Ginsenoside Rg3 suppresses ovarian cancer cell proliferation and invasion by inhibiting the expression of IncRNA H19

Lingqin Zhao1,2,4, Wei Sun1,2,4, Aiwen Zheng1,2, Yingli Zhang1,2, Chenyan Fang1,2 and Ping Zhang1,2*

1 Department of Gynecologic Oncology, Cancer Hospital of the University of Chinese Academy of Sciences, Zhejiang Cancer Hospital, Gongshu District, Hangzhou City, Zhejiang Province, 310000, China; 2 Institute of Cancer and Basic Medicine, IBMC, Chinese Academy of Sciences, China

Ovarian cancer (OC) is the most malignant disease of the female reproductive system and accounts for a large proportion of gynecological cancer-related deaths. Emerging evidence has indicated that ginsenoside Rg3, one of the tetracyclic triterpenoid saponins in ginseng, plays crucial roles in regulating cancer progression, yet its role and mechanisms in regulating the proliferation and invasion of OC are still elusive. In this study, the cell viability, proliferation, migration and invasion of OC were assessed by using methyl thiazol tetrazolium (MTT), colony formation, wound healing and Transwell assays, respectively. The protein levels of E-cadherin and N-cadherin were analyzed by Western blot assay. The expression of long noncoding RNA (IncRNA) H19 was analyzed by quantitative real-time polymerase chain reaction (RT-qPCR). The results revealed that ginsenoside Rg3 significantly inhibited the viability of OC cells (SKOV3 and A2780) in a concentration-dependent manner. Ginsenoside Rg3 (50 μg/ml) had almost no significant effect on the activity of human ovarian epithelial cells (HOSEpiCs). Thus, this dose was selected for the subsequent experiments. Furthermore, Rg3 markedly decreased the colony formation, migration and invasion of OC cells. In addition, the expression of N-cadherin was downregulated, and the expression of E-cadherin was upregulated with Rg3 treatment. Moreover, IncRNA H19 was upregulated in OC cells, and Rg3 negatively regulated H19 expression in a concentration-dependent manner. In terms of the mechanism, knockdown of H19 inhibited cell proliferation, migration and invasion, while overexpression of H19 reversed the inhibitory effect of Rg3 on the OC cells. In conclusion, ginsenoside Rg3 suppresses the proliferation, migration and invasion of OC cells by partially inhibiting the expression of IncRNA H19.

Keywords: ovarian cancer, ginsenoside Rg3, IncRNA H19, proliferation, migration, invasion

Received: 15 April, 2020; revised: 26 July, 2020; accepted: 08 September, 2020; available online: 26 May, 2021

*e-mail: ping725020@163.com

The two authors have contributed equally to this article

Acknowledgements of Financial Support: The research was supported by the 2018 Zhejiang Provincial Fund for Outstanding Young Talents in Traditional Chinese Medicine (No. 201802009) and Zhejiang Province Science and Technology Project of Traditional Chinese Medicine (No. 20212B039).

Abbreviations: IncRNA, long noncoding RNA; MTT, methyl thiazol tetrazolium; OC, ovarian cancer

INTRODUCTION

Ovarian cancer is the most malignant tumor of the female reproductive system and is the 7th most common cancer diagnosis and the 8th leading cause of cancer-related death in women (Coburn et al., 2017; Bookman, 2016; Yokoi et al., 2018); its early symptoms are nonspecific, the disease progresses rapidly, and the 5-year survival rate is only approximately 25%, which seriously threatens women’s health (Pisani et al., 2018; Zheng et al., 2019). In 2018, 22,240 new ovarian cancer cases were estimated in the United States (2.5% of all malignancies in females), and there were approximately 14,070 ovarian cancer-related deaths; less than 40% of ovarian cancer patients can achieve long-term relief and cure (Torre et al., 2018). At present, the main clinical treatment method for ovarian cancer is debulking surgery combined with chemotherapy, but chemotherapeutic drugs are largely toxic and have side effects, and the overall treatment effect is not satisfactory (Berns et al., 2012; Cortez et al., 2018). Therefore, the identification of active ingredients from natural medicinal plants with low toxicity and highly effective anti-ovarian cancer activity is urgently necessary.

Ginsenoside Rg3 is a tetracyclic triterpenoid saponin component in ginseng. Studies have shown that ginsenoside Rg3 can inhibit tumor cell proliferation, invasion and metastasis, epithelial–mesenchymal transition, stemness and neovascularization, induce tumor cell apoptosis, and reverse multidrug resistance of tumor cells (Lee et al., 2015; Li et al., 2015; Li et al., 2016; Phi et al., 2019; Tian et al., 2016; Yuan et al., 2017; Wang et al., 2018). In recent years, related studies suggested the anti-ovarian cancer effect of ginsenoside Rg3 (Zheng et al., 2017; Zheng et al., 2018; Liu et al., 2017; Li et al., 2017). Interestingly, our previous results revealed that ginsenoside Rg3 inhibited cell proliferation, migration and invasion but promoted apoptosis in SKOV3 ovarian cancer cells (Zhao et al., 2019). Moreover, ginsenoside Rg3 decreased hypermethylation of CpG islands in the promoter region of tumor suppressor genes (p53, p16 and hMLH1), reduced the expression levels of DNA cytosome methyltransferases (DNMT1, DNMT3a and DNMT3b), and inhibited the activities of histone deacetylases (HDACs) (Zhao et al., 2019). All of these findings suggested that ginsenoside Rg3 may have potential anticancer activity in OC. The regulation of ovarian cancer cells by ginsenoside Rg3 through epigenetics and its specific molecular mechanism deserves further study.

Long noncoding RNA (IncRNA) is a type of RNA with transcripts longer than 200 nt. With the development of genome-wide sequencing technology and the rapid advancement of bioinformatics, the research found that IncRNAs can participate in many important regulatory processes, such as X chromosome silencing, genomic imprinting, chromatin modification, and transcriptional...
activation/repression. LncRNAs play an important role in the occurrence and development of many diseases, such as cancer, neurodegenerative diseases and other major diseases that seriously endanger human health (Khorkova et al., 2015). LncRNA H19, a long noncoding RNA with a length of approximately 2.6 kb, has been shown to promote the development of cancer, including OC (Zhao et al., 2019; Wu et al., 2015). LncRNA H19 was recently identified as a crucial regulator of methylation levels of genomic DNA, mainly through interaction with S-adenosyl-homocysteine hydrolase (SAHH). Ginsenoside Rg3 was found to reduce the expression level of DNMT3b (Zhao et al., 2019), and it was reported that the knockout of H19 gene activated SAHH and increased the methylation of DNMT3b mediated lncRNA H19 encoding gene Netc1 (Zhou et al., 2015). Moreover, ginsenoside Rg3 was recently found to upregulate lncRNA H19 expression (Zheng et al., 2018), we speculated that H19 might be involved in the effects of Rg3 on OC.

In the present study, we explored the anticancer activity of ginsenoside Rg3 in OC cell lines (SKOV3 and A2780). Our results indicated that ginsenoside Rg3 can inhibit the proliferation, migration and invasion of OC cells. Additionally, we observed an upregulation of H19 in OC cells compared to normal controls. Rg3 negatively regulated H19 expression, and we explored the potential mechanism of this effect. This study provided a novel mechanism for the efficacy of ginsenoside Rg3 against OC and showed that ginsenoside Rg3 may serve as an effective drug for OC and other diseases.

MATERIALS AND METHODS

Cell culture and treatment

A human ovarian epithelial cell line (HOSEpiC) and human OC cell lines (SKOV3 and A2780) were obtained from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. The cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% fetal bovine serum (FBS) and antibiotics: penicillin plus streptomycin. The cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with protease inhibitors (50 mM Tris-HCl pH 8, 50 mM NaCl, 0.5% NP-40). The protein concentration was determined by the Bio-Rad (Bradford) protein assay. The cells (500 per well) transfected as indicated were seeded into 6-well plates and exposed to ginsenoside Rg3 for 24 h following transfection. After culturing for 14 days, the colonies were fixed with 4% paraformaldehyde for 15 min and stained with 0.5% crystal violet (Sigma) for 30 min. The colonies were photographed using a light microscope (TE2000-U, Nikon, Japan).

Wound healing assay

Cells (10^4 per well) transacted as indicated were seeded into 6-well plates, cultured to reach 100% confluence and then exposed to ginsenoside Rg3 for 24 h. A straight scratch was made with a pipette tip in each well. The wounds were photographed at 0 h and 24 h using a microscope (Nikon) and the width of the wound was measured.

Cell invasion assay

Cells (1×10^5 per well) resuspended in 200 μl serum-free medium were seeded into the top chamber of an insert (Corning Costar Co., Cambridge, MA, USA) pre-coated with Matrigel. Then, 800 μl of the medium containing 10% FBS was added into the lower chamber. After 48 h, the cells on the membrane were fixed and stained using crystal violet (Takara, Dalian, China).

RT-qPCR

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. The RNA was then reverse transcribed into cDNA using the PrimeScript RT reagent Kit with gDNA Eraser (Takara Bio, Otsu, Japan). mRNA expression was examined using the ABI PRISM 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and the ExScript SYBR green QPCR kit (TaKaRa, Dalian, China). The relative mRNA expression was normalized to the expression of GAPDH using the 2−ΔΔCT method. The primers were as follows: lncRNA H19 (forward): 5’-ACCACGTGCACTACCTGACTCT-3’, (reverse): 5’-CCGCAGGGGGTGGCCATGAA-3’; GAPDH (forward): 5’-CAGTACGGTGTCACTCAGG-3’, (reverse): 5’-CTCTTTATGTGCACCGCAGAT-3’.

Western blot analysis

Cells were harvested and lysed in RIPA buffer supplemented with protease inhibitors (50 mM Tris-HCl pH 8, 50 mM NaCl, 0.5% NP-40). The protein concentration was determined by the Bio-Rad (Bradford) protein assay (Bio-Rad, Hercules, CA, USA), and 50 μg samples of total protein were separated by denaturing 12% SDS-PAGE and transferred onto a PVDF membrane (Amersham, Little Chalfont, UK). After blocking with 5% nonfat milk in a 0.1% TBST solution for 1 h at room temperature, the membranes were first incubated with primary antibodies (all from Abcam, Cambridge, MA, USA) against E-cadherin (ab15148; 1:1000), N-cadherin (ab76057; 1:1000), and GAPDH (ab181602, 1:1000)
overnight at 4°C. After washing, blots were incubated with HRP-conjugated goat anti-rabbit (ab6721, 1:2000) or HRP-conjugated goat anti-mouse (ab205719, 1:2000) secondary antibodies for 1 h. Immunostaining of the protein bands was visualized using an enhanced chemiluminescence (ECL) detection system. The protein levels were determined by normalization against GAPDH.

Statistical analysis

All the results were presented as the mean ± S.D. and were analyzed using GraphPad Prism 7.0 (GraphPad Software Inc., San Diego, CA, USA) and SPSS software (IBM SPSS 22.0, Chicago, IL). Two-tailed Student’s t-test and one-way ANOVA were used to compare the differences between two groups and among multiple groups, respectively. **p<0.01 was considered as an indicator of statistical significance.

RESULTS

Ginsenoside Rg3 inhibits the proliferation of ovarian cancer cells

HoSEpiC, SKOV3 and A2780 cells were treated with different concentrations of Rg3 for 24 h, and cell viability was determined by MTT assay. The data indicated that Rg3 showed almost no cytotoxicity toward HoSEpiC cells but significantly decreased the viability of SKOV3 and A2780 cells in a concentration-dependent manner with IC50 values of 119.8 µg/ml and 64.9 µg/ml, respectively (Fig. 1A). Therefore, the concentration of Rg3 used in the subsequent experiments was 50 µg/ml. Moreover, colony formation assays also confirmed that 50 µg/ml Rg3 significantly inhibited the proliferation of SKOV3 and A2780 cells (Fig. 1B).

Ginsenoside Rg3 inhibits ovarian cancer cells’ migration and invasion

Strong migration and invasion are critical steps in cancer metastasis (Yamaguchi et al., 2007). Therefore, we evaluated the effect of Rg3 on the migratory and invasion abilities of OC cells through wound healing and Transwell assays. The results showed that Rg3 decreased the cell migration speed and the number of invading cells in both SKOV3 and A2780 cells (Fig. 2A–B). Tumor metastasis is usually accompanied by epithelial-mesenchymal transition (EMT). EMT is a physiological process in which epithelial cells lose their polarity and cell-to-cell adhesion and acquire a mesenchymal phenotype, which is characterized by upregulation of N-cadherin and downregulation of E-cadherin (Lamouille et al., 2014). Western blot assay data indicated that Rg3 increased E-cadherin levels but decreased N-cadherin levels in SKOV3 and A2780 cells (Fig. 2C), indicating that Rg3 might reverse the EMT process in OC cells.

Ginsenoside Rg3 inhibits the expression of lncRNA H19 in ovarian cancer cells

In view of the above results and recent studies showing that Rg3 can downregulate the expression of H19 (Zheng et al., 2018), we determined whether the inhibitory effect of Rg3 on OC cells is exerted by affecting the expression of H19. As shown in Fig. 3A, aberrantly high expression of H19 was observed in SKOV3 and A2780 cells when compared to HOSEpiC cells. However, the expression of H19 was significantly downregulated by Rg3 pretreatment at different concentrations and times.
Figure 2. Ginsenoside Rg3 inhibits ovarian cancer cells’ migration and invasion.
(A–B) Wound healing and Transwell assays were used to determine the effect of Rg3 on the migration and invasion of SKOV3 and A2780 cells. (C) Western blot assay was used to detect the effect of Rg3 on the protein levels of E-cadherin and N-cadherin in SKOV3 and A2780 cells. Data were presented as the mean ± S.D. of at least three independent experiments. **p<0.01.

Figure 3. Ginsenoside Rg3 inhibits the expression of lncRNA H19 in ovarian cancer cells.
(A) The expression of H19 in HOSEpiC, SKOV3 and A2780 cells was measured by RT-qPCR. (B) The expression of H19 in SKOV3 and A2780 cells treated with different concentrations of Rg3 was determined by RT-qPCR. (C) The expression of H19 in SKOV3 and A2780 cells treated with Rg3 for different times was determined by RT-qPCR. Data were presented as the mean ± S.D. of at least three independent experiments. **p<0.01, ***p<0.001.
Ginsenoside Rg3 suppresses ovarian cancer cells’ proliferation and invasion

in a concentration-dependent manner (Fig. 3B–C), which suggests that Rg3 has the potential to downregulate H19 expression and that H19 might be involved in the anticancer effects of Rg3 on OC cells.

Downregulation of the expression of lncRNA H19 inhibits the proliferation, migration and invasion of ovarian cancer cells

Numerous studies demonstrated that lncRNA H19 plays an important role in promoting ovarian cancer progression (Wu et al., 2019; Ji et al., 2019; Zheng et al., 2016). Here, we knocked down H19 by transfection with sh-H19 in SKOV3 and A2780 cells. RT-qPCR assays showed that when compared to the sh-NC group, the expression level of H19 in the sh-H19 group was significantly decreased, indicating that H19 was successfully knocked down in the transfected cells (Fig. 4A). Consistent with the literature research, knockdown of H19 distinctly decreased the cell viability, colony number, migration and invasion of SKOV3 and A2780 cells (Fig. 4B–E). Moreover, downregulation of lncRNA H19 expression increased E-cadherin and decreased N-cadherin expression (Fig. 4F). Taken together, these data indicate that H19 serves as an oncogene to promote cell proliferation, migration and invasion in OC progression.

Ginsenoside Rg3 exerts its function in ovarian cancer cells at least partially through regulating H19

Since Rg3 can negatively regulate the expression of H19, the anticancer mechanism of Rg3 may partly inhibit the oncogenic function of H19 by reducing its expression. To verify our hypothesis, SKOV3 and A2780 cells were transfected with pcDNA3.1-H19 to overexpress H19 and treated with Rg3 to detect the effects on cell proliferation, migration and invasion. As shown in Fig. 5A, compared to the PBS+Vector group, the expression level of H19 in the PBS+H19 group was significantly increased; that is, H19 was successfully overexpressed in the transfected cells, which was verified using RT-qPCR assay. The results indicated that overexpression of H19 promoted the cell proliferation, migration, invasion and EMT of OC cells compared to that in the PBS+Vector group, while the oncogenic effects of H19 were inhibited by simultaneous treatment with Rg3 (Fig. 5B–F). Similarly, the anticancer effect of Rg3 was also reversed by the simultaneous upregulation of H19.
Therefore, Rg3 may target and negatively regulate H19 as part of its anticancer mechanism.

DISCUSSION

Ovarian cancer is a gynecological malignant tumor that is mainly treated by surgery combined with chemotherapy, radiation therapy, and some immunomodulators (Zheng et al., 2016). However, resistance to chemotherapeutics (cisplatin/paclitaxel doublet) often severely limits the efficacy and outcome of the treatment, even leading to a high recurrence rate (Coukos et al., 1998). Traditional Chinese herbs can increase the effectiveness of chemotherapy and reduce its side effects. Therefore, the combination of Chinese herbal medicine and chemotherapeutic drugs is a very common and promising approach in the clinical treatment of cancer, especially in China and South Korea. Ginsenoside Rg3, as the main chemical component of ginseng, has been identified as a therapeutic agent for many lethal cancers (Coukos & Rubin, 1998). In the present study, we found that ginsenoside Rg3 can specifically target OC cells and is not toxic to noncancerous cells, making it a valuable chemotherapeutic. We explored the potential of ginsenoside Rg3 to inhibit the proliferation, migration and invasion of OC cells, possibly by inhibiting the expression of H19.

There is growing evidence demonstrating that ginsenoside Rg3 participates in important cellular activities, showing anticancer properties in various cancers. Consistently, we confirmed that ginsenoside Rg3 inhibited OC progression by decreasing cell proliferation, migration and invasion. Moreover, for the first time, we revealed that Rg3 regulates the levels of EMT-related proteins (Fig. 5B–F). Therefore, Rg3 may target and negatively regulate H19 as part of its anticancer mechanism.
Ginsenoside Rg3 suppresses ovarian cancer cells' proliferation and invasion

proteins in OC cells by increasing E-cadherin levels and decreasing N-cadherin levels, suggesting a possible mechanism for inhibiting ovarian cancer metastasis. Usually, Rg3 was reported to play an antitumor role by regulating a large number of protein-coding genes or crucial cancer-related signaling pathways. Tian and others (Tian et al., 2016) revealed that ginsenoside Rg3 inhibits EMT and lung cancer cell invasion by inhibiting FUT4. Yuan and others (Yuan et al., 2017) reported that ginsenoside Rg3 promotes the cytoxicity of paclitaxel in triple-negative breast cancer by inhibiting the NF-κB pathway and regulating Bax/Bcl-2 expression. Additionally, Rg3 was shown to inhibit PI3K-AKT (Xie et al., 2017), ERK (Tang et al., 2018), AMPK (Yuan et al., 2010) and other signaling pathways. Rg3 was also shown to be engaged in a wide range of mechanisms and have numerous targets by which it exerts its important cellular activities, but the mechanism of its lncRNA-mediated role is still poorly understood and remains to be fully elucidated.

LncRNA has no protein-coding function and is involved in regulating protein-coding genes in the form of RNA at various levels (epigenetic regulation, transcriptional regulation, post-transcriptional regulation, regulation of miRNA, etc.) (Yuan et al., 2010). Recent studies showed that abnormal changes in lncRNAs are a new mechanism of drug response regulation during tumor progression. Xiong and others (Xiong et al., 2019) indicated that chronic oxymatrine therapy induces drug resistance and EMT by targeting lncRNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) in colorectal cancer cells. Geng and others (Geng et al., 2018) reported that resveratrol suppressed cell proliferation, migration and invasion by downregulating the expression of lncRNA NEAT1 in myeloma cells. In addition, the role of lncRNAs in Rg3-mediated regulation of tumor progression was studied. For example, Rg3 was confirmed to inhibit cancer progression by downregulating the expression of CCAT1 and RFX3-AS1 and upregulating the expression of STXBPs-1 and CASC2 (Li et al., 2019; Ham et al., 2019; Zou et al., 2020). Moreover, a new study suggested that Rg3 decreases the level of 67 lncRNAs in ovarian cancer, of which H19 is one of the most downregulated lncRNAs (Zheng et al., 2018). Rg3 was also confirmed to abrogate the competitive inhibition of miR-324-5p by H19, thus enhancing the inhibition of the Warburg effect and the occurrence of OC (Zheng et al., 2018). Nevertheless, it is still unclear whether the dysregulation of H19 expression is the molecular mechanism by which Rg3 affects the proliferation and metastasis of OC. In this study, H19 was upregulated in OC cells but downregulated by Rg3 treatment, which is consistent with the literature (Ji et al., 2019; Wu et al., 2019; Zheng et al., 2018). Hence, a loss-of-function assay was employed to investigate the effect of H19 in OC cells. We discovered that knockdown of H19 significantly inhibited cell proliferation, migration and invasion. Knockdown of H19 also inhibited the EMT of OC cells; that is, it increased the level of E-cadherin and decreased the level of N-cadherin. Moreover, a rescue assay was performed by simultaneous H19 overexpression and Rg3 treatment, and it was found that the tumor-suppressive function of Rg3 could be partially reversed by H19 overexpression. All these data imply that Rg3 exerts an anticancer effect in OC cells by negatively regulating H19.

Although the regulatory effect of Rg3 on lncRNA H19 was identified in the present study, the specific downstream mechanism by which Rg3 affects H19 has not been fully determined. H19 may intervene in OC pathogenesis by sponging with miR-370-3p, thus acting as a ceRNA (Li et al., 2018), or may directly affect the expression of IGFI2, EZH2, SAHH and other genes to participate in cancer progression (Murphy et al., 2006; Zhou et al., 2015; Luo et al., 2013). Therefore, the mechanism by which H19 exhibits its oncogenic functions in the treatment of ovarian cancer with Rg3 is worth further investigation.

In conclusion, we revealed the function of Rg3 and for the first time demonstrated that Rg3 suppresses OC cell proliferation, migration and invasion by negatively regulating lncRNA H19. Our study provides preliminary confirmation of the potential use of Rg3 as an antitumor agent, which may represent a new nontoxic alternative to traditional cancer treatment.

Acknowledgments

Not applicable.

Competing interests

The authors state that there are no conflicts of interest to disclose.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors’ contributions

PZ conceived and designed the experiments; LQZ and WS analyzed and interpreted the results of the experiments; and AWZ, YLZ and CYF performed the experiments.

Ethics approval and consent to participate

Not applicable.

REFERENCES

Li J, Lu J, Ye Z, Han X, Zheng X, Hou H, Chen W, Li X, Zhao (2017) 20(S)-Rg3 blocked epithelial-mesenchymal transition through


