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Inhibition of Enhancer of Zeste Homolog 2 (EZH2) expression is associated with decreased tumor cell proliferation, migration and invasion in endometrial cancer cell lines

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Abstract

Objective—To investigate the impact of Enhancer of Zeste Homolog 2 (EZH2) expression on endometrial cancer cell line behavior.

Methods/materials—EZH2 expression levels were compared between the non-malignant endometrial cell line T-HESC, and 3 endometrial cancer cell lines, ECC-1, RL95-2 and HEC1-A. Stable EZH2 knockdown cell lines were created and the impact on cellular proliferation, migration and invasion were determined. Fluorescent activated cell sorting was used to examine effects of EZH2 silencing on cell cycle progression. EZH2 expression in endometrial cancer tissue specimens was examined using immunohistochemistry. Comparison of differences between control and shEZH2 cell lines was performed using student's *t* test and Fischer's exact test.

Results—EZH2 protein expression was increased in all 3 cancer cell lines, and human endometrial cancer tissue specimens relative to control. RNA interference of EZH2 expression in ECC-1, RL95-2, and HEC1-A significantly decreased cell proliferation, migration and invasion. Down regulation of EZH2 expression resulted in a significant increase in the proportion of cells arrested in G2/M. RNA interference of EZH2 expression was associated with an increase in the expression of Wnt pathway inhibitors sFRP1 and DKK3, and a concomitant decrease in β -catenin. EZH2 expression in human tissue samples was significantly associated with increased stage, grade, depth of invasion and nodal metastasis.

Conclusions—EZH2 expression is associated with tumor cell proliferation, migration and invasion in 3 endometrial cancer cell lines, as well as increased stage, grade, depth of invasion and nodal metastasis in human cancer tissue specimens. Further investigation into this potential therapeutic target is warranted.

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Keywords

EZH2; endometrial cancer; pathology

Introduction

Endometrial carcinoma (EC) is the most common gynecologic malignancy in the United States, with 47,130 new cases and 8,010 deaths projected in 2012⁽¹⁾. Most women (80–85%) present with early stage disease, and surgery in the form of hysterectomy and bilateral salpingo-oophorectomy is curative. Unfortunately, a proportion of patients will present with advanced disease, or develop disease recurrence, with associated poor survival⁽²⁾.

Currently available cytotoxic therapies for the treatment of advanced stage, progressive or recurrent disease, have shown limited success. In the setting of metastatic recurrence, 5-year survival rates are less than 15%⁽²⁾. Few effective treatment options are available once the disease has spread beyond the pelvis, and while recent Phase II trials have shown some promise with novel biologic agents (mTOR inhibitors, bevacizumab), none have shown a response rate over 25%^(3–5). This trend represents an unmet need in endometrial cancer care.

Enhancer of zeste homolog 2 (EZH2) is a histone methyltransferase that mediates gene silencing by catalyzing trimethylation on lysine 27 of histone H3 (H3K27Me3)⁽⁶⁾. It is a member of the polycomb group of genes (PcG), and has been implicated in nucleosome modification, chromatin remodeling, and interaction with various transcription factors⁽⁷⁾. EZH2 is over-expressed in several different types of cancer, and has been correlated with aggressiveness and poor prognosis in breast, prostate, gastric, oral squamous cell carcinomas and cutaneous melanomas^(8–12).

The role of EZH2 in EC has not been elucidated. Although increased EZH2 expression has been shown in clear cell and serous EC specimens, its impact on a cellular, mechanistic, level is poorly understood^(8, 13). Zhou et al detailed that overexpression of EZH2 was significantly associated with high tumor grade, angiolymphatic invasion, lymph node metastasis and a decrease in overall survival in a study exploring EZH2 expression in archived EC tissue specimens⁽¹³⁾. In this article, we investigate the potential role of EZH2 on EC cell line proliferation, migration and invasion and examine the relationship between EZH2 and Wnt pathway inhibitors. In addition, correlation between EZH2 expression in clinical cancer specimens and pathologic variables/outcomes was performed.

Materials and Methods

Institutional review board approval was obtained from the University of California, Irvine prior to the initiation of research.

Cell lines, compounds and reagents

The human EC cell lines utilized in this study, ECC-1, RL95-2, HEC1-A and the non-malignant immortalized human endometrial cell line, T-HESC were purchased from American Type Culture Collection (ATCC, Manassas, VA). ECC-1 was grown in RPMI-1640 medium supplemented with 5% FBS; T-HESC was cultured in a phenol-free DMEM-F12 1:1 mixture supplemented with 1% ITS + premix, and 10% charcoal treated FBS; RL95-2 was grown in DMEM-F12 medium supplemented with 10% FBS and 0.005 mg/ml insulin; HEC1-A cell lines were grown in McCoy's 5A media supplemented with 10% FBS. All cells were supplemented with penicillin (100 units/mL) and streptomycin (100 µg/mL), and maintained at 37°C in a humidified atmosphere of 5% CO₂. Medium was replaced

every 2–3 days as indicated. Antibodies for β -actin, E-cadherin and EZH2 (used in western blot) were purchased from Cell Signaling Technology (Danvers, MA). EZH2 antibodies used in immunohistochemistry (IHC) were purchased from Abgent (San Diego, CA). Antibody against DKK-3 was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Lastly, SFRP1 antibody was purchased from Abcam, Inc. (Cambridge, MA). Thymidine, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was obtained from Sigma (Saint Louis, MO). RNAzol B was purchased from Tel-Test (Friendswood, TX). The Reverse Transcription System kit utilized was from Applied Biosystems (Carlsbad, CA).

EZH2 short hairpin (sh)RNA stable transfection

Four individual 29mer EZH2 shRNA constructs, as well as a scrambled negative control non-effective shRNA (scEZH2), packaged in a retroviral green fluorescent protein (GFP) vector were purchased from OriGene Technologies, Inc. (Rockville, MD). ECC-1, HEC1-A and RL95-2 cell lines, at approximately 60% confluence, were transfected with Lipofectamine® 2000 reagent (Invitrogen, Life Technologies, Grand Island, NY) according to the manufacturer's instructions. Stable clones were selected with puromycin (1 μ g/mL) starting at 48 h after transfection. All transfected cell lines were then assayed for knockdown of the target gene (EZH2) via western blot analysis. Stable transfectants were then propagated and maintained in their respective media with puromycin selection pressure (1 μ g/mL).

MTT assay⁽¹⁴⁾

Briefly, both control and shEZH2 knock down cells were plated onto 24 well plates at a density of 2×10^4 cells in 500 μ l of growth medium. After incubation for 72 hours, 500 μ l of MTT solution was added to each well and plates were incubated at 37°C for 3 hours. The MTT solution was then extracted and 500 μ l of dissolving buffer was added to each well. Cell viability was assessed by measuring absorbance at 570 nm in a micro-plate reader (Bio-Rad, Hercules, CA).

Cell migration and invasion assays

Analysis of cell migration was performed using a BD Falcon HTS 24-Multiwell insert system (Bedford, MA). Cells were plated in the top chamber of the insert system, with an 8 micron pore size, at a seeding density of 1×10^5 cells/cm² in appropriate serum-free media. The inserts were then placed into the bottom chamber wells of a 24 well plate containing media supplemented with 30% FBS supplemented media. After 24 h of incubation, cells remaining on the inserts' top layers were removed by cotton swab scrubbing $\times 2$; cells on the lower surface of the membrane were fixed in 100% methanol for 15 min, followed by staining with crystal violet solution. The cell numbers in five random fields (100 $\times$ magnification) at the center and periphery of the membrane were counted microscopically for each chamber, and the average value was calculated. Percent migration for negative control cell lines was set at 100%.

For invasion assays, a BD BioCoat™ Matrigel™ Invasion Chamber was used (Bedford, MA). Inserts were rehydrated with DMEM for 2 hours at room temperature prior to use. Control and knockdown cells (seeding density of 1×10^5 cells/well) suspended in 0.5 mL of medium were plated in the top chamber, whereas the bottom chambers were filled with medium containing 30% FBS as a chemo-attractant. After 22 h of incubation at 37°C and 5% CO₂, the number of migrated cells (lower side of the membrane) was counted as described above. A percent invasion was determined by dividing the number of cells invading through the Matrigel™ insert by the number of cells invading through the control insert.

FACS analysis of Cell Cycle

Cells were trypsinized and washed 3 times with ice-cold PBS solution, and re-suspended in 900 μ l of 95% ethanol. Cells were then fixed at 4°C overnight. Subsequently, the fixed cells were pelleted and re-suspended in 1 ml of staining solution containing propidium iodide (PI), 1 mg/mL of RNase A (Qiagen, Valencia, CA) and 1 $\times$ PBS. Cells were then analyzed by a fluorescence-activated cell sorter (FACSort, BD Biosciences, San Jose, CA), with cell cycle profiles analyzed using WinMDI 2.8 (Mannheim, Germany), publicly available software. For each sample, at least 50,000 events were recorded. Samples were run in triplicate and each experiment was repeated three times.

Protein Isolation and Western Blot Analysis

Cell extracts were prepared in RIPA lysis buffer containing protease inhibitors (Sigma, St. Louis, MO). Cell lysates were centrifuged at 12,000 $\times g$ for 15 minutes and the supernatant was collected. The BCA assay was used to determine protein concentration⁽¹⁵⁾. Volumes of clarified protein lysate containing equal amounts of protein (30 μ g) were then separated on 10–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically (90 min at 100 Volts) transferred to a Hybond-ECL membrane (GE Healthcare, Piscataway, NJ). Blots were then blocked for 1 hour in TBST (10mM Tris-HCL, pH 8.0, 150 mM NaCL, and 0.05% Tween-20) containing 5% blocking grade non-fat dry milk (Bio-Rad, Hercules, CA), and then incubated overnight with primary antibody at 4°C. Blots were then washed 3 times in TBST and incubated for 1.5 hours at room temperature with HRP-conjugated goat anti-rabbit or anti-mouse IgG secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactive bands were visualized using an enhanced chemiluminescence detection system (Thermo Scientific, Rockford, IL).

Real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from all cell lines using the TRizol reagent (Invitrogen, Carlsbad, CA). Complementary DNA was then synthesized from 2 μ g of total RNA using a High Capacity cDNA Reverse Transcription kit per protocol (Applied Biosystems, Foster City, CA). Real time PCR amplification reactions for EZH2 were then carried out using the CFX Connect™ system (Bio-Rad) as previously described by Tang et al⁽¹⁶⁾. EZH2, sFRP1, DKK3, β -catenin, and E-cadherin primers were obtained from Qiagen (Valencia, CA) with primer sequences available upon request. Data was then analyzed using the C_t method as previously described⁽¹⁷⁾. Each experiment was carried out in triplicate.

Immunohistochemical staining and scoring

Immunohistochemistry (IHC) assays were performed on formalin-fixed, paraffin-embedded tissue sections to detect EZH2. Staining was performed using an automated IHC stainer (DAKO Autostainer Plus, DAKO, Carpinteria, CA) with appropriate positive and negative controls for each run. Antigen retrieval was performed using steam heat in 0.01 mol/L sodium citrate buffer (pH 6) for 20 minutes. Antibodies were incubated for 1 h at room temperature (primary antibody dilution of 1:50). The EnVision Plus Detection system (DAKO, Carpinteria, CA) was used for antigen detection. Sections were then lightly counterstained with hematoxylin. Tissues in which nuclei were stained for EZH2 protein were considered positive. Stained slides were scored for EZH2 expression by 2 investigators (RW and BY) blinded to the clinic-pathologic data. No staining (score 0) was defined as absence of any nuclear or cytoplasmic stain. A score of 1+ was defined as < 25 % nuclear staining. A 2+ score was defined as > 25% but < 50% nuclear staining. Strong staining (score of 3+) was defined as > 50% nuclear staining. Images of all immunostained slides were digitized at a 0.5 μ m resolution. Acquired images were digitally sharpened for evaluation. All specimens were stained and evaluated in triplicate.

Statistical Analysis

The data are presented as means $\pm$ standard errors (SE) where applicable. Comparison of differences between control and knockdown populations was performed using student's *t* test and paired *t* test where applicable. The association between EZH2 expression levels and patient characteristics was evaluated using the Fisher exact test for categorical variables and the Kruskal-Wallis test for continuous variables. All statistical tests were 2 sided, and the level of significance was set at a *p* value < 0.05 . Data analysis was conducted using SAS 9.2 (SAS Institute, Inc., Cary, NC).

Results

EZH2 is overexpressed in endometrial cancer cell lines relative to normal human endometrial cells

Expression of EZH2 was examined by both western blot and PCR in 3 separate endometrial cancer cell lines (ECC-1, HEC1-A and RL95-2) as well as the normal endometrial cell line T-HESC. When compared to T-HESC, EZH2 was expressed at higher levels (5–20 fold) in all cancer cell lines (Fig. 1a and 1b). Following confirmation of differential expression, stably transfected knock down clones were created using a retroviral green fluorescent protein (GFP) vector. For each cancer cell line, a negative control (scEZH2) and knock down clone (shEZH2) was isolated. The knockdown efficacy of EZH2 was confirmed by Western blotting (Fig. 1c)

EZH2 knockdown inhibits endometrial cancer cell line proliferation, migration and invasion in in-vitro models

Previous investigation has shown EZH2 expression to correlate with a high proliferation index⁽¹⁸⁾. We sought to determine the effects of EZH2 knockdown on proliferation of EC cell lines. Compared with controls, EZH2 knockdown significantly reduced cell proliferation as indicated by MTT assays (Fig. 2a).

Furthermore, EZH2 has been implicated in cell invasion in various cancer cell lines^(9, 19, 20). We sought to determine the effects of EZH2 knockdown on cell migration and invasion in the ECC-1, HEC1-A and RL95-2 endometrial cancer cell lines. Control and shEZH2 expressing cell lines were evaluated for their ability to migrate through uncoated membranes as well as Matrigel™ coated membranes. Compared to controls, EZH2 knockdown cell lines exhibited significantly decreased migration and invasion. This was observed in all tested endometrial cancer cell lines (Fig. 2b and 2c).

EZH2 knockdown results in G2/M accumulation and cell cycle arrest

We also examined whether EZH2 knockdown was associated with cell cycle arrest⁽²¹⁾. As shown in Figure 3, EZH2 knockdown resulted in a marked increase in the number of cells arrested at the G2/M phase in ECC-1, HEC1-A and RL95-2 cell lines. These findings indicate that EZH2 knockdown mitigates the G2/M transition in EC cells, and may explain the inhibition of cell proliferation seen on MTT assay⁽¹⁰⁾.

EZH2 knockdown results in increased Wnt pathway inhibitor expression, and is associated with increased E-cadherin expression

Crosstalk between EZH2 and the Wnt pathway/ β -catenin has been previously described⁽²²⁾. Furthermore, canonical Wnt pathway activation has been correlated with adverse clinico-pathologic outcomes in patients with endometrial cancer⁽²³⁾. Thus, we sought to explore the relationship between EZH2 knockdown and Wnt pathway inhibitor expression. EZH2 silencing was associated with increased Wnt pathway inhibitor (DKK3 and SFRP1)

expression, as well as decreased β -catenin expression as confirmed by western blot and PCR (Fig. 4A). Furthermore, transcriptional silencing of E-cadherin was reversed in all 3 EZH2 knockdown cell lines⁽²⁰⁾ (Fig. 4B). These mechanisms further explain the manner in which EZH2 may impact endometrial cancer cell survival and promote proliferation and invasion.

EZH2 expression is positively correlated with tumor stage, grade, depth of invasion and nodal status

Lastly, we sought to determine the correlation between EZH2 expression and clinicopathologic features of human endometrial cancer specimens. A total of 40 endometrial cancer specimens were evaluated, with investigators blinded to clinical data at the time of staining. There was a significant positive correlation between EZH2 expression and tumor stage ($p = 0.008$), grade ($p = 0.0007$), depth of invasion ($p = 0.0022$) and node positive status ($p = 0.0049$), confirming the findings of previous authors (Table 1)⁽¹³⁾. There were insufficient recurrences and deaths within this patient subset to evaluate impact of EZH2 expression on prognosis. Representative strong positive, weak positive and negatively staining specimens are shown (Fig. 5)

Discussion

It is hypothesized that EZH2, a member of the polycomb repressive complex (PRC2), hypermethylates H3K27, epigenetically silencing tumor suppressor genes. Detailed knowledge regarding the role of EZH2 in EC is limited. Two previous publications exploring EZH2 expression in EC illustrated that increased EZH2 (via immunohistochemistry) was associated with pathologic risk factors, increased tumor cell proliferation (as indicated by Ki-67 staining), and was an independent prognostic factor^(8, 13). In the present study, we explored the expression patterns of EZH2 in 3 endometrial cancer cell lines, and focused on the mechanistic impact of EZH2 silencing on cell proliferation, migration and invasion. Furthermore, we studied the association between EZH2 expression in EC tissue specimens and several clinic-pathologic variables known to predict clinical outcome⁽²⁴⁾.

Silencing of EZH2 expression via short hairpin RNA interference was associated with decreased proliferation, migration, invasion, as well as increased G2/M cell fraction in 3 EC cell lines. In addition, EZH2 knockdown cell lines showed increased Wnt inhibitor expression, and a concomitant decrease in β -catenin. Examination of EC tissue specimens showed correlation between EZH2 expression and higher stage, depth of invasion, tumor grade, lymph-vascular space involvement and lymph node metastasis.

In ovarian, breast, prostate, colon, gastric and oral cancer as well as melanoma, EZH2 expression has been associated with adverse clinical outcomes^(8–12, 18, 19, 25–28). Our data supports previously published literature implicating EZH2 as a maker for tumor aggressiveness, invasion and proliferation. Previous experiments have also associated loss of E-cadherin expression with increased EZH2 activity. Cao et al. described a novel mechanism by which E-cadherin is down-regulated in EZH2-overexpressing cells through histone H3K27 trimethylation at the E-cadherin promoter⁽²⁰⁾. Our results confirmed the repressive effects of EZH2 on E-cadherin. Following shRNA mediated EZH2 silencing, increased E-cadherin expression was noted, with an associated decrease in invasion. This is independent of the proposed effects of EZH2 on actin polymerization, which may also play a role in cancer cell motility and invasion⁽²⁸⁾.

An alternate pathway implicated in EC pathogenesis is the Wnt pathway. Dysregulation of the Wnt pathway has been associated with a variety of human malignancies^(29, 30). DKK-3, a Wnt pathway inhibitor is down-regulated in gastrointestinal, breast, prostate and renal

carcinoma and its role as a tumor suppressor has been investigated in both non-small-cell lung cancer and osteosarcoma. In addition, SFRP-1 down-regulation has been shown in microsatellite unstable EC tissue specimens. Our results point to an association between EZH2 and Wnt inhibitor expression. Following EZH2 silencing, an increase in DKK-3 and SFRP-1 expression was noted, along with a decrease in β -catenin expression. Taken together, these results point towards cross-talk between EZH2 and components of the Wnt pathway.

Effective therapies for patients with advanced or recurrent EC are limited. With cytotoxic agents, the greatest response rate, 25%, was seen in platinum naïve patients treated with single agent paclitaxel. In addition, multiple targeted therapies including anti-angiogenic agents, mTOR inhibitors as well as fibroblast growth factor receptor (FGF) inhibitors have been evaluated in the recurrent setting, with response rates ranging from 7–25%⁽³⁾. Given the lack of effective options in patients with recurrent disease, exploration into alternate pathways is warranted. The PcG proteins have recently been identified as potential candidates for targeted therapy. Our findings point towards EZH2 as a marker for EC aggressiveness, and support further investigation into this possible therapeutic target. It is known that EZH2 activity requires an intact SET domain and histone deacetylase (HDAC) activity, and that inhibition of HDAC activity blocks EZH2 mediated transcriptional repression⁽⁹⁾. HDAC inhibitors (HDACi) have shown promising anti-tumor effects in melanoma, breast, prostate and liver cancer cell lines primarily via mitochondrial injury and promotion of apoptosis⁽³¹⁾. Specifically, inhibition of HDAC activity resulted in inactivation of Bcr/Abl and repression of c-Myc^(32, 33). Furthermore, HDACi led to differentiation and re-programming in melanoma cancer cell lines, and are now being studied in phase I/II clinical trials as single agents or in combination with other cytotoxic agents^(34, 35). These findings, taken together, may support the investigation of HDAC inhibitors as therapeutic agents in EZH2 overexpressing EC patients.

Acknowledgments

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Figure 1.

EZH2 expression in 3 endometrial cancer cell lines (ECC-1, HEC1-A, RL95-2) and the normal endometrium cell line T-HESC. (A) Real time PCR (B) Western Blot (C) ECC-1, HEC1-A and RL95-2 cell lines were transfected with shEZH2 or control (scEZH2). Drug selected cells were examined for expression of EZH2 and β -actin by western blotting. As indicated, stable knock down clones were created and isolated for all cell lines. All experiments were completed in triplicate with representative images shown.

Figure 2.

EZH2 knockdown inhibited cell proliferation, migration and invasion. (A) Equal number of control and shEZH2 knock down cells were assayed for proliferation via MTT assay. A significant decrease in proliferation was noted. (B, C) Control and shEZH2 cell lines were assayed for migration through control and Matrigel™ coated membranes. Cells that had migrated through the membrane were stained with 1% crystal violet in PBS and counted. All experiments were completed in triplicate

Figure 3.

EZH2 silencing induced G2/M phase accumulation. Cell cycle assays illustrate an increase in the G2/M fraction in shEZHZ2 HEC1-A, ECC-1 and RL95-2 cell lines relative to control ($\diamond = p < 0.05$). The image is representative of 3 separate experiments.

Figure 4. EZH2 silencing via RNA interference induces the expression of SFRP1, DKK3 and E-cadherin, and decreases β -catenin expression. A) Protein levels of the Wnt inhibitors SFRP1 and DKK3, down stream β -catenin as well as E-Cadherin are shown in both control and EZH2 knockdown cell lines. β -Actin was used as a loading control in these experiments. B) Real time PCR expression of the above markers is also shown. A representative blot was shown from 3 independent experiments.

Figure 5. Expression of EZH2 in human endometrial cancer tissue samples. (A and B) Strong glandular and stromal staining. (C and D) Weak glandular and stromal staining. (Magnification $\times 400$)

Table 1

Correlation between EZH2 expression and clinico-pathologic variables

Endometrial Adenocarcinoma Specimens					
Patient Characteristics		EZH2 Expression			P Value
		N	0–1+	2–3+	
Age (Range: 31–86 y)	≤54	11	8	3	0.6525
	55–69	7	14	3	
	≥70	12	10	2	
BMI (kg/m ²)	≤24	5	4	1	0.4678
	25–34	14	13	1	
	≥35	21	15	6	
Stage	1	20	20	0	0.008***
	2	11	8	3	
	3 to 4	9	4	5	
Tumor grade	Low (I)	27	25	1	0.0007
	High (II–III)	13	6	7	
Tumor penetration	Inner 1/2	25	24	1	0.0022**
	Outer 1/2	12	7	5	
	Serosa	3	1	2	
LVSI	Absent	25	22	3	0.1256
	Present	15	10	5	
Nodal Status *	Negative	21	19	2	0.0049
	Positive	3	0	3	
Disease recurrence	Yes	4	2	2	0.1723
	No	36	30	6	
Death	Yes	1	0	1	0.2
	No	39	32	7	

* 16 patients did not have lymph node sampling at the time of their surgical procedure

** Inner 1/2 vs. outer 1/2 + serosa

*** Stage 1 and 2 vs. stage 3 and 4