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Silencing Bmi1 expression suppresses cancer stemness and enhances chemosensitivity in endometrial cancer cells

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ABSTRACT

Background: Bmi1, a polycomb group gene, is essential for self-renewal of stem cells and is frequently upregulated in various cancer cells. We aimed to investigate the effect of Bmi1 silencing on cancer stemness and chemosensitivity in endometrial cancer using targeted siRNA approach in HEC1A and Ishikawa cells.

Methods: Cell viability after treatment with Bmi1 siRNA was assessed using the MTT assay, and cell apoptosis was visualized using the TdT-mediated dUTP nick-end labeling (TUNEL) method. Western blotting, migration assays and invasion assays were performed to detect changes in the stem-like properties of cancer cells. To evaluate the anticancer effect of Bmi1 silencing, HEC1A and Ishikawa cells were treated with 100 nM Bmi1 siRNA and/or 40 μM cisplatin.

Results: In the MTT assay, compared to control, viability of HEC1A and Ishikawa cells significantly decreased after Bmi1 siRNA treatment in a dose-dependent manner. Bmi1 silencing using siRNA increased the expression of cleaved caspase-3 and cleaved poly adenosine diphosphate-ribose polymerase polymerase (PARP) as observed in the western blot analysis. Apoptosis significantly increased in the HEC1A and Ishikawa cells treated with 100 nM Bmi1 siRNA for 48 h than in the control cells in TUNEL assay. SOX2 and Oct4 expression decreased in the HEC1A and Ishikawa cells treated with Bmi1 siRNA, while E-cadherin expression increased. Further, migratory and invasive properties were significantly inhibited by Bmi1 siRNA treatment in both cell lines. Notably, viability of HEC1A and Ishikawa cells decreased more when they were concurrently treated with Bmi1 siRNA and cisplatin compared to when they were treated with Bmi1 siRNA or cisplatin alone.

Conclusion: Bmi1 silencing suppresses cancer stemness in HEC1A and Ishikawa cells. Concurrent treatment with Bmi1 siRNA and cisplatin resulted in additive anticancer effect with a cell line-specific pattern, which was higher than that shown by cisplatin treatment alone.

1. Introduction

Endometrial cancer is the most common gynecologic malignancy, and more than 61,000 women were diagnosed with this cancer in 2017 in the United States [1]. Endometrial cancer is confined to the uterine corpus in two-thirds of cases at diagnosis. In these cases, the 5-year overall survival rate is estimated to be more than 90%; however, that for advanced stage endometrial cancer which extends beyond the uterine corpus is less than 70% [2]. In advanced stage disease, systemic therapy and/or radiotherapy is recommended as the standard adjuvant therapy after surgical staging. Residual or recurrent disease also commonly requires systemic therapy, generally applied using "platinum-based chemotherapy" [3]. However, cisplatin as the first platinum-based alkylating agent induces apoptosis only in sensitive cells, and not in their resistant counterparts. Response rates to cisplatin as the first-line chemotherapeutic agent in endometrial cancer were reported to be only 20–42% [4]. The underlying molecular mechanism for chemoresistance is not well understood. Therefore, overcoming the resistance to platinum in advanced stage disease remains a therapeutic challenge in the field of cancer research.

B-cell-specific Moloney murine leukemia virus integration site 1 (Bmi1) gene was first identified as a proto-oncogene by van Lohuizen in 1991 [5]. Bmi1, a polycomb group gene, regulates the proliferative activity of normal stem cells and their progenitors. It is also essential for

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the self-renewal of neural and hematopoietic stem cells [6]. Bmi1 is frequently upregulated in a variety of cancers, including ovarian cancer, and its correlation with high grade, advanced stage, lymph node metastasis and poor prognosis has been reported [7]. Particularly, it is known that Bmi1 helps tumor cells escape apoptotic cell death and is also responsible for the failure of chemotherapy [8]. However, the role of Bmi1 in regulation of stem-like properties related with epithelial–mesenchymal transition (EMT) and chemosenstivity in endometrial cancer is still unclear. Further research is needed to clarify the potential of Bmi1 as an effective therapeutic target for treating chemoresistant tumors.

The aim of the present study was to investigate the correlation between Bmi1 Levels and endometrial cancer stemness, and validate the anticancer effect of Bmi1 silencing using targeted siRNA approach. Further, we also explored the effects of Bmi1 silencing on chemosensitivity towards cisplatin in HEC1A endometrial cancer cells.

2. Material and methods

2.1. Cell culture and reagents

The human endometrial cancer cell line HEC1A and Ishikawa cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and kindly donated from CEFO Research Center (Seoul, Korea), respectively. The HEC1A cells were cultured in McCoy's 5 A (HyClone, Logan, UT) with sodium pyruvate (supplemented with 10% fetal bovine serum [FBS] and 1% penicillin–streptomycin [Invitrogen]) in a humidified chamber with 5% CO_2 at 37 °C. The Ishikawa cells were cultured in MEM (Welgene, Kyungsan, Korea) with 2 mM Glutamine, 1% non-essential amino acids and 10% FBS, respectively, in a humidified chamber with 5% CO2 at 37 °C.

Cisplatin was purchased from Sigma Chemical Company (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO, USA). The final concentration of cisplatin in the culture medium never exceeded 0.2%.

2.2. siRNA transfection

Bmi1 (SC-390443) and control (SC-37007) siRNAs were purchased from Santa Cruz Biotechnology. Transient transfection experiments with siRNAs were performed using Lipofectamine RNAi MAX™ (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions.

2.3. Cell proliferation assay

Cell survival analysis was performed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium reduction (MTT) assay (Cell Titer 96 Aqueous Cell Proliferation Assay kit; Promega, Madison, WI, USA). Briefly, the cells were cultured with Bmi1 siRNA. Next, 10 μL of 4 mg/mL MTT solution was added to each well of a 96-well plate. Cells were incubated for 4 h in the dark, at which point they showed an even distribution with 5×10^3 cells in each well, as observed under a microscope. Absorbance at 490 nm was measured using a microplate reader, and the results were expressed in terms of the percentage of control values.

2.4. TdT-mediated dUTP nick-end labeling (TUNEL) assay

HEC1A and Ishikawa cells were plated in an 18-mm cover glass with McCoy's 5 A at ~70% confluence and incubated for 24 h at 37 °C. Next, the cells were treated with 100 nM Bmi1 siRNA for 48 h. They were fixed in ice-cold 2% paraformaldehyde (PFA), washed with PBS, and stained using the TUNEL kit components. TUNEL was subsequently performed using the In Situ Cell Death Detection kit (Roche, Penzberg, Germany), following the manufacturer's instructions. Data were represented as the fold change in TUNEL-positive nuclei compared to control.

2.5. Western blot analysis

The cells were resuspended in ice-cold cell lysis buffer (Cell Signaling Technology, Beverly, MA, USA) supplemented with Protease Inhibitor Cocktail (cOmplete, Mini Tablet; Roche). Protein concentrations were determined using a bicinchoninic acid assay kit (Pierce, IL, USA), according to the manufacturer's instructions. Proteins (20 μg/well) were separated using 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The membranes were blocked using 5% non-fat milk. Next, they were incubated with anti-Bmi1 (Cell signaling systems), anti-cleaved caspase-3 (Cell signaling systems), anti-cleaved poly ADP ribose polymerase (PARP; Cell signaling systems), anti-E-cadherin (Cell signaling systems), anti-SOX2 (Cell signaling systems), anti-Oct4 (Cell signaling systems), and anti-alpha-tubulin (Sigma) antibody individually. Finally, the membranes were incubated with HRP-conjugated IgG secondary antibody (Invitrogen) and visualized using Super Signal West Pico Chemiluminescent Substrate (Pierce, IL, USA).

2.6. Migration assays

A sterile tip was used to wound the cell layer, and the cells were treated with Bmi1 siRNA. For each time point, photographs were obtained using a microscope. The monolayers were scratched with a 200-μL pipette tip, which was washed with the medium to remove the detached cells. The wounded areas were imaged after incubating for the indicated time period.

2.7. Transwell invasion assay

Cell invasion was measured using a Transwell chamber. Briefly, 2×10^5 cells were added to each Transwell invasion chamber coated with 1 mg/mL Matrigel (reconstituted basement membrane; BD Biosciences, Mississauga, ON, Canada). Next, the cells were treated with Bmi1 siRNA. Non-invading cells in the upper chamber were removed using a cotton swab. The remaining cells on the membrane were fixed for 10 min in methanol, stained with 1% crystal violet solution, and washed with PBS. The number of invading cells was counted in five fields/field of view at 200 × magnification.

2.8. Statistical analyses

Data are shown as mean, along with standard deviation. Student's *t*-test was used for comparing the means between two groups. A *p*-value < 0.05 was considered to be statistically significant.

3. Results

3.1. Effects of Bmi1 silencing on the viability and apoptosis of HEC1A and Ishikawa cells

First, we performed the silencing of Bmi1 gene using a targeted siRNA in both cell lines (Fig. 1). The HEC1A and Ishikawa cells were treated with 50 nM or 100 nM Bmi1 siRNA for 48 h, and the proportion of surviving cells was determined using the MTT assay. Compared to control, viable cells significantly decreased in a dose-dependent manner after siRNA treatment in both cell lines (HEC1A cells:77.8% for 50 nM ($p = 0.0004$) and 61.3% for 100 nM ($p = 0.0001$); Ishikawa cells: 85.5% for 50 nM ($p < 0.0001$) and 71.0% for 100 nM ($p < 0.0001$).

In both cell lines, cleaved caspase-3 and cleaved PARP, which are apoptosis-related markers, were observed to be highly expressed 48 h post-treatment with 50 or 100 nM Bmi1 siRNA evident by the western blot analysis. In TUNEL assays, performed to confirm the apoptotic