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The role of EZH2 and DNA methylation in hMLH1 silencing in epithelial ovarian cancer

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ABSTRACT

Enhancer of zeste homolog 2 (EZH2) is overexpressed in various malignancies and associated with poor prognosis and drug-resistance. A recent study suggested that there is a link between EZH2 expression and the mediation of gene silencing in association with aberrant DNA methylation. In the present study, we showed an inverse correlation between EZH2 and human mutL homolog 1 gene (hMLH1) expression in 30 epithelial ovarian cancer (EOC) tissues. Moreover, we found that EZH2 downregulation could induce the re-expression of the unmethylated, basally expressed hMLH1 gene without affecting DNA methylation in the hMLH1 promoter. These results suggest that EZH2 can modulate the transcription of basally expressed hMLH1 via a non-DNA-methylation-dependent pathway, but it has no effect on hMLH1 silencing that is mediated by DNA hypermethylation.

1. Introduction

Enhancer of zeste homolog 2 (EZH2) is a core component of the polycomb repressive complex 2 (PRC2), which is a highly conserved histone methyltransferase that catalyzes the trimethylation of histone H3 lysine 27 (H3K27me3). EZH2 plays a critical role in tumorigenesis and cancer progression through epigenetic gene silencing and chromatin remodeling [1]. Increasing evidence suggests that the EZH2 gene is overexpressed in a variety of human malignancies, including breast, prostate, oral, esophageal, gastric, colon, hepatocellular, bladder, and endometrial cancers [2–4]. Our previous studies have shown that EZH2 is upregulated in ovarian cancer and that its expression is significantly associated with an advanced International Federation of Gynecology and Obstetrics (FIGO) stage and resistance to cisplatin. Furthermore, loss of EZH2 suppresses proliferation and migration of ovarian cancer cells and resensitizes cisplatin-resistant cells *in vitro* and *in vivo* [5,6]. However, the mechanisms of EZH2 involvement in ovarian cancer have not been studied.

Mismatch repair (MMR) deficiency is a distinct carcinogenic mechanism. Following its discovery in hereditary non-polyposis colorectal cancer (HNPCC) syndrome in the early 1990s [7], MMR deficiency was identified as a potential cancer-initiating pathway in cancers of other organs, including the ovaries [8]. Promoter hypermethylation of the MMR gene mutL homolog 1 (hMLH1), an epigenetic mechanism, is known to cause inactivation of the MMR system [9–11]. DNA methylation occurs on cytosine bases linked to guanine bases, forming CpG dinucleotide pairs known as islands [12]. Promoter hypermethylation of the hMLH1 gene has been observed in sporadic microsatellite instability-high (MSI-H) cancers, including colorectal and endometrial cancers [13]. In addition, studies in ovarian cancer have reported a frequency of hMLH1 promoter hypermethylation that ranged from 6% to 12.5% [11,12,14,15]. More recently, preclinical data suggested a relationship between hMLH1 deficiency and resistance to cisplatin and carboplatin [16,17].

EZH2 can recruit the DNA methyltransferases DNMT1, DNMT3A, and DNMT3B [18], resulting in DNA methylation and subsequent gene silencing in certain circumstances [19]. A previous study showed that trimethylation of H3K27 and EZH2 binding were enriched along the hypermethylated and silenced hMLH1 promoter in colon cancer cells [20]. In addition, Vire et al. proposed that EZH2 directly controls both the initiation and maintenance of DNA methylation [18]. Thus, we hypothesized the existence of a link between EZH2 overexpression and hMLH1 silencing in association with DNA methylation in ovarian cancer. However, the findings we now present suggest that although EZH2 may function to hold hMLH1 in a basal transcription state in the absence of DNA hypermethylation, it is not solely responsible for the maintenance of transcriptional repression of the heavily methylated hMLH1 gene.

2. Materials and methods

2.1. Cell culture

The epithelial ovarian cancer (EOC) cell lines HO8910, SKOV3 and ES2 were obtained from the Center for Type Culture Collection

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of the Chinese Academy of Sciences (CTCCCAS, Shanghai, China), A2780 was obtained from the China Center for Type Culture Collection (CCTCC, Wuhan, China), and OV2008 and C13* were obtained from Dr. Rakesh Goel of the Ottawa Regional Cancer Center, Canada. Cells were cultured at 37 °C in RPMI 1640 (Gibco, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, USA) in a 5% CO_2 atmosphere.

2.2. Western blot analysis

The total protein from each sample was separated on a 10% SDS–PAGE gel and subsequently transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, USA). The membranes were incubated overnight with the following primary antibodies: rabbit anti-EZH2 antibody (1:1000; Invitrogen, USA), mouse anti-hMLH1 antibody (1:500; BD Pharmingen, USA), rabbit anti-H3K27me3 antibody (1:1000; Cell Signaling Technology, USA), rabbit anti-HDAC1 antibody (1:1000; Anbo Technology, China), and rabbit anti-GAPDH antibody (1:1000; Santa Cruz, USA). The primary antibodies were detected using anti-rabbit or anti-mouse secondary antibodies (1:5000 dilution; Santa Cruz, USA), which were incubated with the membrane for 2 h at room temperature and visualized using an ECL system (Beyotime, China) via exposure to an X-ray film in accordance with the manufacturer's instructions.

2.3. Tissue specimens

Thirty epithelial ovarian cancer (EOC) tissue samples were obtained from patients by surgical resection at the Department of Gynecology (Untion Hospital, Tongji medical college, Huazhong Science and Technology) between January 2005 and October 2010. These samples included ten serous cystadenocarcinomas, three mucinous cystadenocarcinomas, eight endometrioid carcinomas, seven clear cell tumors and two undifferentiated tumors. Each sample was divided into two portions; one was immediately frozen in liquid nitrogen after resection, and the other was paraffin-embedded after fixation by 10% formalin. The patients were between 42 and 73 years of age, with a median age of 53 years. The histological diagnosis was confirmed by two pathologists in all cases, and none of the patients had any preoperative treatment, such as radiation or chemotherapy. The study was approved by the ethics committee of Huazhong University of Science and Technology, Wuhan, China.

2.4. Immunohistochemistry

The immunohistochemistry for EZH2 (1:100; Invitrogen, USA) and hMLH1 (1:200; BD Pharmingen, USA) was performed on formalin-fixed, paraffin-embedded tissue sections using steam heat-induced epitope retrieval and the DAB chromogen (Boster, China). Specimens were scored as positive for overexpression of EZH2 when >21% cells were positive, and specimens were scored as negative for overexpression when only 0–20% of the cells were positive. For hMLH1 expression, samples were considered positive if >10% of cells had positive staining.

2.5. RNA isolation and real-time RT-PCR

Total RNA from the cell lines was isolated with TRIzol Reagent (Invitrogen, USA) and reverse transcribed to cDNA with the ExScript RT (Takara, China). The PCR primer pairs used had the following sequences: EZH2, upstream 5′-TTGTTGGCGGAAGCGTGT AAAATC-3′ and downstream 5′-TCCCTAGTCCCGCGCAATGAGC-3′; hMLH1, upstream 5′-CTGAAGGCACTTCCGTTGAG-3′ and downstream 5′-TGGCCGCTGGATAACTTC-3′; GAPDH, upstream 5′-GCA CCGTCAAGGCTGAGAAC-3′ and downstream 5′-ATGGTGGTG AAGACGCCAGT-3′. All reactions were performed on an Applied Biosystems Step One Plus Real-Time System (Applied Biosystems, USA). Real-time fluorescence monitoring of the PCR products was performed with SYBR Green Realtime PCR Master MIX (TOYOBO, China).

2.6. EZH2 siRNA transient transfection

A2780, SKOV3, ES2, HO8910, OV2008 and C13* cells were transfected with either a non-targeting control or an EZH2-targeting small interfering RNA (siRNA) (GenePharma, Shanghai, China) using Lipofectamine 2000 (Invitrogen, USA). At various time points after transfection, cells were harvested and subjected to several assays.

2.7. EZH2 shRNA stable transfection

A non-targeting or EZH2-targeting short hairpin RNA (shRNA) was cloned into the SuperSilencing shRNA expression vector (Genepharma, Shanghai, China) as previously shown [6]. C13* and A2780 cells were transfected with Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. The transfected cells were selected with G418, and individual clones were isolated.

2.8. Immunocytochemical staining

Immunocytochemistry for EZH2 (1:100; Invitrogen, USA) and hMLH1 (1:200; BD Pharmingen, USA) was performed on 4% paraformaldehyde-fixed cancer cells. The cells were subsequently stained using the DAB chromagen (Boster, China). The coverslips were viewed with a microscope (IX71, Olympus, Japan) equipped with a CCD camera (Olympus, Japan) and IP-lab software.

2.9. Pyrosequencing analysis

The methylation status of specific cytosine residues in the hMLH1 gene promoter was determined by pyrosequencing following bisulfite modification of DNA extracted from cells and frozen EOC tissues. The tumor specimens selected for DNA isolation had high tumor cellularity. The DNA from cells and frozen tissues was extracted with a Genomic DNA Extraction Kit (Tiangen Biotech, China). Bisulfite modification was performed using a BisulFlash™ DNA Modification Kit (Epigentek, USA). The modified DNA was amplified by PCR with the following primers: forward primer: 5′-TTTAGGAGTGAAGGAGGT-3′ and reverse primer: Biotin-5′-BIOTIN-CCCTATACCTAATCTATC-3′. After PCR, the biotinylated strand was captured on streptavidin-coated beads (Biotage AB, USA) and incubated with a sequencing primer: 5′-GTTTTGAYG-TAGAYGTTTTATTAGGGT-3′. A 50-μl PCR product was used for pyrosequencing with a Pyro Gold SNP Reagent Kit (Biotage AB, USA) and a PyroMark Q96 ID (Qiagen, Germany) according to the manufacturer's instructions.

2.10. Statistical analysis

Statistical analyses, including the *t*-test, Pearson correlation, and Fisher's exact test, were performed using SPSS for Windows version 13.0 (SPSS Inc, Chicago, IL, USA). A *P*-value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. hMLH1 promoter methylation is inversely associated with hMLH1 expression but not with EZH2 expression in EOC cell lines

The expression of EZH2 and hMLH1 were investigated in six ovarian cancer cell lines (A2780, ES2, OV2008, C13*, SKOV3,

HO8910). The Western blot data showed that EZH2 protein was detectable in all cell lines tested, whereas hMLH1 protein was expressed in ES2, C13*, SKOV3 and HO8910 and was not expressed in A2780 or OV2008 (Fig. 1A). The levels of EZH2 and hMLH1 mRNA in these cell lines, was judged by real-time RT-PCR, were well correlated with their respective protein levels. All cell lines expressed a higher level of EZH2 mRNA but showed varied expression levels of hMLH1 mRNA. The levels of hMLH1 mRNA were especially low in the A2780 and OV2008 cell lines (Fig. 1B).

DNA methylation levels in the promoter region of hMLH1 were measured by pyrosequencing. A2780 and OV2008 cells had significantly higher methylation levels (94.67 ± 1.53% and 94.63 ± 0.82%, respectively). The mRNA expression of hMLH1 was significantly inversely correlated with its DNA methylation level (Fig. 1C, Pearson correlation, $r = -0.943$, $P = 0.005$), suggesting an important role for cytosine methylation in controlling hMLH1 expression. However, the expression of EZH2 mRNA and the level of hMLH1 methylation were not correlated (Fig. 1D, Pearson correlation, $r = 0.599$, $P = 0.209$).

3.2. EZH2 overexpression correlates with the loss of hMLH1 in EOC tissues

To investigate the association between EZH2 expression, hMLH1 expression, and DNA methylation in the hMLH1 promoter region in tissues of EOC patients, we performed immunohistochemical staining for EZH2 and hMLH1 as well as pyrosequencing for hMLH1 methylation on 30 EOC tissue specimens. EZH2 and hMLH1 were predominantly expressed in the nuclei of the epithelial cancer cells; the staining intensity in the cytoplasm was minimal. Positive EZH2 staining was found in 20 (66.7%) cases. Positive hMLH1 staining was observed in 14 (46.7%) cases, eight of which (57.14%) were EZH2 negative. Among the 16 cases without detectable hMLH1 expression, 14 (87.5%) were EZH2 positive (representative results shown in Fig. 2A). The correlation between the expression of EZH2 and hMLH1 was significant (Table 1, Fisher's exact test, $P = 0.019$). These findings suggest that EZH2 expression inversely correlates with hMLH1 expression.

The methylation of hMLH1 occurred more frequently in hMLH1-negative tumors (33.99 ± 16.00%) compared to those with positive hMLH1 expression (19.43 ± 11.43%; Student's *t*-test, $P = 0.007$; Fig. 2B). This finding suggests a correlation between the promoter methylation and gene silencing of hMLH1. However, the hMLH1 methylation levels did not correlate with EZH2 protein expression (Fig. 2C, Student's *t*-test, $P = 0.435$).

3.3. Downregulation of EZH2 activates the transcription of unmethylated, basally expressed hMLH1

To determine whether EZH2 can independently affect hMLH1 gene expression, we downregulated EZH2 in cell lines using siRNA. A marked decrease in EZH2 protein level was detected by Western blot in all six EOC cell lines 72 h after transfection with EZH2-targeted siRNA. This decrease was accompanied by a decrease in the levels of the EZH2-specific chromatin H3K27me3 marks and the levels of histone deacetylase 1 (HDAC1) in all cell lines. Additionally, an increased level of hMLH1 protein was found in C13*, ES2, HO8910, and SKOV3 cells, but not in OV2008 and A2780 cells, in which the hMLH1 promoter is hypermethylated (Fig. 3A). Real-time RT-PCR revealed similar results on the mRNA level. The level of EZH2 mRNA was markedly decreased in all six cell lines after transfection with EZH2-targeted siRNA (Fig. 3B). Furthermore, the level of hMLH1 mRNA increased from 1.5-fold to 2.3-fold after EZH2 downregulation in the four unmethylated cell lines C13*, ES2, HO8910, and SKOV3 (C13*, 2.1-fold; ES2, 2.3-fold; HO8910, 1.5-fold; SKOV3, 1.7-fold) (Fig. 3C). In contrast, the mRNA expression of hMLH1 was unaffected in OV2008 and A2780 cells. Moreover, immunocytochemical staining demonstrated that the level of hMLH1 protein expression was restored in the nucleus after EZH2 depletion in ES2 cells (Fig. 3D), confirming that EZH2 overexpression downregulates hMLH1 protein expression in ovarian cancer cells.

Given that the transient downregulation of EZH2 might not allow enough time for gene re-expression or sufficient depletion of EZH2 to fully affect its catalyzed mark, we also stably downregulated EZH2 in C13* and A2780 cells using a SuperSilencing shRNA

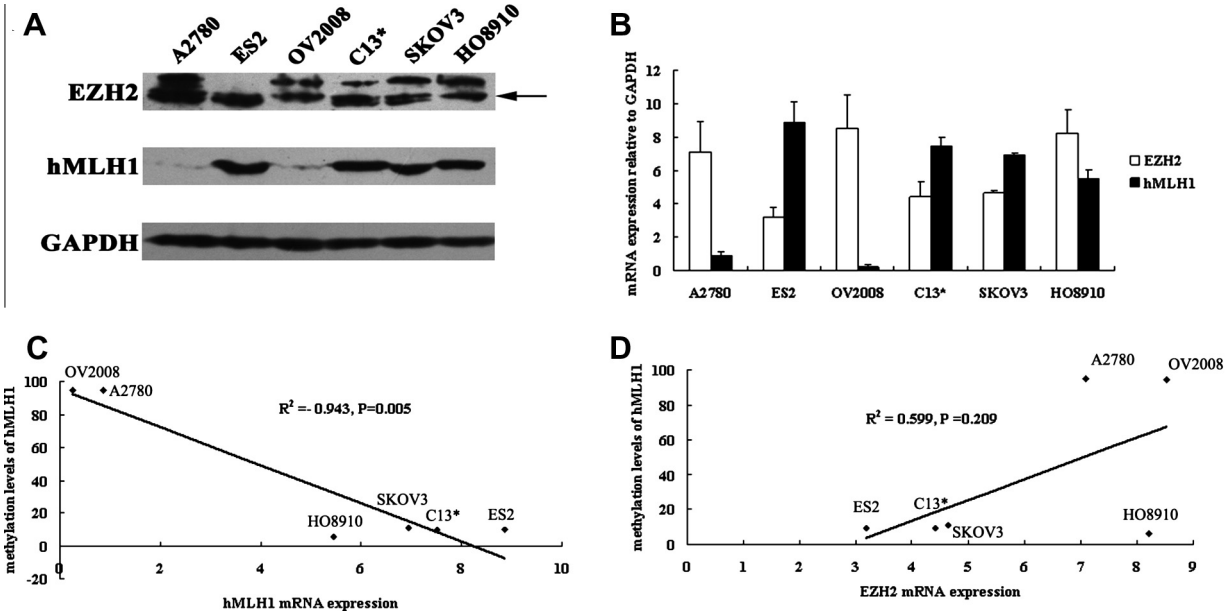

Fig. 1. Expression of EZH2 and hMLH1 in EOC cell lines. (A) Western blot analysis of EOC cell lines for EZH2 and hMLH1 with GAPDH as a loading control. (B) Real-time RT-PCR analysis of EZH2 and hMLH1 expression in EOC cell lines. (C) Association of hMLH1 methylation levels and mRNA expression in EOC cell lines (Pearson correlation, $r = -0.943$, $P = 0.005$). (D) Association of hMLH1 methylation levels and EZH2 mRNA expression in EOC cell lines (Pearson correlation, $r = 0.599$, $P = 0.209$, ns).

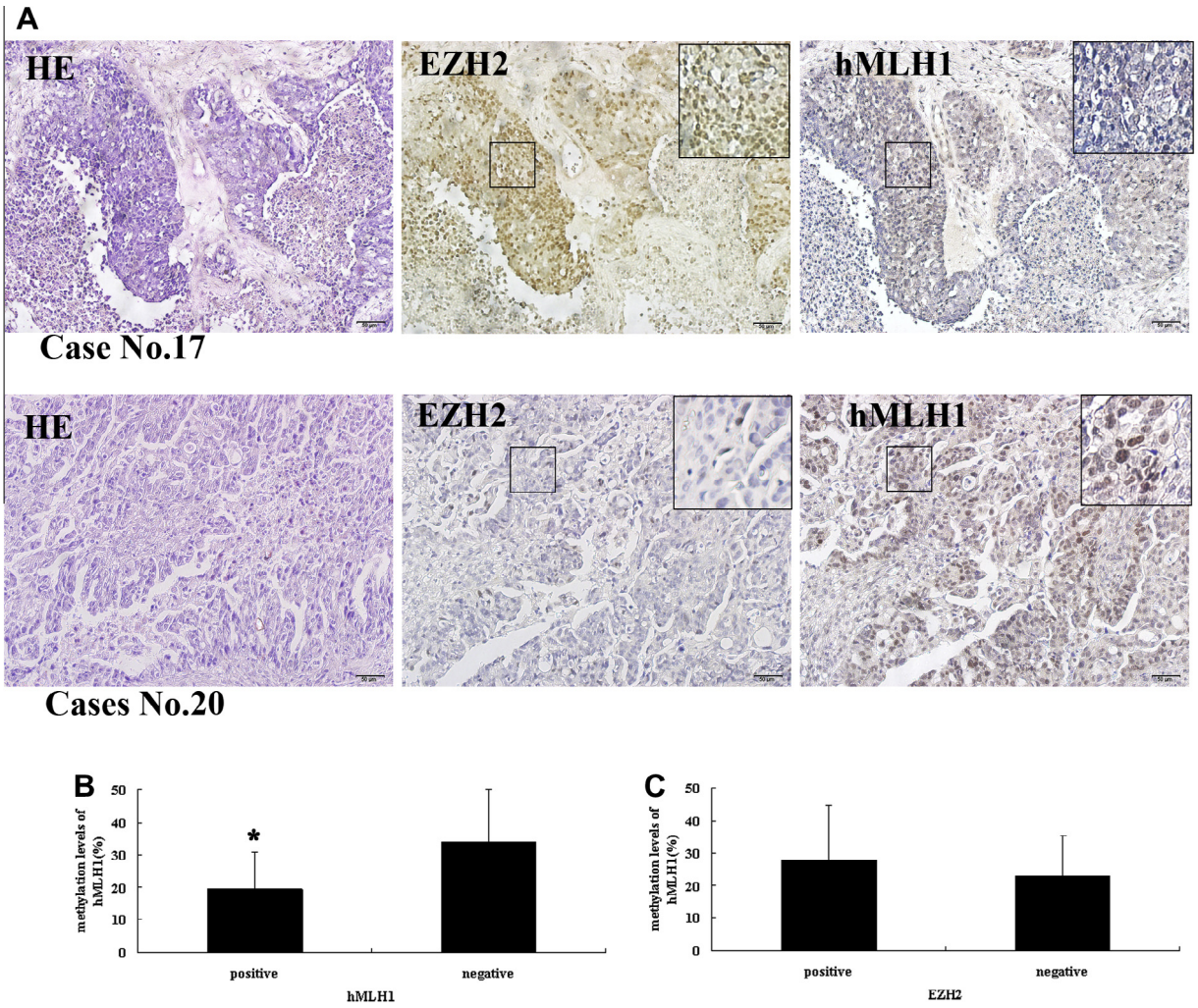

Fig. 2. EZH2 overexpression correlates with the loss of hMLH1 expression in EOC patients. (A) hMLH1 protein detected by immunohistochemical staining in tissue specimens from EOC patients. Histological appearance of the EOC specimens stained with H&E (hematoxylin and eosin) and immunohistochemistry for EZH2 and hMLH1 proteins were performed using two continuous thin tissue sections to show the inverse correlation of the expression of EZH2 and hMLH1 proteins in individual cancer cells. In case 17, EZH2 overexpression is observed in EOC cancer cells when hMLH1 expression was negative. Conversely, hMLH1 was expressed when EZH2 was negative in case 20. (B) Methylation levels of hMLH1 in EOC patients with positive and negative hMLH1 staining (Student's *t*-test, $P = 0.007$). (C) Methylation levels of hMLH1 in EOC patients with positive and negative EZH2 staining (Student's *t*-test, $P = 0.435$, ns).

Table 1
Association between hMLH1 and EZH2 protein expression in EOC patients.

	hMLH1		P^*
	Positive	Negative	
EZH2			
Positive	6(30.0%)	14(70.0%)	0.019
Negative	8(80.0%)	2(20.0%)	

* *P* values were based on Fisher exact tests.

expression vector with a short hairpin loop that targets EZH2. Western blot and real-time RT-PCR analyses showed that downregulation of EZH2 induced a significant increase of hMLH1 in C13* cells, which lack DNA methylation, but not in hypermethylated A2780 cells. These results are similar to those obtained using the transient approach (Fig. 3A, E and F).

3.4. Downregulation of EZH2 does not affect DNA methylation levels of the hMLH1 promoter

Finally, we were interested in whether the DNA methylation status of hMLH1 accounts for the hMLH1 expression mediated by EZH2 downregulation. However, we failed to observe any significant reduction in DNA methylation at the hMLH1 promoter in cell lines after either the transient (Fig. 4B) or the stable (Fig. 4A–C) downregulation of EZH2, suggesting downregulation of EZH2 is not sufficient to affect the DNA methylation levels of the hMLH1 promoter.

4. Discussion

Loss of MMR capacity may represent an important tumor-initiating mechanism in ovarian cancer. Previous studies reported that hMLH1 expression is reduced in up to 47.6% of ovarian cancers [11,21,22], whereas hMLH1 promoter methylation is only responsible for hMLH1 reduction in 6–12.5% of sporadic ovarian cancers [15,23]. The discovery of novel strategies to restore hMLH1 expression and function is highly desirable for developing therapies for women with hMLH1-deficient tumors. The mechanism of hMLH1 regulation by EZH2 demonstrated above may shed some light on this topic.

Epigenetics is emerging as an essential aspect of cell-lineage determination and maintenance mechanisms. DNA methylation and histone modification control epigenetic phenomena, particularly histone methylation. Recently, Vire et al. reported that EZH2

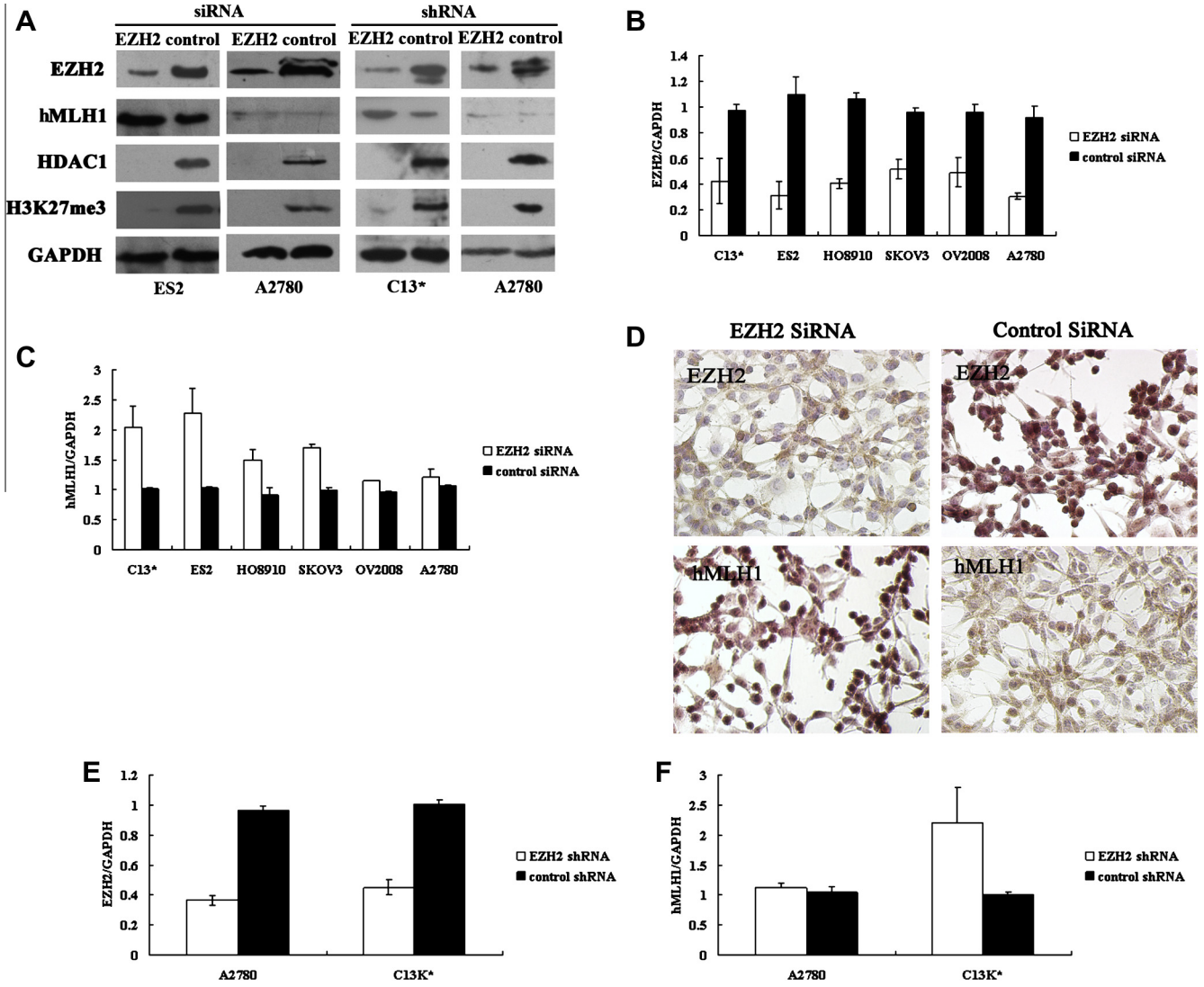

Fig. 3. Downregulation EZH2 leads to re-expression of hMLH1 when the promoter is not associated with dense DNA methylation in EOC cell lines. (A) Western blot analysis. Left panels, protein extracts were probed for EZH2, hMLH1, H3K27me3, HDAC1 and GAPDH in ES2 and A2780 cells transiently transfected with EZH2 siRNA or control siRNA; right panels, protein extracts were probed for EZH2, hMLH1, H3K27me3, HDAC1 and GAPDH in C13* and A2780 cells stably transfected with either EZH2 shRNA or control shRNA. (B and C) Real-time RT-PCR analysis of EZH2 and hMLH1 in C13*, ES2, HO8910, SKOV3, OV2008 and A2780 cells transiently transfected with EZH2 siRNA or control siRNA. GAPDH was used as a loading control. EZH2 mRNA expression levels are shown in B, and hMLH1 mRNA expression levels are shown in C. (D) Immunocytochemical staining of EZH2 and hMLH1 in ES2 cells transiently transfected with either EZH2 siRNA or control siRNA. E and F, real-time RT-PCR analysis of EZH2 and hMLH1 in C13* and A2780 cells stably transfected with either EZH2 shRNA or control shRNA. GAPDH was used as a loading control. EZH2 mRNA expression levels are shown in E, and hMLH1 mRNA expression levels are shown in F.

can directly control DNA methylation; EZH2 interacts with DNMTs and can affect DNMT activity *in vivo*, and knockdown of EZH2 or DNMTs markedly increases the expression of their target genes. These effects permit the two processes that can repress gene expression, histone methylation and DNA methylation, to act in concert [18]. Although EZH2 is required for DNA methylation initiation, it is not essential for maintaining DNA methylation and gene silencing. Kelly et al. showed that although depletion of EZH2 could induce the re-expression of its nonmethylated target genes, it could not affect the expression of fully silenced and hypermethylated genes. Contrary to Vire's results, Kelly et al. failed to observe a significant reduction of DNA methylation at the promoters regions of EZH2-taget genes MYT1 and WNT1 after EZH2 knockdown [24]. Similarly, Kondo et al. found that downregulation of EZH2 restored expression of the H3K27me3 target genes alone or in synergy with histone deacetylase inhibition without affecting promoter methylation and restored expression of genes silenced by DNA hypermethylation [25]. Consistent with Kelly and Knodo's studies, we found that EZH2 downregulation resulted in increased expression of unmethylated, basally expressed hMLH1, but did not induce re-expression of the silent hMLH1 gene, which was frequently DNA-methylated in the EOC cell lines A2780 and OV2008. Furthermore, reduction of EZH2 did not significantly affect the levels of hMLH1 promoter methylation, suggesting that EZH2 has cell- and gene-specific gene silencing mechanisms. When these genes are not regulated by DNA methylation and when the expression is at basal levels, depletion of EZH2 can induce increased transcription. Additionally, reduction of EZH2 does not significantly affect the levels of hMLH1 promoter methylation, indicating that EZH2 plays a key role in the downregulation of hMLH1, independent of promoter methylation. Therefore, EZH2 likely regulates hMLH1 through another pathway.

Fujii et al. showed that the levels of H3K27me3 and HDAC1 bound to the promoter of the EZH2-target gene RUNX3 were significantly reduced after knockdown of EZH2, and the decreases in H3K27me3 and HDAC1 were inversely correlated with the increase

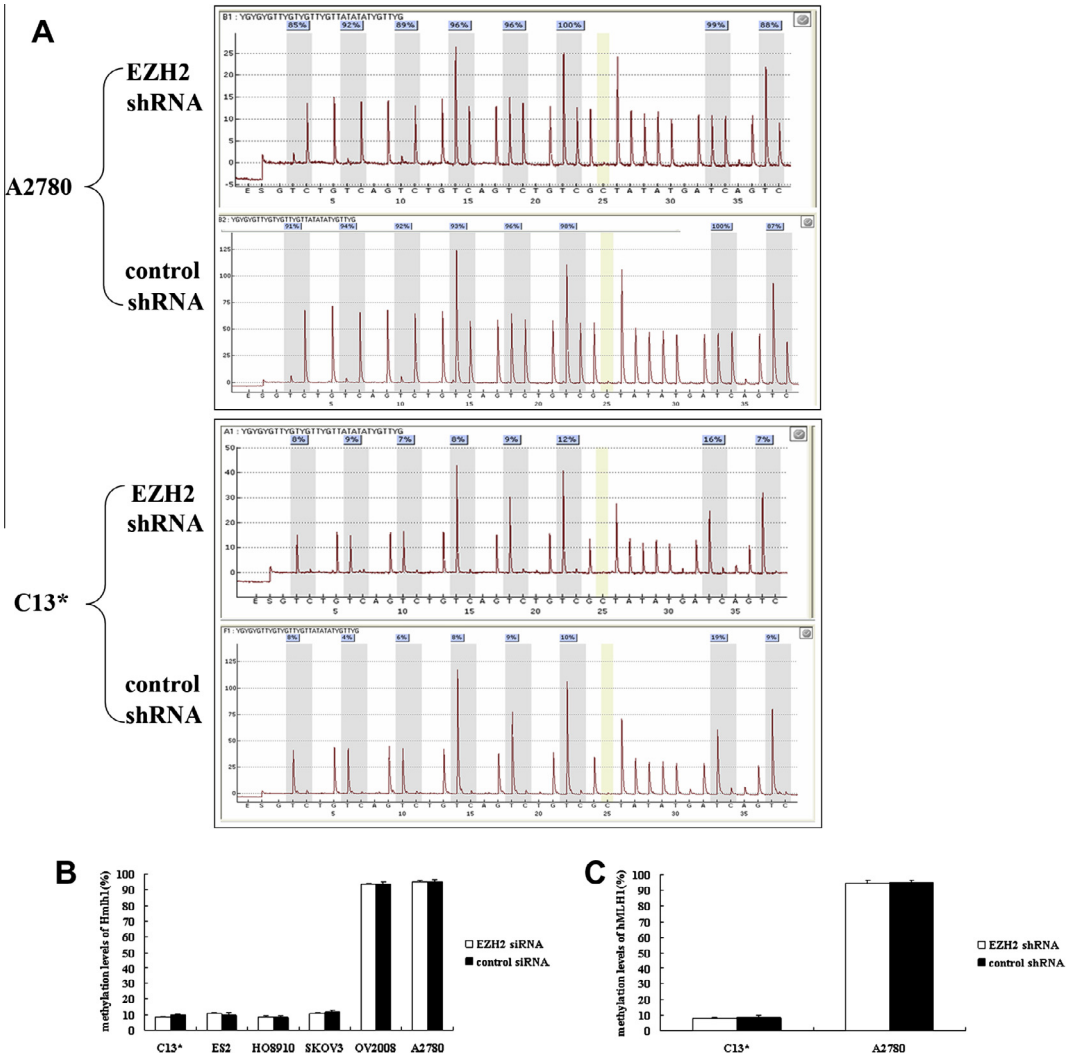

Fig. 4. EZH2 depletion does not reduce hMLH1 promoter methylation. (A) Pyrosequencing of the hMLH1 promoter in A2780 and C13* cells stably transfected with EZH2 shRNA or control shRNA. (B) DNA methylation levels of the hMLH1 promoter in C13*, ES2, HO8910, SKOV3, OV2008 and A2780 cells transiently transfected with either EZH2 siRNA or control siRNA, evaluated by pyrosequencing analysis. (C) DNA methylation levels of hMLH1 promoter in C13* and A2780 cells stably transfected with either EZH2 shRNA or control shRNA, evaluated by pyrosequencing analysis.

in expression of the RUNX3 gene. In their study, EZH2 knockdown restored the RUNX3 transcript levels without any change in the DNA methylation status of the RUNX3 promoter region. RUNX3 expression also increased upon treatment with the deacetylase inhibitor TSA. These findings strongly indicate that EZH2 and HDAC1 act synergistically to downregulate RUNX3 expression. Similarly, we found that downregulation of EZH2 was associated with a global decrease in H3K27me3 and HDAC1, suggesting that downregulation of hMLH1 may be mediated by both H3K27me3 and histone deacetylation, although further studies are needed to verify this hypothesis.

The regulatory mechanisms of EZH2 towards its target genes have been increasingly studied. Studies have shown that EZH2 overexpression is sufficient for activation of the phosphoinositide 3-kinase/Akt (PI3K/Akt) pathway, specifically through the activation of Akt isoform 1, which in turn results in dysregulation of BRCA1 expression and genomic stability [26]. Moreover, several noncoding RNAs (ncRNAs) have been shown to interact with EZH2 and may facilitate its recruitment to some target genes, such as HOTAIR and Xist [27,28]. Another significant study reported that EZH2 targets many components of key signaling pathways through the epigenetic silencing of tumor suppressor microRNAs (miRNAs) in hepatocellular carcinoma (HCC). For example, DVL1, a member of the Wnt signaling pathway, and CACNG3, a member of the MAPK/ERK signaling pathway, are predicted targets of miR-139, a well-characterized EZH2-target miRNA [29]. Although various functions of EZH2 have been identified, its modulatory mechanisms are complex and have not yet been fully explored.

In this study, we described, for the first time, that EZH2 can function as a modulator of hMLH1 expression when the promoter of hMLH1 is not associated with frequent DNA methylation. Our results suggest that downregulation of hMLH1 may be one pathway by which EZH2 affects tumor progression and response to chemotherapy in ovarian cancer.

Acknowledgments

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References

[1] R. Cao, L. Wang, H. Wang, L. Xia, H. Erdjument-Bromage, P. Tempst, R.S. Jones, Y. Zhang, Role of histone H3 lysine 27 methylation in Polycomb-group silencing, Science 298 (2002) 1039–1043.

[2] Y. Matsukawa, S. Semba, H. Kato, A. Ito, K. Yanagihara, H. Yokozaki, Expression of the enhancer of zeste homolog 2 is correlated with poor prognosis in human gastric cancer, Cancer Sci. 97 (2006) 484–491.

[3] R.J. Bryant, N.A. Cross, C.L. Eaton, F.C. Hamdy, V.T. Cunliffe, EZH2 promotes proliferation and invasiveness of prostate cancer cells, Prostate 67 (2007) 547–556.

[4] K. Collett, G.E. Eide, J. Arnes, I.M. Stefansson, J. Eide, A. Braaten, T. Aas, A.P. Otte, L.A. Akslen, Expression of enhancer of zeste homologue 2 is significantly associated with increased tumor cell proliferation and is a marker of aggressive breast cancer, Clin. Cancer Res. 12 (2006) 1168–1174.

[5] J. Guo, J. Cai, L. Yu, H. Tang, C. Chen, Z. Wang, EZH2 regulates expression of p57 and contributes to progression of ovarian cancer in vitro and in vivo, Cancer Sci. 102 (2011) 530–539.

[6] S. Hu, L. Yu, Z. Li, Y. Shen, J. Wang, J. Cai, L. Xiao, Z. Wang, Overexpression of EZH2 contributes to acquired cisplatin resistance in ovarian cancer cells in vitro and in vivo, Cancer Biol. Ther. 10 (2010) 788–795.

[7] S.N. Thibodeau, G. Bren, D. Schaid, Microsatellite instability in cancer of the proximal colon, Science 260 (1993) 816–819.

[8] M.A. Murphy, N. Wentzensen, Frequency of mismatch repair deficiency in ovarian cancer: a systematic review This article is a US Government work and, as such, is in the public domain of the United States of America, Int. J. Cancer 129 (2011) 1914–1922.

[9] C.E. Bronner, S.M. Baker, P.T. Morrison, G. Warren, L.G. Smith, M.K. Lescoe, M. Kane, C. Earabino, J. Lipford, A. Lindblom, et al., Mutation in the DNA mismatch repair gene homologue hMLH1 is associated with hereditary non-polyposis colon cancer, Nature 368 (1994) 258–261.

[10] N. Papadopoulos, N.C. Nicolaides, Y.F. Wei, S.M. Ruben, K.C. Carter, C.A. Rosen, W.A. Haseltine, R.D. Fleischmann, C.M. Fraser, M.D. Adams, et al., Mutation of a mutL homolog in hereditary colon cancer, Science 263 (1994) 1625–1629.

[11] J.P. Geisler, M.J. Goodheart, A.K. Sood, R.J. Holmes, M.A. Hatterman-Zogg, R.E. Buller, Mismatch repair gene expression defects contribute to microsatellite instability in ovarian carcinoma, Cancer 98 (2003) 2199–2206.

[12] E. Gras, L. Catasus, R. Arguelles, G. Moreno-Bueno, J. Palacios, C. Gamallo, X. Matias-Guiu, J. Prat, Microsatellite instability, MLH-1 promoter hypermethylation, and frameshift mutations at coding mononucleotide repeat microsatellites in ovarian tumors, Cancer 92 (2001) 2829–2836.

[13] F. Vikhanskaya, G. Colella, M. Valenti, S. Parodi, M. D'Incalci, M. Broggini, Cooperation between p53 and hMLH1 in a human colocarcinoma cell line in response to DNA damage, Clin. Cancer Res. 5 (1999) 937–941.

[14] G. Strathdee, K. Appleton, M. Illand, D.W. Millan, J. Sargent, J. Paul, R. Brown, Primary ovarian carcinomas display multiple methylator phenotypes involving known tumor suppressor genes, Am. J. Pathol. 158 (2001) 1121–1127.

[15] A. Wiley, D. Katsaros, H. Chen, I.A. Rigault de la Longrais, A. Beeghly, M. Puopolo, R. Singal, Y. Zhang, A. Amoako, D. Zelterman, H. Yu, Aberrant promoter methylation of multiple genes in malignant ovarian tumors and in ovarian tumors with low malignant potential, Cancer 107 (2006) 299–308.

[16] S. Aebi, B. Kurdi-Haidar, R. Gordon, B. Cenni, H. Zheng, D. Fink, R.D. Christen, C.R. Boland, M. Koi, R. Fishel, S.B. Howell, Loss of DNA mismatch repair in acquired resistance to cisplatin, Cancer Res. 56 (1996) 3087–3090.

[17] J.A. Plumb, G. Strathdee, J. Sludden, S.B. Kaye, R. Brown, Reversal of drug resistance in human tumor xenografts by 2'-deoxy-5-azacytidine-induced demethylation of the hMLH1 gene promoter, Cancer Res. 60 (2000) 6039–6044.

[18] E. Vire, C. Brenner, R. Deplus, L. Blanchon, M. Fraga, C. Didelot, L. Morey, A. Van Eynde, D. Bernard, J.M. Vanderwinden, M. Bollen, M. Esteller, L. Di Croce, Y. de Launoit, F. Fuks, The Polycomb group protein EZH2 directly controls DNA methylation, Nature 439 (2006) 871–874.

[19] M. Rush, R. Appanah, S. Lee, L.L. Lam, P. Goyal, M.C. Lorincz, Targeting of EZH2 to a defined genomic site is sufficient for recruitment of Dnmt3a but not de novo DNA methylation, Epigenetics 4 (2009) 404–414.

[20] K.M. McGarvey, J.A. Fahrner, E. Greene, J. Martens, T. Jenuwein, S.B. Baylin, Silenced tumor suppressor genes reactivated by DNA demethylation do not return to a fully euchromatic chromatin state, Cancer Res. 66 (2006) 3541–3549.

[21] G. Colella, F. Vikhanskaya, A.M. Codegoni, C. Bonazzi, M. D'Incalci, M. Broggini, HMLH1 and hMSH2 expression and BAX frameshift mutations in ovarian cancer cell lines and tumors, Carcinogenesis 19 (1998) 691–694.

[22] E.M. Swisher, R.M. Gonzalez, T. Taniguchi, R.L. Garcia, T. Walsh, B.A. Goff, P. Welcsh, Methylation and protein expression of DNA repair genes: association with chemotherapy exposure and survival in sporadic ovarian and peritoneal carcinomas, Mol. Cancer 8 (2009) 48.

[23] G. Strathdee, M.J. MacKean, M. Illand, R. Brown, A role for methylation of the hMLH1 promoter in loss of hMLH1 expression and drug resistance in ovarian cancer, Oncogene 18 (1999) 2335–2341.

[24] K.M. McGarvey, E. Greene, J.A. Fahrner, T. Jenuwein, S.B. Baylin, DNA methylation and complete transcriptional silencing of cancer genes persist after depletion of EZH2, Cancer Res. 67 (2007) 5097–5102.

[25] Y. Kondo, L. Shen, A.S. Cheng, S. Ahmed, Y. Boumber, C. Charo, T. Yamochi, T. Urano, K. Furukawa, B. Kwabi-Addo, D.L. Gold, Y. Sekido, T.H. Huang, J.P. Issa, Gene silencing in cancer by histone H3 lysine 27 trimethylation independent of promoter DNA methylation, Nat. Genet. 40 (2008) 741–750.

[26] M.E. Gonzalez, M.L. DuPrie, H. Krueger, S.D. Merajver, A.C. Ventura, K.A. Toy, C.G. Kleer, Histone methyltransferase EZH2 induces Akt-dependent genomic instability and BRCA1 inhibition in breast cancer, Cancer Res. 71 (2011) 2360–2370.

[27] R. Bonasio, S. Tu, D. Reinberg, Molecular signals of epigenetic states, Science 330 (2010) 612–616.

[28] M.C. Tsai, O. Manor, Y. Wan, N. Mosammaparast, J.K. Wang, F. Lan, Y. Shi, E. Segal, H.Y. Chang, Long noncoding RNA as modular scaffold of histone modification complexes, Science 329 (2010) 689–693.

[29] S.L. Au, C.C. Wong, J.M. Lee, D.N. Fan, F.H. Tsang, I.O. Ng, C.M. Wong, Enhancer of zeste homolog 2 epigenetically silences multiple tumor suppressor microRNAs to promote liver cancer metastasis, Hepatology 56 (2012) 622–631.