Inhibition of enhancer of zeste homolog 2 increases the expression of p16 and suppresses the proliferation and migration of ovarian carcinoma cells in vitro and in vivo

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Received April 15, 2017; Accepted November 16, 2017

DOI: 10.3892/ol.2017.7712

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Key words: ovarian cancer, enhancer of zeste homolog 2, p16, epigenetic regulation

Abstract. Enhancer of zeste homolog 2 (EZH2) is a histone methyltransferase, which targets histone H3 lysine 27. Studies have reported that EZH2 is involved in the development of several types of tumor, including ovarian cancer. p16, a well-known cell cycle regulator, has been demonstrated to be a tumor suppressor gene in a variety of malignant cells. However, the regulatory association between EZH2 and p16 in ovarian cancer remains to be fully elucidated. The present study aimed to determine whether EZH2 is involved in the development of ovarian cancer by regulating the expression of p16. An EZH2 short hairpin RNA (shRNA) lentiviral vector was constructed and used for transducing A2780 and SKOV3 ovarian cancer cell lines. The expression levels of EZH2 and p16 in the ovarian cancer cells were detected using a reverse transcription-quantitative polymerase chain reaction and western blot analyses, respectively. The function of the inhibition of EZH2 in cell proliferation and migration were determined using a CCK-8 assay and Transwell assay. In addition, a nude mouse xenograft model was used to determine the function of EZH2 and p16 in the formation of ovarian cancer in vivo. The results revealed that the inhibition of EZH2 increased the expression of p16, and suppressed the proliferation and migration capabilities of ovarian cancer in vitro. The downregulated expression of EZH2 suppressed ovarian tumor formation in vivo. The results of the study revealed that p16 was negatively regulated by EZH2 in ovarian cancer, and that p16 and EZH2 are important in the tumorigenesis of ovarian cancer. EZH2 and p16 represent potential biomarkers for the diagnosis of ovarian cancer and as targets for ovarian cancer gene therapy.

Introduction

Ovarian cancer remains one of the leading causes of cancer-associated mortality, representing a serious threat to the health and lives of women globally (1). However, the exact molecular mechanisms leading to ovarian tumorigenesis remain to be fully elucidated. Despite advances in the diagnostic and therapeutic methods associated with ovarian cancer, there have been no significant changes in the 5-year survival rate of patients due to a lack of specific symptoms in the early stage and chemotherapy resistance (2). Therefore, it is important to identify early diagnostic markers and novel therapeutic targets for the treatment of ovarian cancer.

Enhancer of zeste homolog 2 (EZH2), an epigenetic regulator, functions as a histone methyltransferase specific to histone H3 lysine 27 (H3K27), and is an important component of polycomb repressive complex 2. Studies have reported that EZH2 is involved in the progression and development of several types of cancer via its effects on promoting cell proliferation, migration and invasion (3). Ectopic expression of EZH2 has been observed in pancreatic cancer (4), breast cancer (5) and colon cancer (6), and it is significantly associated with the poor prognosis of patients. Our previous study indicated that the mRNA and protein levels of EZH2 were significantly increased in ovarian cancer, compared with those in normal tissues. Furthermore, the inhibition of EZH2 repressed the proliferation and migration of cancer cells in vitro and in vivo (7).

P16 is a well-known cell cycle regulator, which controls the G1-to-S transition by inhibiting cyclin-dependent kinases 4 and 6, and is a critical tumor suppressor (8). Aberrant expression of p16 may interfere with the normal cell cycle, induce uncontrolled cell proliferation and, finally, result in tumorigenesis (9). Cui et al demonstrated that DNA methylation at the p16 promoter, particularly at the CpG islands, directly inactivated its transcription and facilitated
cancer migration and invasion due to the inhibition of p16 (10). In addition, the upregulation of p16 in ovarian cancer was demonstrated to decrease the translation of eukaryotic translation elongation factor 1α2 protein and reduce the proliferation of tumor cells, including PA-1, SKOV3 and OVCAR3 cells, in vitro (11). Kong et al. reported that the silencing of EZH2 using short hairpin RNA (shRNA) increased the mRNA and protein levels of p16 in gastric cancer cells (12). Therefore, it has been hypothesised that p16 may be one of the target genes of EZH2 in ovarian cancer. The present study aimed to elucidate the function of EZH2 in the regulation of p16, and its functions in the progression of ovarian cancer.

Materials and methods

Cell culture. Human A2780 and SKOV3 ovarian cancer cell lines were purchased from the China Center for Type Culture Collection (Wuhan, China). The two cell lines were grown in RPMI-1640 media (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and maintained in 5% CO₂ at 37°C in incubators with suitable humidity.

Lentivirus construction and transduction. The specific shRNA-targeted EZH2 was synthesised according to the EZH2 gene sequences obtained from GenBank (accession no. NM_004456). Following an annealing reaction in a polymerase chain reaction (PCR) instrument, in which the complementary DNA fragments of shEZH2 and shNC were dissolved in annealing buffer (Beyotime Institute of Biotechnology Co., Ltd., Shanghai, China) and placed in a water bath at 90°C for 15 min, then cooled to room temperature, the shEZH2 and negative control (shNC) fragments were cloned into the shRNA expression vector (GeneChem Co., Ltd., Shanghai, China) and used for lentiviral packaging. Lentiviral transduction of the A2780 and SKOV3 cells was performed according to the manufacturer's protocol. The sequences of EZH2 siRNA oligonucleotides were as follows: 5'-GAAATCTTAAACCAAGAAT-3'. The sequences of NC siRNA oligonucleotides were as follows: 5'-TTCTCCGAACGTGTCACTG-3'.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR) analysis. Total RNAs of the A2780 and SKOV3 were extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The RT reaction was then performed using a PrimeScript™ RT reagent kit (Takara Bio, Inc., Kyoto, Japan). The EZH2 primer sequences were as follows: 5'-TGTTGCGGGAAGCCTGTAAAATC-3' for the forward primer and 5'-TCTCTAGTCCCGCGGATTGACG-3' for the reverse primer. The p16 primer sequences were as follows: 5'-CCCTTTGTATCCGCAAGCTG-3' for the forward primer and 5'-CCTCTAGGACCTTCGTTGA-3' for the reverse primer. The β-actin primer sequences were as follows: 5'-TGTCACCGAAATGCTTCTATG-3' for the forward primer and 5'-TGCTGACCTCACCAGGTTTG-3' for the reverse primer. An Applied Biosystems 7300 Real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used for the RT-qPCR analysis. Each reaction sample was composed of 10 µl of SYBR-Green Real-time PCR master mix (Rox; Roche Diagnostics GmbH, Mannheim, Germany), 7.3 µl of RNase-free water, 0.6 µl of 10 µM primer and 1.5 µl of cDNA sample. The reactions were performed according to the following cycling conditions: Initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. The relative mRNA levels of EZH2 and p16 were calculated using the comparative quantification cycle (Cq) method (2-ΔΔCq) (13) normalised by the expression of β-actin. All experiments were repeated three times.

Protein isolation and western blot analysis. The A2780 and SKOV3 cell lysates were collected using radioimmuno-precipitation assay buffer (EMD Millipore, Billerica, MA, USA). The total proteins (40 µg) were boiled in a water bath for 5 min following detection of the protein concentrations of each sample using a bicinchoninic acid assay (Beyotime Institute of Biotechnology Co., Ltd.). Following this, the denatured proteins were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The proteins were then transferred onto a polyvinylidene difluoride membrane. The membrane was blocked for 2 h with freshly prepared 5% bovine serum albumin (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China), and the membrane then was incubated with rabbit anti-EZH2 polyclonal antibody (1:1,000 dilution; cat. no. ab3748; Abcam, Cambridge, MA, USA) or rabbit anti-p16 monoclonal antibody (1:2,000 dilution; cat. no. ab51243; Abcam) overnight at 4°C. The fluorescent dye-conjugated anti-rabbit secondary antibody (1:15,000 dilution; cat. no. 5566; Cell Signaling Technology, Danvers, MA, USA) was used for detecting the primary antibodies for 1 h at room temperature. Following washing three times with TBST, the protein bands were visualised using an Odyssey infrared fluorescence scanning imaging system (Odyssey; LI-COR Biosciences, Lincoln, NE, USA).

Cell proliferation assay. A CCK-8 kit (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was used to examine the proliferation ability of the A2780 and SKOV3 cells following the downregulation of EZH2. In brief, 48 h following lentiviral transduction, the ovarian cancer cells (3,000/well) were seeded into 96-well plates with six replicates and cultured at 37°C in a 5% CO₂ atmosphere. Every 24 h for 4 consecutive days, CCK-8 was added to one of the 96-well plates and incubated for 1 h, following which the optical densities of each well were detected at 450 nm with a microplate reader. Cell growth curves were drawn according to the average optical densities of each day.

Cell migration assay. A Transwell migration assay was performed to analyse the migration capability of the ovarian cancer cells. The A2780 and SKOV3 cells were resuspended with 100 µl of serum-free RPMI-1640 medium at a density of 1x10⁶ cells/well, and then seeded into the upper chambers of the Transwell inserts (Corning Costar, Cambridge, MA, USA). The lower chambers were filled with 600 µl complete medium. Following incubation for 24 h, cells adhering to the lower surface of the membrane were fixed with methanol and stained with 0.1% crystal violet. The numbers of migrated cells were counted in five randomly selected high-power
fields (magnification, x400) under an optical microscope. The experiment was repeated three times, and the average numbers of cells were used for assessing the migration ability.

**In vivo growth of ovarian cancer in a xenograft model.** For the cancer formation experiment, the present study used A2780 cells, which can be readily used for stable transfection. Briefly, 10 five-week-old female nude mice were randomly divided into two groups, with each group containing five nude mice. The BALB/C nude mice were obtained from the Animal Experimental Centre of Guangxi Medical University. The room temperature was between 24‑25˚C, and a 12-h light-dark diurnal cycle was used. The mice were housed under specific pathogen-free conditions in an animal facility with free access to a rodent diet, including sunflower seeds and egg yolks, which were sterilized. Access to water was ad libitum. The mice in each group were subcutaneously injected with 1x10⁶ shEZH2-A2780 cells or shNC-A2780 cells into the dorsal flank. The tumor volumes were measured every week, and the xenografts were removed for further analysis when the mice had developed symptoms of cachexia. Each tumor was analysed for mRNA levels of EZH2 and p16 via RT-qPCR analysis. The present study was approved by the Animal Care and Welfare Committee of the Department of Laboratory Animal Science of Guangxi Medical University (Nanning, China).

**Statistical analyses.** Data processing was performed using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). Significant differences among multiple groups were evaluated using one-way analysis of variance, and the Student's t-test was used for analysing differences between two groups. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Inhibition of EZH2 enhances the expression of p16 in A2780 and SKOV3 cells.** To investigate the expression of p16 following the downregulation of EZH2, the present study selectively decreased the expression of EZH2 in the A2780 and SKOV3 ovarian cancer cell lines via an EZH2 shRNA lentiviral expression vector. The EZH2 interference efficiency and levels of p16 were then detected using RT-qPCR and western blot analyses. The shNC lentiviral expression vector was used as a negative control, and the untransduced cells were used as a blank control. The results of the RT-qPCR analysis indicated that the mRNA expression level of EZH2 in the shEZH2-A2780 cells was 27.14±3.6% of that in the blank control, which was significantly lower than the results for shNC-A2780 and untransduced A2780 cells (P<0.001). By contrast, the mRNA expression of p16 increased to 131.78±8.0% in the shEZH2-A2780 cells, compared with that in the untransduced A2780 cells (P=0.001), whereas no significant difference was identified between levels in the shNC-A2780 cells and the blank control (P>0.05; Fig. 1A and B). In the shEZH2-SKOV3 cells, the mRNA level of EZH2 was reduced to 26.34±3.42% (P<0.001). The mRNA expression of p16 was increased to 131.31±2.61% following EZH2-knockdown (P<0.001). No significant differences were observed in the mRNA levels of EZH2 and p16 between the shNC-SKOV3 and untransduced SKOV3 cells (P>0.05; Fig. 1A and B).

The results of the western blot analysis confirmed that the protein level of EZH2 was reduced to 25.15±2.74% in the shEZH2-A2780 cells, which was significantly lower, compared with levels in the shNC-A2780 and untransduced A2780 cells (P<0.001). In addition, the protein level of p16 in the shEZH2-A2780 cells was increased to 142.55±2.82% following EZH2-knockdown (P<0.001). No statistically significant difference was revealed between the shNC-A2780 cells and the blank control (P>0.05; Fig. 1C-E). In the shEZH2-SKOV3 cells, the protein expression of EZH2 was 22.60±2.78% of that in the blank control, which was reduced, compared with the expression in the negative and blank controls (P<0.001; Fig. 1C and F). By contrast, EZH2-knockdown increased the protein level of p16 to 131.87±3.07% in the SKOV3 cells (P<0.001), whereas no significant differences were observed in the protein levels of p16 between the shNC-SKOV3 and untransduced SKOV3 cells exhibited no significant differences (P>0.05; Fig. 1E and F). These results revealed that the inhibition of EZH2 enhanced the expression mRNA and protein expression levels of p16 in the A2780 and SKOV3 ovarian cancer cells.

**EZH2-knockdown reduces the proliferation capability of ovarian cancer cells in vitro.** A CCK-8 assay was used for evaluating the effect of the inhibition of EZH2 on cell proliferation. The cell growth curves in the CCK-8 assay results demonstrated that, compared with the negative and blank controls of the A2780 and SKOV3 cells, the absorbance values of the shEZH2-A2780 and shEZH2-SKOV3 cells were reduced on days 2, 3 and 4. These differences were statistically significant (P<0.001 vs. negative control; P<0.001 vs. blank control; Fig. 2A and B). This result indicated that the proliferation of ovarian cancer cells was decreased following EZH2-knockdown.

**EZH2-silencing decreases ovarian cancer cell migration in vitro.** To evaluate the migration capability of the A2780 and SKOV3 cells, a Transwell migration assay was performed following the inhibition of EZH2. It was observed that the number of migrated shEZH2-A2780 cells was significantly lower, compared with the number of migrated untransduced A2780 cells (P<0.001; Fig. 3A and B). However, no significant difference was revealed between the shNC-A2780 cells and the blank controls (P>0.05).

In the SKOV3 ovarian cancer cell line, the number of migrated shEZH2-SKOV3 cells was markedly reduced, which was statistically significant, compared with the number of migrated shNC-SKOV3 and untransduced SKOV3 cells (P<0.001 vs. shNC-SKOV3 cells and P<0.001 vs. untransduced SKOV3 cells; Fig. 3C and D). These data demonstrated that EZH2-silencing was involved in regulating the migration of ovarian cancer cells.

**EZH2 depletion suppresses ovarian tumor formation in vivo.** The present study further examined the function of EZH2 in tumor development by inducing the growth of A2780 ovarian cancer cells in a xenograft model. In brief, shNC-A2780 or shEZH2-A2780 cells were injected into the dorsal flanks of five BALB/C nude mice obtained from the Animal Experimental Centre of Guangxi Medical University. The room temperature was between 24‑25˚C, and a 12-h light-dark diurnal cycle was used. The mice were housed under specific pathogen-free conditions in an animal facility with free access to a rodent diet, including sunflower seeds and egg yolks, which were sterilized. Access to water was ad libitum. The mice in each group were subcutaneously injected with 1x10⁶ shEZH2-A2780 cells or shNC-A2780 cells into the dorsal flank. The tumor volumes were measured every week, and the xenografts were removed for further analysis when the mice had developed symptoms of cachexia. Each tumor was analysed for mRNA levels of EZH2 and p16 via RT-qPCR analysis. The present study was approved by the Animal Care and Welfare Committee of the Department of Laboratory Animal Science of Guangxi Medical University (Nanning, China).
nude mice each, and the tumor sizes of each nude mouse were measured continuously for 4 weeks. After the 4 weeks, it was found that the growth of tumors in the shEZH2-A2780 group was suppressed (P<0.001; Fig. 4A and B). The results of the RT-qPCR analysis revealed that the mRNA expression of p16 in the shEZH2-A2780 cells was increased to 121.44±6.41%, compared with shNC-A2780, and this difference was statistically significant (P<0.001; Fig. 4C). These data indicated that EZH2 depletion suppressed ovarian cancer formation in vivo.

Discussion

Ovarian cancer is one of three types of malignant tumor of the female reproductive system; it is also the most life threatening gynaecological malignancy. Patients who are diagnosed with advanced ovarian cancer are more likely to have a poor prognosis; however, the exact pathogenesis of ovarian cancer remains to be fully elucidated. Tumorigenesis involves oncogene activation and the inhibition of tumor suppressor genes depending on different physiological and pathological processes. Gene expression is regulated by epigenetic mechanisms through DNA methylation (14), histone modifications (15), chromatin remodelling (16) and non-coding RNA regulation (17). Epigenetic alterations may result in aberrant gene expression, eventually promoting tumor development.

EZH2 is a critical histone methyltransferase, which inhibits transcription by catalysing the trimethylation of H3K27 at the promoters of target genes (18,19). Several studies have revealed...
Figure 2. EZH2-knockdown reduces the proliferation capability of ovarian cancer cells in vitro. (A) Growth curves of A2780, shNC-A2780 and shEZH2-A2780 cells on days 1, 2, 3 and 4. (B) Growth curves of SKOV3, shNC-SKOV3 and shEZH2-SKOV3 cells on days 1, 2, 3 and 4. EZH2, enhancer of zeste homolog 2; sh, short hairpin RNA; NC, negative control.

Figure 3. EZH2-silencing decreases ovarian cancer cell migration in vitro. (A) Cell migration rates of A2780, shNC-A2780 and shEZH2-A2780 cells. (B) Migrated A2780, shNC-A2780 and shEZH2-A2780 cells (magnification, x400). (C) Cell migration rates of SKOV3, shNC-SKOV3 and shEZH2-SKOV3 cells. (D) Migrated SKOV3, shNC-SKOV3 and shEZH2-SKOV3 cells (magnification, x400). EZH2, enhancer of zeste homolog 2; sh, short hairpin RNA; NC, negative control.

Figure 4. EZH2 depletion suppresses ovarian tumor formation in vivo. (A) u volumes of the shNC-A2780 and shEZH2-A2780 groups in a nude mouse xenograft model. (B) Tumor volume of the shEZH2-A2780 group in a nude mouse xenograft model was significantly decreased, compared with the volume of tumors developing in the shNC-A2780 group at 3 and 4 weeks. (C) mRNA expression of P16 was increased in the shEZH2-A2780 group, compared with that in the shNC-A2780 group. EZH2, enhancer of zeste homolog 2; sh, short hairpin RNA; NC, negative control.
that EZH2 functions as an oncogene and contributes to cancer genesis by promoting cell proliferation, invasion and tumor drug resistance (3-21). In the case of prostate cancer, Zhang et al revealed that EZH2 epigenetically silenced the expression of proapoptotic genes, namely microRNA (miR)-31 and miR-205, and eventually reduced tumor cell apoptosis (22). The protein level of EZH2 was higher in colon cancer, compared with that in paracancer tissues and was associated with a poor prognosis for patients. The downregulation of EZH2 by specific siRNA has been revealed to significantly decrease the proliferation and migration ability of colon cancer cells in vitro (6). Zhou et al confirmed that the high expression of EZH2 was involved in maintaining the resistance of A549/DDP cells and AGS/DDP cells to cisplatin, and was partly mediated by its epigenetic regulation of the multidrug resistance 1 gene (21). The present study indicated that the proliferation and migration of ovarian cancer cells were significantly suppressed in vitro and in vivo following the downregulation of EZH2, which was consistent with our previous study (7).

As a member of the INK family, p16 is important in several physiological and pathological processes, including cell cycle control, tumor suppression and the induction of apoptosis (23). Loss of the expression of p16 often leads to cell cycle dysregulation, the increase of mitosis, and finally tumorigenesis. In a nude mouse model, Chang et al reported that orthotopic pancreatic cancer was induced by the inactivation of p16 (24). In the present study, it was found that the increased expression of p16, which was induced by the inhibition of EZH2, suppressed the growth of ovarian cancer cells in vitro and in vivo, and reduced the migration ability of tumor cells in vitro. These results indicated that p16 may act as a tumor suppressor in the progression of ovarian cancer. However, previous studies have demonstrated the tumor-promoting function of p16 by revealing that viral protein E7 encoded by human papilloma virus (HPV) may indirectly increase the expression of p16, and the two are involved in the development of HPV-induced cervical cancer (25). Therefore, it appears that p16 has different effects on different tissues.

A previous study based on lymphoma revealed that the EZH2 protein is recruited at the promoter region of the p16 gene, which is accompanied by a high level of H3K27me3 at the p16 promoter; these results revealed that EZH2 maintained malignant tumor phenotypes (26). In the present study, the expression of EZH2 was selectively inhibited in ovarian cancer cells via an EZH2 shRNA lentiviral vector. The results of subsequent RT-qPCR and western blot analyses indicated that the mRNA and protein levels of p16 were increased following the down-regulation of EZH2, which suggested that the p16 gene may be epigenetically regulated by EZH2 in ovarian cancer cells.

In conclusion, EZH2 depletion promoted the expression of p16, which may contribute to the repression of ovarian cancer proliferation in vitro and in vivo, and to the inhibition of cell migration in vitro. The results of the present study indicated the effect of EZH2 on the epigenetic regulation of p16 in ovarian cancer, suggesting a novel therapeutic approach for ovarian cancer treatment.

Acknowledgements

The present study was funded by the National Natural Science Foundation of China (grant no. 81360389) and the National Natural Science Foundation of Guangxi (grant no. 2015jjAA40440).

References


