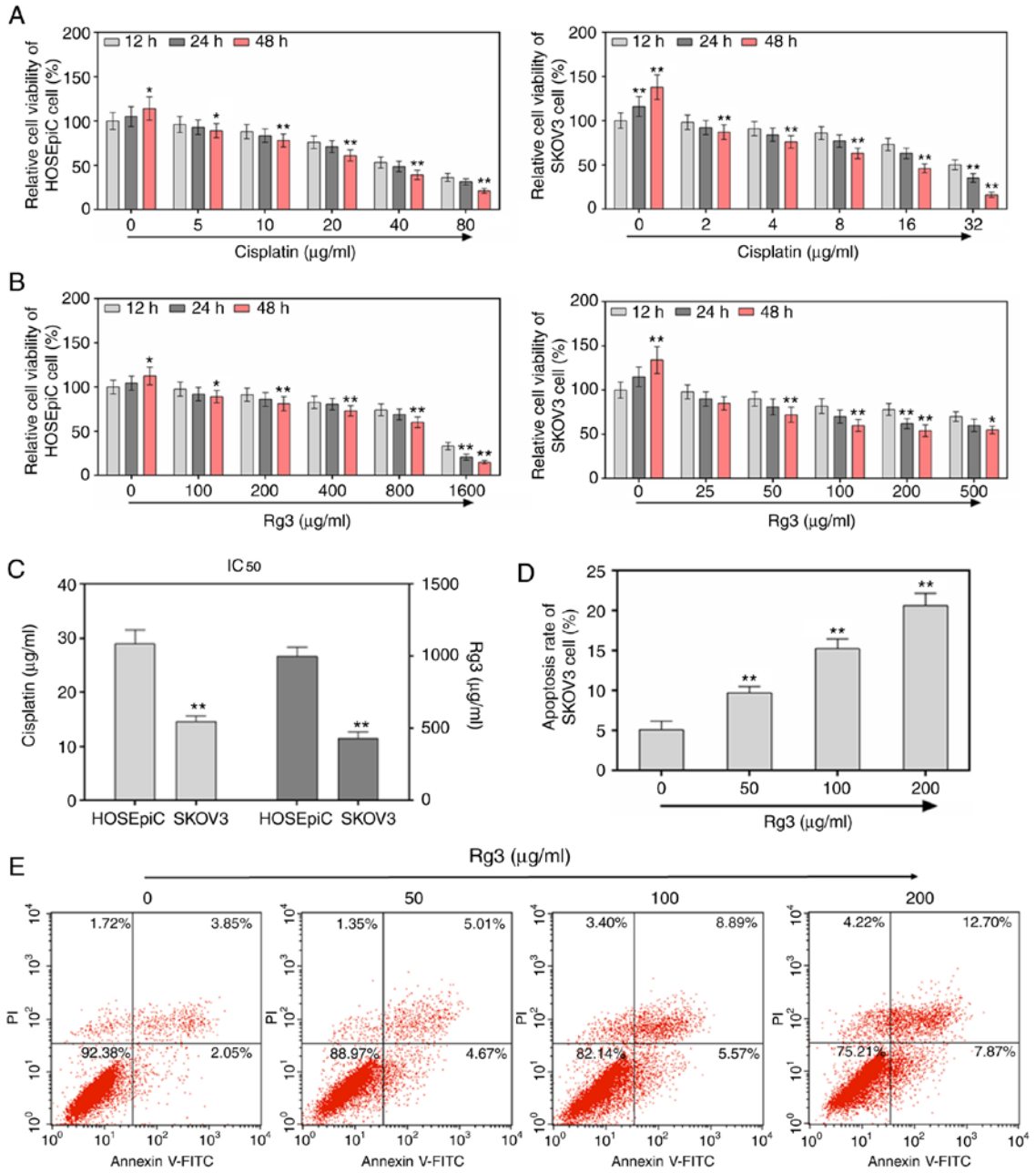

Figure 1. Ginsenoside Rg3 inhibits cell proliferation and promotes apoptosis of ovarian cancer cells. Cell viabilities were detected by treatment with different concentrations of classical anticancer drugs (A) cisplatin and (B) Rg3 in human normal ovarian epithelial cells HOSEpiC and human ovarian cancer SKOV3 cells at 12, 24 and 48 h. *P<0.05 and **P<0.01 vs. 12 h of each concentration. (C) The IC_{50} of cisplatin and Rg3 in human normal ovarian epithelial cells HOSEpiC and human ovarian cancer SKOV3 cells at 48 h. *P<0.05 and **P<0.01 vs. HOSEpiC cells. (D and E) The cell apoptosis rates were revealed to be significantly promoted by ginsenoside Rg3 treatment (50, 100 and 200 μg/ml). *P<0.05 and **P<0.01 vs. ovarian cancer cells without ginsenoside Rg3 treatment.

cancer cells was determined respectively by performing Transwell and wound healing assays. The Transwell images with 200-fold amplification demonstrated that the invasion rates of SKOV3 ovarian cancer cells significantly decreased as concentration of ginsenoside Rg3 increased from 50, to 100 and to 200 μg/ml (P<0.01, Fig. 2A and B). The wound healing images revealed that the metastatic rates of SKOV3 ovarian cancer cells also significantly decreased as concentration of ginsenoside Rg3 increased from 50, to 100 and to 200 μg/ml, both at 12 and 24 h. (P<0.01, Fig. 2C and D).

Ginsenoside Rg3 inhibits methylation levels in ovarian cancer cells. By performing MSP detection, the methylation levels of p53, p16 and hMLH1 were revealed to be significantly decreased by ginsenoside Rg3 treatment (0, 50, 100 and 200 μg/ml), while the un-methylation levels were significantly increased (Fig. 3A-C). In addition, the mRNA and protein levels of p53, p16 and hMLH1 were respectively assessed by carrying out RT-qPCR and western blot assays. The data demonstrated that the expression of p53, p16 and hMLH1 were significantly increased by ginsenoside Rg3 treatment, compared to those in the control group (cells not treated with ginsenoside Rg3) (P<0.05, Fig. 3D-J). In addition, treating 5-aza-dc as a positive control, the activity of DNA methyltransferases (DNMTs) was assessed using EpiQuik DNMT Activity Assay Kit. The results revealed that ginsenoside

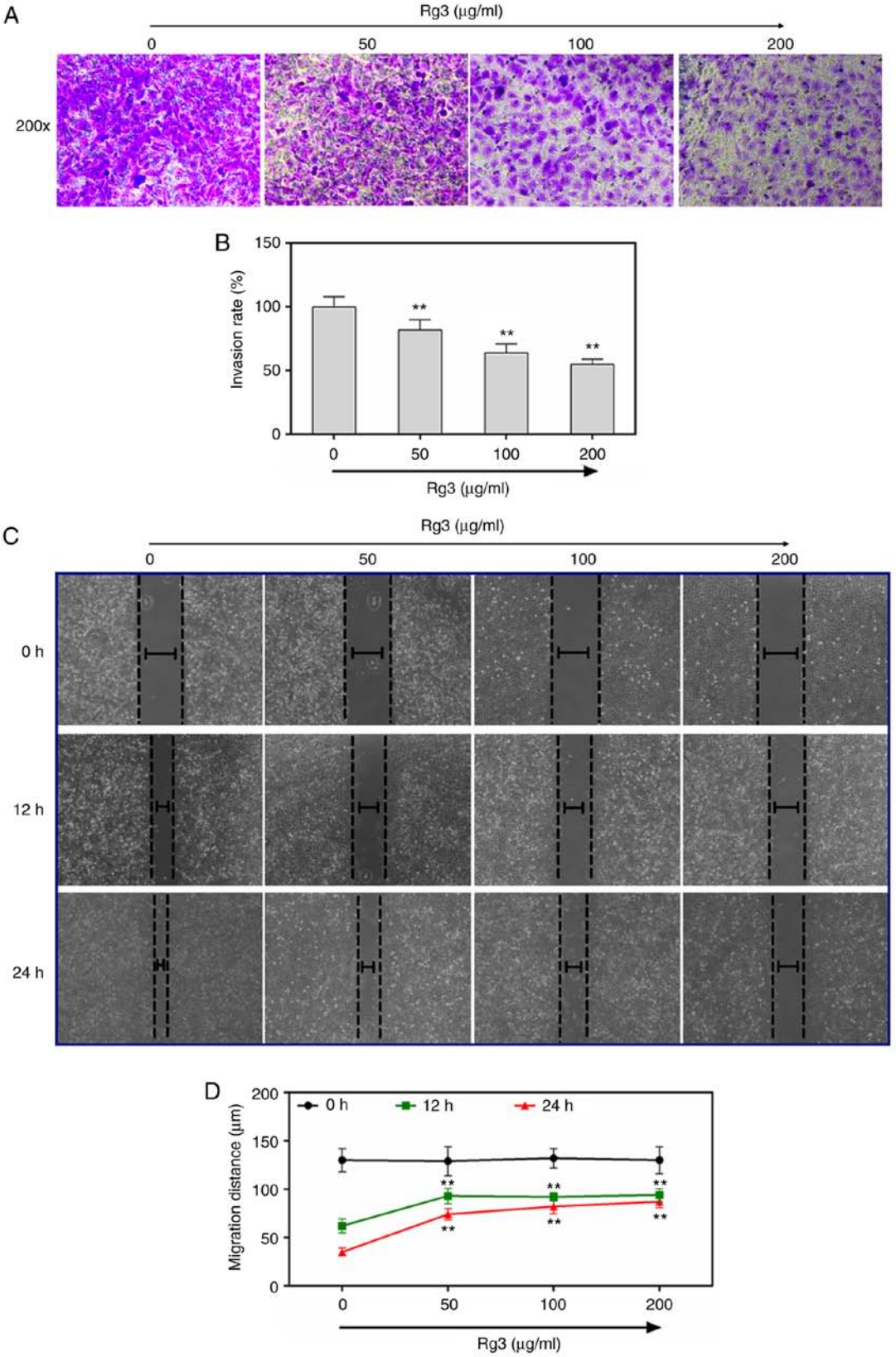

Figure 2. Ginsenoside Rg3 inhibits cell invasion and metastatic abilities of ovarian cancer cells. (A and B) The invasion rates of SKOV3 ovarian cancer cells significantly decreased as concentration of ginsenoside Rg3 increased from 50, to 100 and to 200 μg/ml. (C and D) The metastatic rates of SKOV3 ovarian cancer cells significantly decreased as concentration of ginsenoside Rg3 increased from 50, to 100 and to 200 μg/ml. **P<0.01 vs. ovarian cancer cells without ginsenoside Rg3 treatment.

Rg3 significantly decreased the DNMT activities in SKOV3 ovarian cancer cells (P<0.05, Fig. 4A). The mRNA and protein levels of DNMT1, DNMT3a and DNMT3b were also determined by performing RT-qPCR and western blot assays,

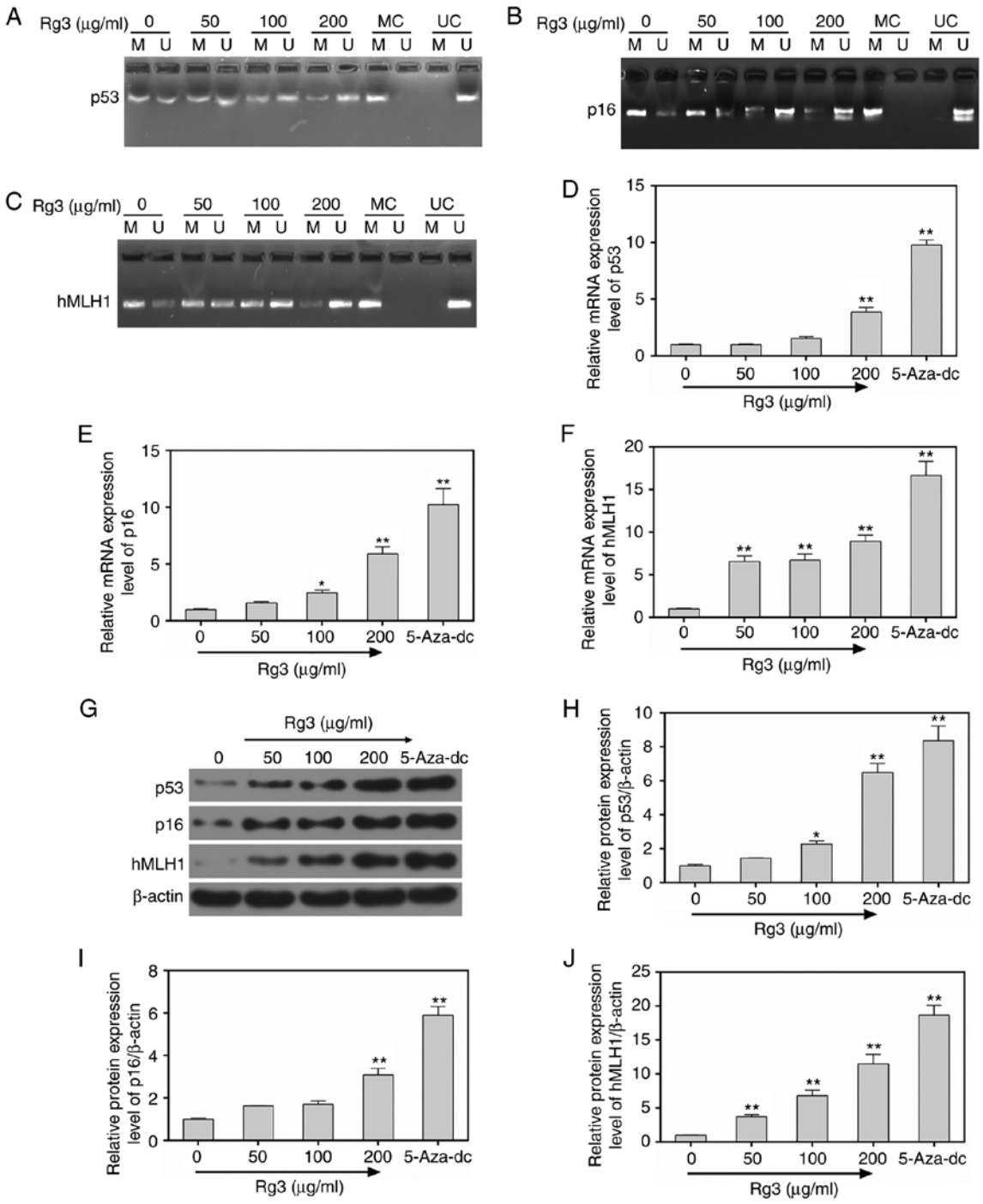

Figure 3. Ginsenoside Rg3 inhibits methylation levels in ovarian cancer cells. (A-C) The methylation levels of p53, p16 and hMLH1 were detected using MSP and the results revealed that the levels were significantly decreased by ginsenoside Rg3 treatment (0, 50, 100 and 200 μg/ml). The (D-F) mRNA and (G-J) protein levels of p53, p16 and hMLH1 were respectively determined by performing real-time quantitative polymerase chain reaction and western blotting. *P<0.05 and **P<0.01 vs. ovarian cancer cells without ginsenoside Rg3 treatment. MSP, methylation specific PCR; hMLH1, human mutL homolog-1.

and it was revealed that the expression of DNMT1, DNMT3a and DNMT3b were significantly decreased by ginsenoside Rg3 (P<0.05, Fig. 4B-H).

Ginsenoside Rg3 promotes acetylation levels in ovarian cancer cells. The activity of deacetylases HDAC was detected by chemical colorimetry, and the results revealed that HDAC activity was markedly decreased by ginsenoside Rg3 treatment (0, 50, 100 and 200 μg/ml) in SKOV3 ovarian cancer cells (P<0.05, Fig. 5A). TSA was treated as a positive control. Subsequently, the protein levels of acetylated H3 K14 and K9 were assessed by western blot assays. The results revealed that acetylated H3 K14 and K9 were significantly increased when SKOV3 ovarian cells were treated with 0, 50, 100 and 200 μg/ml ginsenoside Rg3 (P<0.05, Fig. 5B-D). The protein levels of acetylated H4 K12, K5 and K16 were significantly increased when SKOV3 ovarian cells were treated with 0, 50, 100 and 200 μg/ml ginsenoside Rg3 (P<0.05, Fig. 5E-H).

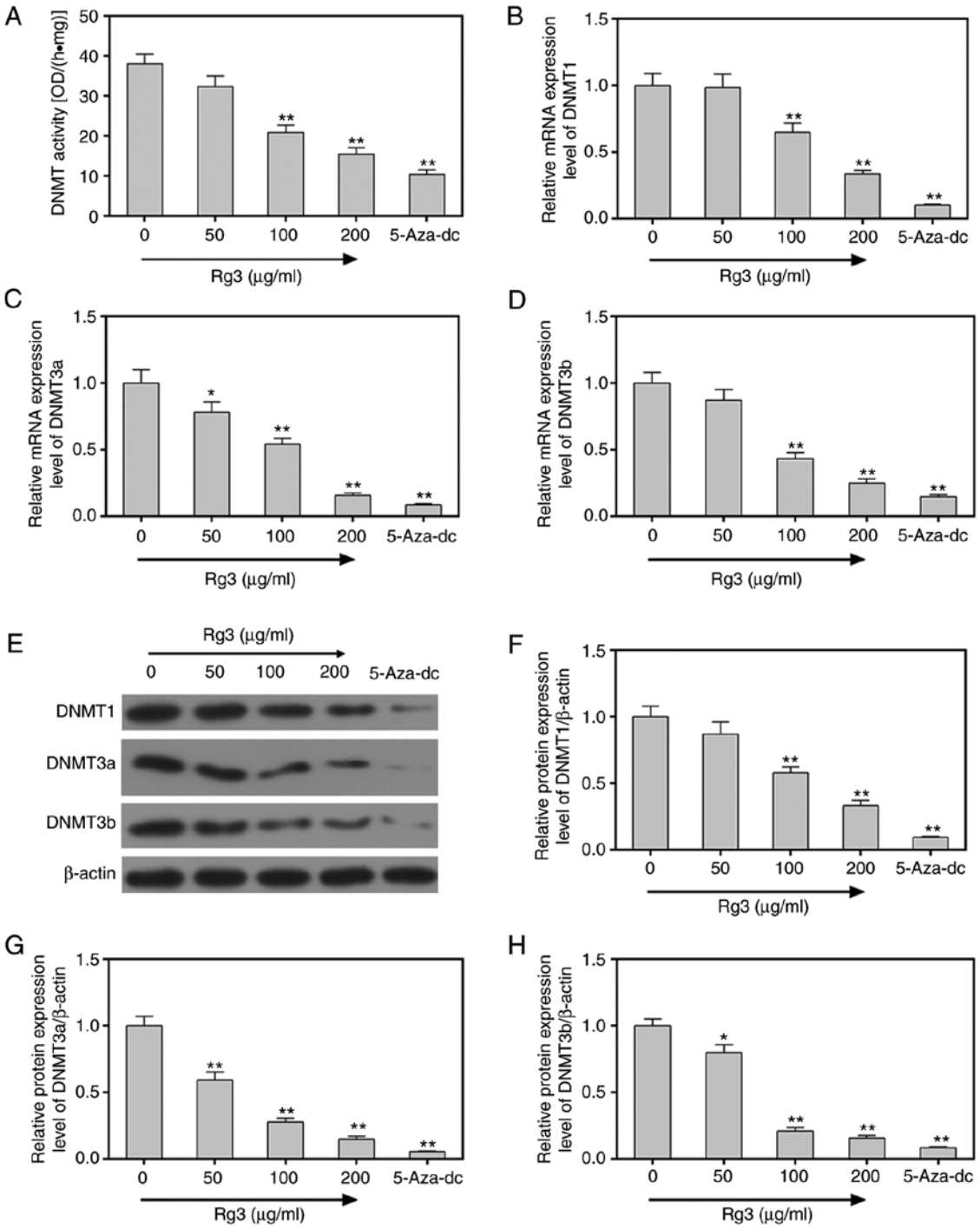

Figure 4. Ginsenoside Rg3 inhibits DNMT activities in ovarian cancer cells. (A) The activity of DNMT was determined by EpiQuik DNMT Activity/Inhibition Assay Ultra Kit in ovarian cancer cells treated with ginsenoside Rg3 (0, 50, 100 and 200 μg/ml) or 50 μg/ml 5-aza-dc (as a negative control). The (B-D) mRNA and (E-H) protein levels of DNMT1, DNMT3a and DNMT3b were respectively determined by performing RT-qPCR and western blotting, and were revealed to be significantly decreased. *P<0.05 and **P<0.01 vs. ovarian cancer cells without ginsenoside Rg3 treatment. DNMT, DNA methyltransferase; 5-aza-dc, 5-aza-2'-deoxycitydine; RT-qPCR, real-time quantitative polymerase chain reaction.

Discussion

Despite surgery and chemotherapy that have been applied in the treatment of ovarian cancer, the overall survival rate among ovarian cancer patients has not significantly improved (19) and the incidence and mortality of cancer is still increasing (20). Thus, the diagnosis and treatment of ovarian cancer is still a research hotspot.

As a ginsenoside monomer, ginsenoside Rg3 has been reported to be helpful for ovarian cancer treatment. In this research, we studied whether the effect of ginsenoside Rg3 on ovarian cancer cells was related to the molecular mechanism of methylation and histone acetylation. It was first ascertained that ginsenoside Rg3 had almost no obvious cytotoxicity effect, and then it was revealed that Rg3 inhibited cell proliferation, invasion and metastasis and promoted cell apoptosis

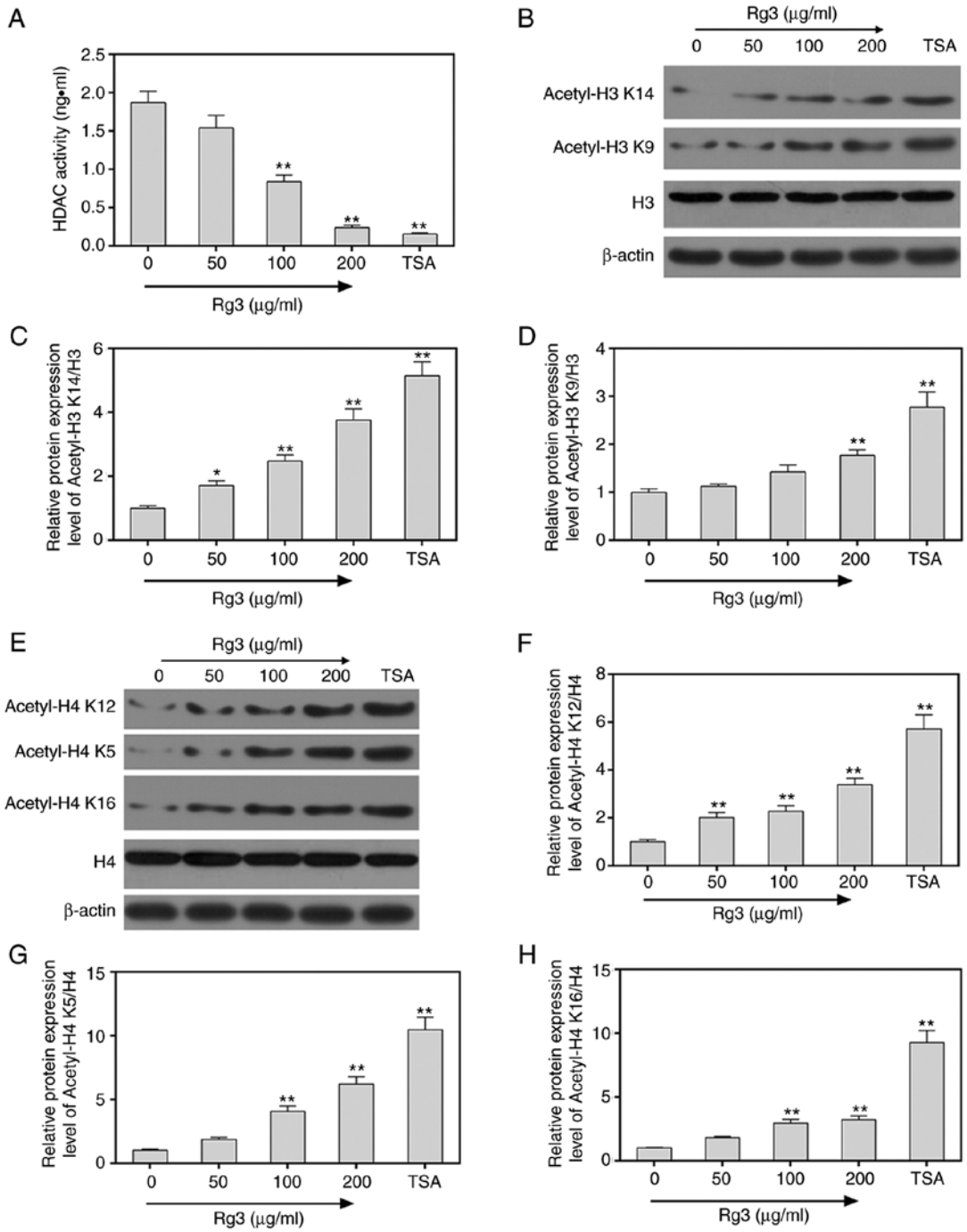

Figure 5. Ginsenoside Rg3 promotes acetylation levels in ovarian cancer cells. (A) The activity of HDAC was determined using Epigenase HDAC Activity/Inhibition Direct Assay Kit in ovarian cancer cells treated with ginsenoside Rg3 (0, 50, 100 and 200 μg/ml) or 500 ng/ml HDAC inhibitor TSA. (B-D) The protein levels of acetylated H3 K14 and K9 were determined by performing western blotting and were revealed to be significantly increased. (E-H) The protein levels of acetylated H4 K12, K5 and K16 were determined by performing western blotting and were revealed to be significantly increased. *P<0.05 and **P<0.01 vs. ovarian cancer cells without ginsenoside Rg3 treatment. HDAC, histone deacetylase; TSA, trichostatin A.

in a dose-dependent manner and the effects of Rg3 on migration/invasion were not completely due to the effects on cell viability. 5-Aza-dc is a commonly used demethylation drug in clinical treatment of many cancers. However, 5-aza-dc also produces severe side effects. In the present study the de-methylation function of ginsenoside Rg3 with 5-aza-dc was compared in ovarian cancer cells.

As two tumor suppressor genes, p53 and p16 (alternatively named multiple tumor suppressor 1 (MTS1) or cyclin-dependent kinase inhibitor 2A (CDKN2A) are highly efficient transcription factors that not only bind to 300 promoter elements in the genome, but also extensively alter gene expression patterns (21). Normal p53 and p16 proteins act as cell cycle checkpoints and participate in the process of DNA repair and apoptosis (22). The activities of p53 and p16 are regulated by post-translational modifications, for example, methylation, acetylation or phosphorylation (23,24). The inactivation of p53 or p16 is considered to be one of the main

features of malignant tumors (25). MLH1 is responsible for DNA mismatch repair, and the methylated hMLH1 has been frequently identified in many cancers, for instance, colorectal and esophageal cancer (26,27). In this study, an MSP assay was used to detect the methylation levels in the promoter region of p53, p16 and hMLH1. It was revealed that the methylation levels of CpG islands in p53, p16 and hMLH1 decreased, and that the levels of p53, p16 and hMLH1 significantly increased when ovarian cancer cells were treated with ginsenoside Rg3 or 5-aza-dc. This indicated that ginsenoside Rg3 promoted tumor suppressor function of p53, p16 and hMLH1 in ovarian cancer cells by inhibiting promoter-methylation and expression levels.

The enzymes, which catalyze the DNA methylation reaction, mainly include DNMT1, DNMT3a and DNMT3b. DNMT1 is the main enzyme that maintains the methylation reaction. After DNA replication is completed, DNMT1 catalyzes the transfer of methyl groups to newly synthesized DNA duplexes. DNMT3a and DNMT3b are responsible for catalyzing the methylation formation (*de novo* methylation) reaction on DNA duplex (28). In the present study, it was revealed that ginsenoside Rg3 was able to inhibit the expression levels of DNMT1, DNMT3a and DNMT3b. These findings indicated that ginsenoside Rg3 could be used as a potential de-methylation drug in cancer treatment.

Apart from promoter-methylation, histone-deacetylation modification was also frequently found in some cancers such as gastric cancer, colorectal cancer. The balance of histone acetylation and deaetylation is an indispensable condition for tumor development. HDACs are a family of proteases that catalyze the hydrolysis of acetyl groups at the ends of lysine residues in various substrates such as nucleosomal histones (29,30). HDACs are the histone deacetylases which can inhibit gene transcription and promote tumor proliferation (31). TSA is a commonly used HDAC inhibitor, and it can inhibit tumor cell proliferation (32). The effect of ginsenoside Rg3 on deacetylatiion in ovarian cancer cells was investigated by detecting HDAC activity and the acetylation levels of histone H3 and H4, and the results revealed that ginsenoside Rg3 inhibited HDAC activity and increased the levels of H3 K14, H3 K9, H4 K12, H4 K5 and H4 K16. This indicated that ginsenoside Rg3 not only participated in methylation modification, but also in acetylation modification. Collectively, ginsenoside Rg3 functioned as tumor suppressor in ovarian cancer cells. Notably, the antitumor effect of ginsenoside Rg3 on other ovarian cancer cell lines should be investigated and it is also essential to undertake animal experiments to demonstrate the role of ginsenoside Rg3 *in vivo*.

In conclusion, it was revealed that ginsenoside Rg3 inhibited ovarian cancer cell proliferation, metastasis and invasion and promoted cell apoptosis by regulating methylation and acetylation. Thus, ginsenoside Rg3 could be applied as a methylase or histone deacetylase inhibitor to inhibit cell proliferation in ovarian cancer treatment. The methylation levels of tumor suppressor genes, or acetylation levels of histones may be considered as novel markers in diagnosing ovarian cancer.

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

LZ, HS, substantially contributed to the conception and design of the manuscript and drafted the article or critically revised it for important intellectual content. LC, WG, CF and PZ acquired, analyzed and interpreted the data. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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