



Research Article

Expressions of H3K9me2 and E-cadherin Correlate with Cervical Cancer Invasiveness

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Citation: Chen RJ, Chou CH, Shun CT, Wen WF, Yen ML, et al. (2023) Expression of H3K9me2 and E-cadherin Correlate with Cervical Cancer Invasiveness. J Oncol Res Ther 8: 10179. DOI: 10.29011/2574-710X.10179

Received Date: 25 July, 2023; Accepted Date: 04 August, 2023; Published Date: 07 August, 2023

Research Highlights

- E-cadherin expression is suppressed by H3K9me2.
- Low E-cadherin increases cervical cancer invasiveness with poor patient survival.
- Suppression of E-cadherin is mediated by MPP8 and SIRT1 pathways.
- Inhibition of H3K9me2 may result in the reduction of cancer invasiveness.

Abstract

Objective: To investigate whether H3K9me2 is linked to cervical cancer invasiveness.

Methods: A total of 480 cervical cancer tissue array samples and 55 matched cervical cancer patients were enrolled. Immunohistochemical staining was used to analyze E-cadherin, SIRT1, ZEB-1, MPP8 protein expression, while qRT-PCR was used to estimate mRNA transcription. Transwell migration assays were performed to determine cancer cell invasiveness. BIX01294 was used to reverse of histone methyl transferase activity. Small interfering RNA interference strategy used to verify the role of SIRT1. Pro E-cad 178-Luc (-178 to +92) promoter assay was used to verify the transcriptional regulation of E-cadherin.

Results: H3K9me2 protein expression increased step-wise from normal to cancer precursor to invasive cancer. E-cadherin expression inversely correlated with H3K9me2 expression. HeLa and SiHa cells that treated with H3K9me2 reversal (BIX01294) found to inhibit migration capability and increase E-cadherin promoter activities of both of the cervical cancer cell lines. E-cadherin silencing related molecule MPP8 was expressed in both cervical cancer cells and normal epithelium, ZEB-1 was found in mesenchymal cells but not in cancer cells. SIRT1 was expressed in cervical cancer cells. In cervical cancer cell model, the inhibition of SIRT1 by siRNA result in reducing cell migration and increasing E-cadherin mRNA expression. Meanwhile, SIRT1 expression in cervical cancer tissue was associated with lower patient survival.

Conclusion: E-cadherin and SIRT1 expression correlated negatively with poor survival of cervical cancer patients. Our results suggest that using BIX01294 to inhibit H3K9me2 expression is a potential approach for the management of aggressive cervical cancer.

Keywords: H3K9me2; E-cadherin; SIRT1; Invasiveness; Cancer precursors; Cervical cancer

Introduction

Cervical cancer is a common female cancer. Although there are regional inequalities in cervical cancer's global burden [1], in 2018 it was estimated that there would be 570,000 new cases of cervical cancer and a resultant 311,000 deaths from this disease worldwide [2]. Cervical cancer cells spread primarily by direct local invasion, resulting in either a tumor mass and ulceration or an invasion of the lymphatic system resulting in lymphatic dissemination. Measurements of local invasion and lymphatic spread are the main foci for an evaluation of the cancer's severity and clinical stage that is needed to determine treatment modality [3-9]. However, the molecular mechanisms of invasion/metastasis are not yet fully understood.

We have previously characterized the molecular linkage that directs both G9a histone methyl transferase overexpression and angiogenesis in cervical cancer. We found that, among 22 angiogenic factors, although G9a promotes angiogenesis mainly via IL-8, angiogenin and CXCL16, it does not promote production of apoptotic factors [10]. H3K9me2 (dimethylated histone H3 at lysine residue 9) plays an important role in the regulation of gene expression. H3K9me2 synthesis is catalyzed by G9a histone methyltransferase. This synthesis of H3K9me2 can be selectively impaired by treating cells with BIX01294 (diazepin-quinazolin-amine derivative), a specific and biologically active inhibitor against G9a histone methyltransferases. BIX01294 selectively impairs the target enzyme G9a and curtails the generation of H3K9me2 [11]. However, H3K9me2 expression in cervical cancer, and whether H3K9me2 has other tumorigenic properties such as tumor invasion, are not fully understood.

Epithelial cadherin (E-cadherin) belongs to the cadherin family. Its function is to sustain normal tissue structure by maintaining epithelial cell-to-cell adhesion [12]. In bladder cancer, enhancing E-cadherin expression may suppress bladder cancer cell growth and metastasis [13]. The correlation between H3K9me2 and E-cadherin expression is not well known. Since G9a regulates epigenetic gene regulation, and since its expression correlates inversely with E-cadherin, here we explored whether H3K9me2 involves e-cadherin expression and its possible mechanism.

Materials and Methods

Tissue array and cervical cancer tissue

To determine specific protein expression in cervical cancer, we used cancer tissue from both tissue arrays and clinical specimens as previous reported [10]. Briefly, the formalin-fixed arrays of normal, benign, and malignant cervical tissue used were CXC961,

CXC962, CXC1021, CIN481, CIN482, and CIN483 (from Pantomics, Inc., San Francisco, CA), and the paraffin-embedded cancer blocks were from radical hysterectomy specimens. Normal cervical specimens obtained surgically from cases of a benign uterine corpus and normal cervical specimens obtained surgically from cases of a benign ovarian disease were also used.

IHC

The IHC procedure used and the secondary antibodies for IHC staining were the same as reported previously [10]. In this study, the first antibodies used were the anti-Histone H3 (di methyl K9) antibody (Abcam 1220), the E-cadherin antibody (Abcam 40772), the MPP8 antibody (Abcam 122575), the ZEB1 antibody (Abcam 203829), and the SIRT1 antibody (Abcam 110304). The sites of positive staining were in the nucleus for H3K9me2, MPP8, ZEB1 and SIRT1 staining, and was in the intercellular junction for E-cadherin staining. Expression levels were quantified by calculating the ratio of positive staining cells to the total number of cells under a 400X high power field.

Cell culture

Cervical cancer cell lines SiHa (squamous carcinoma, cervix, human) and Hela (epithelioid carcinoma, cervix, human) were used in this study. The source, growth and maintenance of these cell lines were as reported previously [10].

Chemicals reagents

BIX01294 was purchased from Sigma (St Louis, MO).

E-cadherin promoter assay

Transfection of proE-cad 178-Luc (-178 to +92) vectors into cervical cancer cells was performed using the Transfast Transfection Reagent (Promega) method as described previously [14,15]. Briefly, the cells were transfected, serum-starved, and then treated with SIRT1 siRNA or control siRNA, and were then co-transfected with pSV- β -galactosidase. The culture was reproduced in triplicate.

E-cadherin qRT-PCR

Total RNA extraction and cDNA preparation were described previously [10]. The specific oligonucleotide primer pairs for E-cadherin were 5'- CGG GAA TGC AGT TGA GGA TC-3' [16] and 5'- AGG ATG GTG TAA GCG ATG GC -3'; and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-GGGAAGGTGAAGGTC GG-3' and 5-TGGACTCCACGACGTACTCAG-3'. The amplification program used was as previously described [17]. Briefly, it consisted of one cycle of an initial incubation at 61 °C for 20 min, followed by 40 cycles of denaturation at 95 °C for 10 sec, annealing at 55–57°C for 10 sec, and extension at 72 °C for 10 sec. GAPDH mRNA was used for normalization.

RNA interference

The targeted siRNAs for SIRT1 and control siRNA were sc-40986 and sc-37007, respectively. The transfection procedure used was described previously [18]. Briefly, the culture cells were transfected in serum-free Opti-MEM using the Oligofectamine method.

Transwell migration assay

HeLa and SiHa cells were used for transwell migration assay [18]. Briefly, the cells were seeded into Transwell (8- μ m pore size) dishes and permitted to migrate. The migrated cells on the lower surface were digitally photographed and quantified under a high power field.

Statistical analysis

We present the data as mean \pm standard deviation. Student-t test, ANOVA, Post-Hoc (Tukey's) test, and post-test for linear trend are used for statistical analyses of continuous variables. The Kaplan-Meier estimate is used for survival analysis. A p-value of less than 0.05 is considered significant.

Results

Differential expression of H3K9me2 in cancerous and normal cervixes

Comparison of H3K9me2 expression in cancerous and normal cervixes is shown in **Figure 1A**. H3K9me2 had a high expression in cervical cancer cells and a low expression in normal cervixes. In 440 array samples, the H3K9me2 expression rate per 100 cells for cervical cancer (35.7 ± 15.2 , $n=186$) was significantly higher than for either cancer precursor (23.1 ± 15.8 , $n=106$) or normal cervix (14.0 ± 10.8 , $n=148$, ANOVA, $p=0.000$). In surgical specimens obtained from radical hysterectomies from 55 matched cervical cancer patients, the H3K9me2 expression rate was much higher for those who died from cervical cancer (54.167 ± 14.968) than for those who survived (45.307 ± 15.407). Moreover, survival analysis revealed that the survival rate was lower for those with a high grade of H3K9me2 expression (≥ 75 per 100 cells) than for those with a low grade of expression (<75 per 100 cells, **Figures 1B & 1C**).

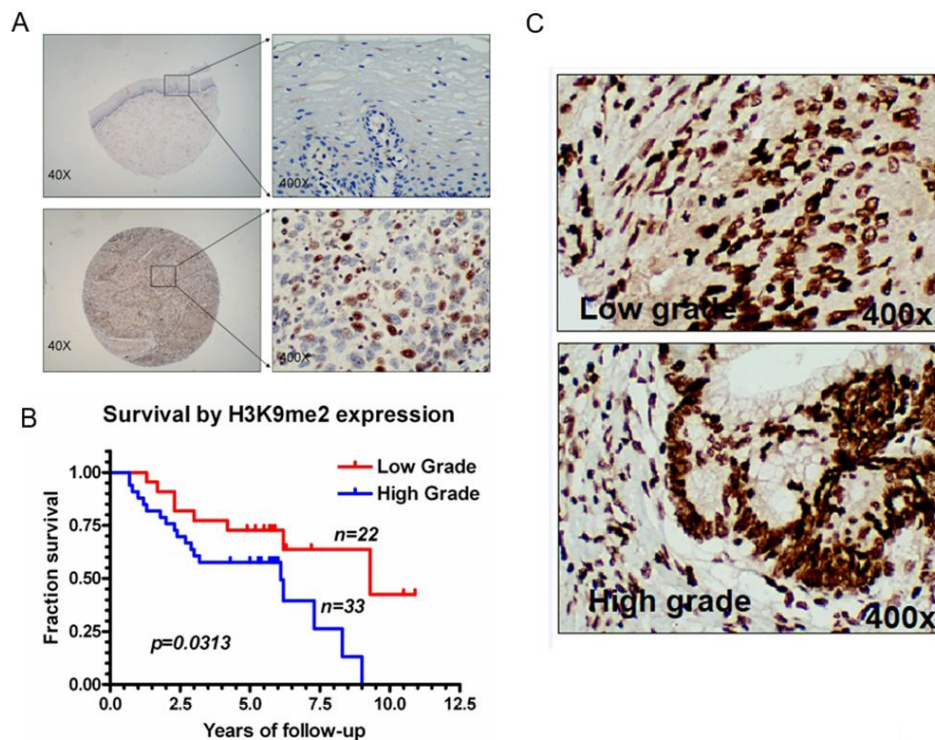


Figure 1: H3K9me2 was overexpressed in cervical cancer tissue and correlated with a poorer prognosis for cervical cancer patients. (A) Overexpression of H3K9me2 was found in carcinoma cells; it was not expressed in normal cervical epithelium. (B) Kaplan-Meier survival curves indicate that a high expression of H3K9me2 in patients correlated with a poorer survival probability. (C) High grade expression and low grade expression.

Inhibition of H3K9me2 resulted in up-regulation of E-cadherin expression

HeLa and SiHa cells were treated with BIX01294 to inhibit H3K9me2. Before treatment, E-cadherin mRNA was regarded as 1. E-cadherin expression was measured at intervals of 6, 16, 24, 48 and 72 hrs after treatment. Our results show that E-cadherin expression increased after H3K9me2 inhibition (Figure 2A).

E-cadherin expression critically involved in migration/invasion capability of cervical cancer cells

In trans-well cell invasion assays for cervical cancer, when HeLa and SiHa cells were serum-deprived cancer cell invasion/migration was significantly lower than when cells were

serum-enriched ($p < 0.05$). The H3K9me2 inhibitor BIX01294 significantly reduced the migration/invasion capability of cervical cancer cells when FBS was used as a chemo-attractant, but not when FBS was absent (Figure 2B).

H3K9me2 inhibitor down-regulated E-cadherin promoter activity of cervical cancer cells

E-cadherin expression in cervical cancer cells was measured by luciferase activity. Cells were first treated with BIX01294 and then transfected with the proE-cad178-Luc (-178 to +92) vector. Luciferase activity increased as incubation time period increased for both SiHa and HeLa cells (Figure 2C).

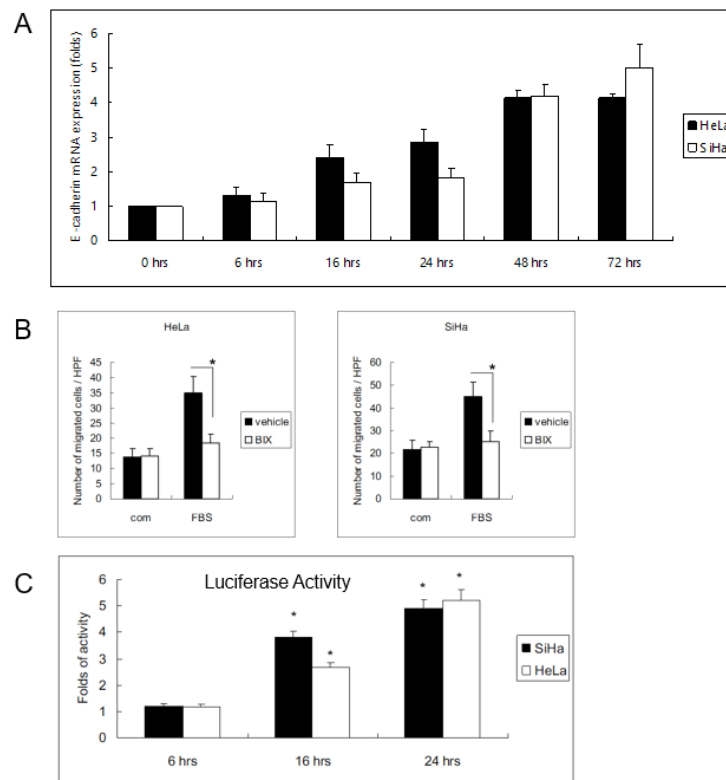


Figure 2: Inhibition of H3K9me2 resulted in the up-regulation of E-cadherin expression and the reduction of the migration/invasion capability of cervical cancer cells. (A) Inhibition of H3K9me2 resulted in the up-regulation of E-cadherin in cervical cancer cells. HeLa and SiHa cells were treated with BIX01294 at indicated times. The expression of E-cadherin mRNA was determined by q-RT-PCR. (B) E-cadherin expression was critically involved in the migration/invasion capability of cervical cancer cells. HeLa and SiHa cells were treated with BIX01294 in the confines of a 16 hour trans-well cell invasion assay. The lower chamber was loaded with chemoattractant. The chemoattractant used in the com group was a serum-free condition medium, while a condition medium containing fetal bovine serum was used in the FBS group. The number of migrated cells was counted in high power field (HPF). Migrated cells are shown in two groups. (C) H3K9me2 inhibitor up-regulated E-cadherin promoter activity. ProE-cad178-Luc vectors were made at sites -178 to +92. HeLa and SiHa cells were transfected with vectors for 24 hours prior to treatment with BIX01294. Cell lysates were processed for luciferase activity at indicated times. * $p < 0.05$.

E-cadherin expression was low in cervical cancer tissue

E-cadherin expression in normal cervical epithelium was high, while in cancer tissue, especially in the invasive tumor front (ITF), it was low, as is shown in **Figure 3A**. A significant difference in E-cadherin expression thus exists between invasive cancer, cancer precursors, and normal epithelium (**Table 1**). We found its expression to be lowest in invasive cancer. Interestingly, squamous cell carcinoma was found to have a lower E-cadherin level than adenocarcinoma.

Characteristics	Case no. or mean ± SD	E-cadherin (%)			p-value
		Grade 1	Grade 2	Grade 3	
Total cases	480	215 (44.8%)	98 (20.4%)	167 (34.8%)	
Age	46.9 ± 10.7	46.5 ± 11.3	46.7 ± 11.1	47.4 ± 9.8	NS*
Histological diagnosis					< 0.0001 [#]
Normal cervix	145	23 (15.9%)	20 (13.8%)	102 (70.3%)	
Cancer precursors	90	35 (38.9%)	21 (23.3%)	34 (37.8%)	
Invasive carcinoma	245	157 (64.1%)	57 (23.3%)	31 (12.7%)	
Tumor stage[†]					NS ^{###}
Stage I	212	134 (63.2%)	50 (23.6%)	28 (13.2%)	
Stage II	33	23 (69.7%)	7 (21.2%)	3 (9.1%)	
Tumor cell type[†]					0.004 ^{###}
Squamous cell carcinoma	202	138 (68.3%)	44 (21.8%)	20 (9.9%)	
Adenocarcinoma	43	19 (44.2%)	13 (30.2%)	11 (25.6%)	
Tumor cell differentiation^{†,§}					< 0.030 [#]
Well-differentiated	12	9 (75.0%)	2 (16.7%)	1 (8.3%)	
Moderately differentiated	64	38 (59.4%)	15 (23.4%)	11 (17.2%)	
Poorly differentiated	91	75 (82.4%)	10 (11.0%)	6 (6.6%)	
*ANOVA [#] Pearson Chi-square test with linear-by-linear association [†] invasive carcinoma ^{###} Pearson Chi-square test [§] information not available for 78 cases NS: non-significant					

Table 1: E-cadherin expression in epithelial cells of normal cervix, cancer precursors and invasive carcinoma of the uterine cervix.

E-cadherin expression correlated negatively with poor patient survival

The 5-year survival rates for patients with low (E-cadherin grade 1), medium (E-cadherin grade 2), and high E-cadherin expression (E-cadherin grade 3) statistically differ (Figures 3B & 3C). A lower expression of E-cadherin is associated with poor survival.

Negative correlation between expressions of E-cadherin and H3Kme2

In the 480 tissue samples for which we have both H3K9me2 and E-cadherin staining available (including the 425 tissue array samples and 55 clinically obtained tissue samples matched to cervical cancer patients), the H3K9me2 expression rate per 100 cells correlated negatively with E-cadherin expression (Figure 3D).

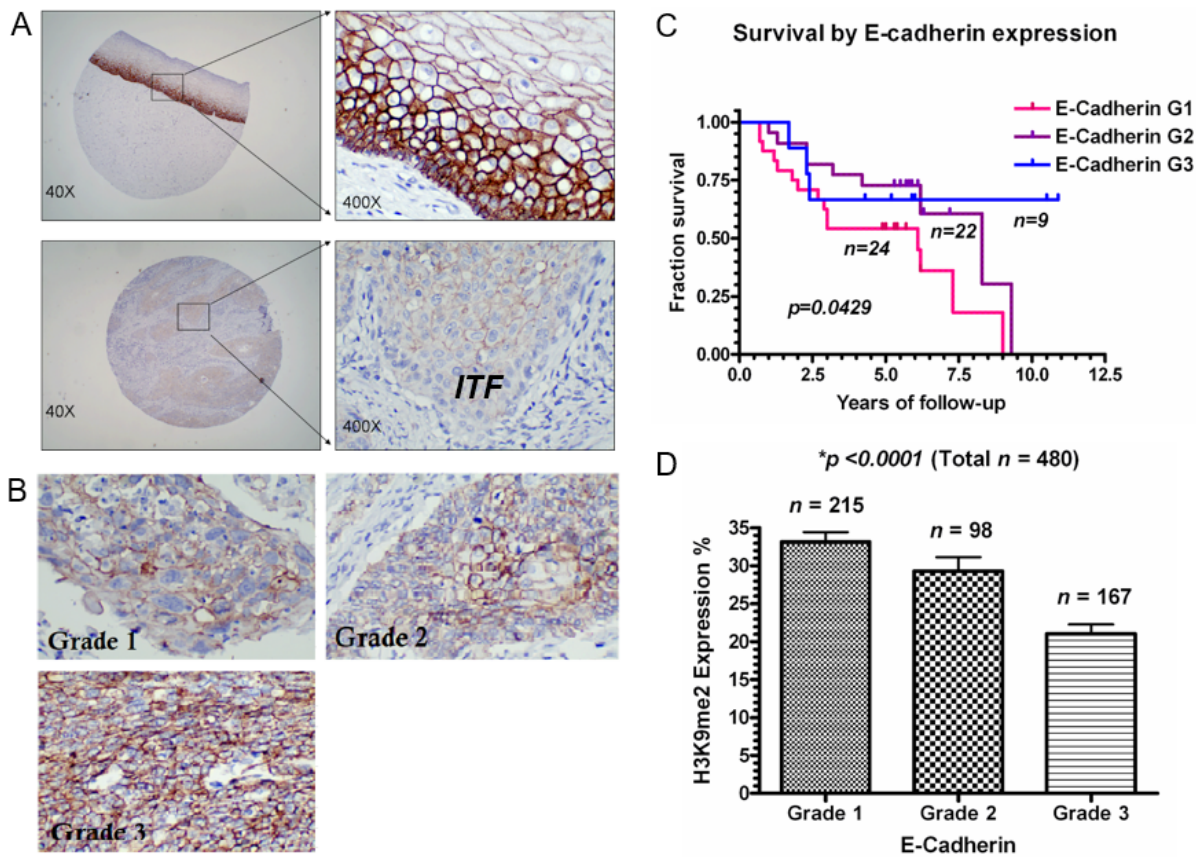


Figure 3: E-cadherin expression in cervical cancer. (A) E-cadherin promoter activity had a low expression in cervical cancer tissue (ITF: invasive tumor front). (B) Histological pictures of various grades of E-cadherin. (C) Expression of E-cadherin inversely correlated with poor survival of cervical cancer patients. (D) Higher E-cadherin expression was associated with lower H3K9me2 expression.

Expression of MPP8, ZEB-1, and SIRT1 in cervical cancer tissue

Expression of the Methyl-H3K9-binding protein MPP8 in cervical cancer, carcinoma in situ and normal epithelium is shown in (Figure 4A). Expression of ZEB-1 was found in mesenchymal cells but not in cancer cells (Figure 4B). Expression of SIRT1 in cervical cancer cells was high (Figure 4C).

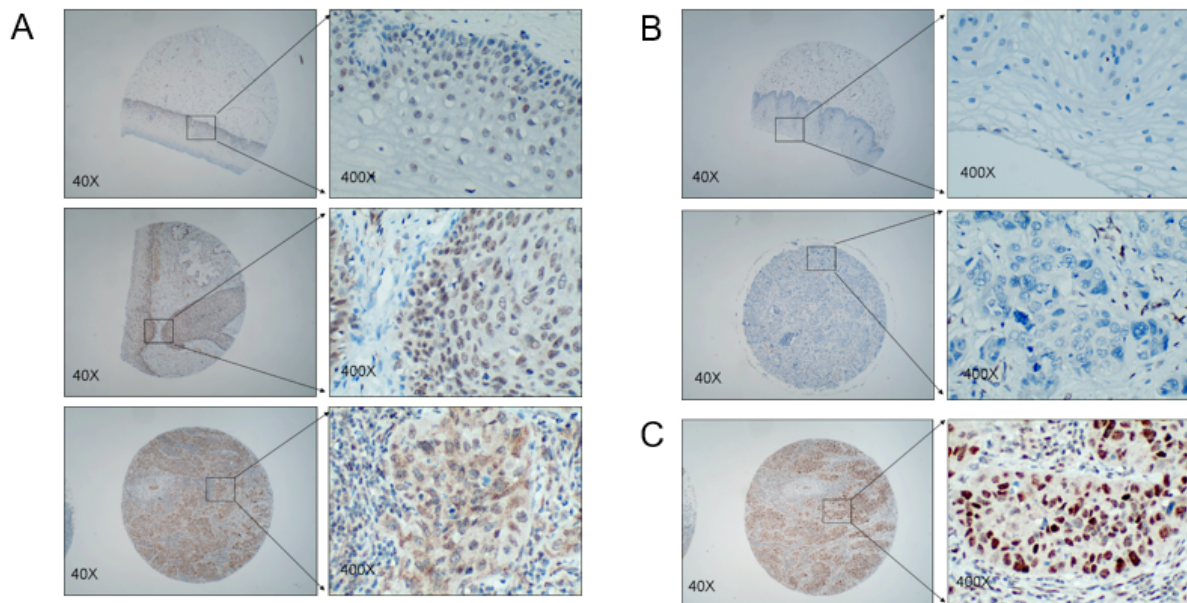


Figure 4: Expression of MPP8, ZEB-1, and SIRT1 in cervical cancer tissue. (A) MPP8 expression was higher in cervical cancer than in carcinoma in situ. MPP8 expression in normal squamous epithelium was low. (B) No ZEB-1 expression was found in normal cervical tissue. In cervical cancer tissue, ZEB-1 expression was found in mesenchymal cells but not in cancer cells. (C) SIRT1 expression in cervical cancer cells was high.

SIRT1 silencing

We used gene silencing (employing the SIRT1siRNA treatment) to suppress SIRT1 (**Figure 5A**). We found that siRNA treatment reduced CC cell invasion/migration and enhanced E-cadherin expression. SIRT1 expression correlated with patient prognosis. Kaplan-Meier analysis revealed that a higher level of SIRT1 was associated with lower patient survival (**Figures 5B & 5C**).

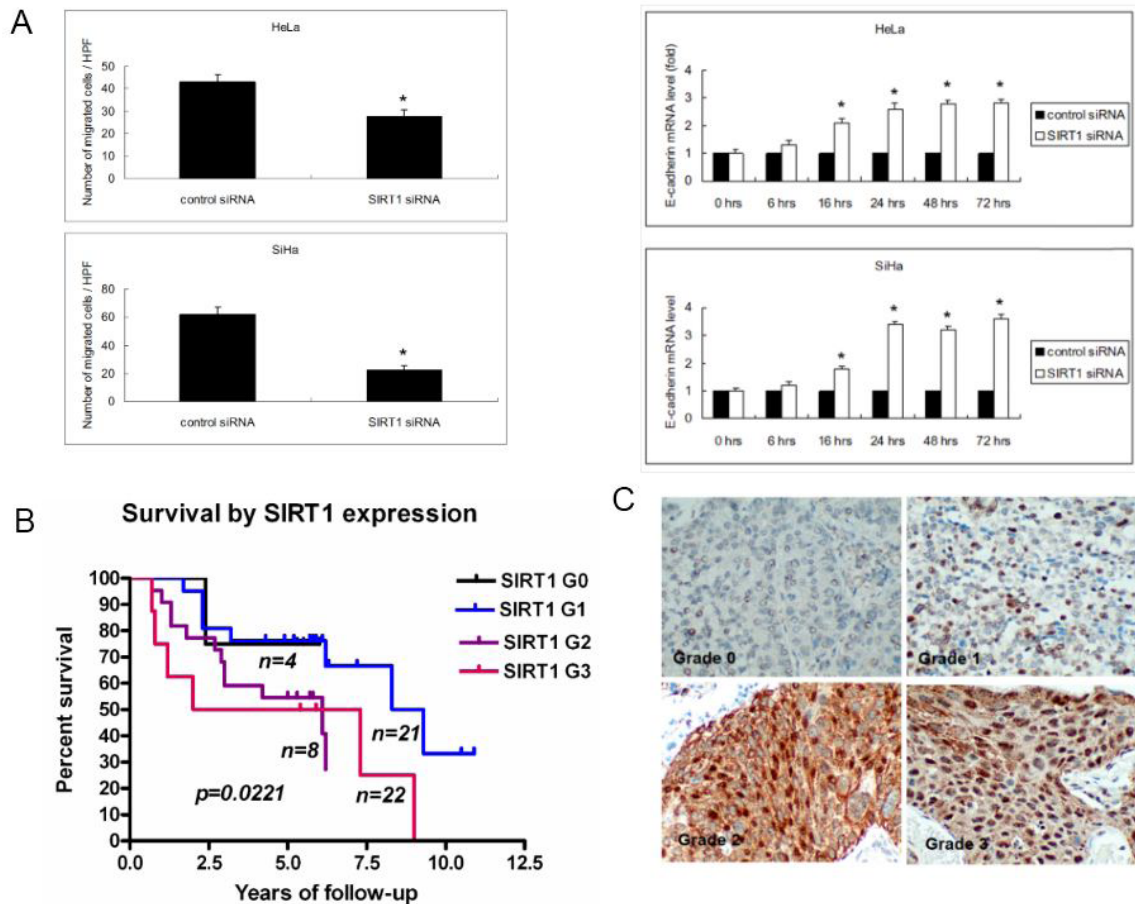


Figure 5: SIRT1 gene suppression and survival. (A) SIRT1 gene silencing by siRNA treatment reduced cancer cell invasion/migration (Left panel) and enhanced E-cadherin expression (right panel). * $p < 0.05$. (B) SIRT1 expression correlated with the prognosis of cervical cancer patients: a higher level of SIRT1 gene suppression entailed lower patient survival. (C) Histological pictures of various grades of SIRT1.

Discussion

In this study, we found that the higher expression of H3K9me2 was associated with lower E-cadherin expression, cancer cell invasiveness and lower patient survival. Both MPP8 and SIRT1 can combine with H3K9me2 and then epigenetically suppress E-cadherin gene expression.

Previous immunohistochemical studies have identified ZEB1 expression in cancer cells from bladder cancers [19] and breast cancers [20]. However, in our study we found ZEB1 expression in mesenchymal cells but not in squamous cancer cells. In a breast cancer cell line model, E-cadherin transcriptional downregulation by promoter methylation was related to an epithelial-to-mesenchymal transition [21]. An epithelial-to-mesenchymal transition is thought to play a central role during tumor metastasis, which involves DNA methylation and several post-translational

histone modifications [22]. In a bladder cancer model, the reversal of this epithelial-to-mesenchymal transition, the mesenchymal-to-epithelial transition, was discerned; it may also facilitate metastatic colonization [23]. Interestingly, in our study ZEB1 expression was found not in squamous cancer cells but instead in mesenchymal cells. Thus, ZEB1 does not seem to be directly involved in the mechanism whereby E-cadherin expression is inhibited in cancer cells. Whether ZEB1 takes part in the epithelial-to-mesenchymal or the mesenchymal-to-epithelial transition to promote cervical cancer invasion merits further study.

E-cadherin is considered to be a molecule integral for cell-cell adhesion. It is also a putative tumor suppressor gene implicated in carcinogenesis [24]. Enhancing E-cadherin expression in a bladder may suppress cancer cell growth and metastasis [13]. In our study, we found its expression in invasive cancer to be lowest

when compared with cancer precursors and normal epithelium. Interestingly, squamous cell carcinoma was found to have a lower expression of E-cadherin than adenocarcinoma. This finding suggests that the squamous cell type of cervical cancer may have a higher local invasiveness than adenocarcinoma. Previously we reported that, in terms of survival rates, for stage I-II cervical cancer radiotherapy is more effective for squamous cell cancer than for adenocarcinoma [25]. Whether differences in local invasiveness between squamous cell cancer and adenocarcinoma makes for differences in treatment needs further study.

In this study, we found that dimethylation of histone H3 lysine-9 (H3K9) and then over-expression of dimethyl H3K9 (H3K9me2) may transcriptionally repress E-cadherin. Low expression of E-cadherin in turn promotes cancer invasiveness and decreases patient survival. Our study suggests that H3K9me2 is essential for the epigenetic promotion of cervical cancer's invasiveness and metastasis. Moreover MPP8, a methyl-H3K9-binding protein, has been found to bind to methylated histone H3K9 [26]. This finding verifies that MPP8 mediates H3K9me2-induced E-cadherin gene silencing.

Invasive cervical cancer involves progression from cancer precursors. Cervical cancer precursors are called cervical intraepithelial neoplasia (CIN). CIN is graded as mild (grade 1, CIN1) -- which denotes the presence of a low grade lesion -- and moderate (grade 2, CIN2) and severe (grade 3, CIN3) -- which refer to high grade lesions. The most advanced CIN is carcinoma in situ (CIN3). SIRT1 was found to be more prominent in high grade than in low grade lesions. In a study of cervical cancer, all 24 cases of invasive and microinvasive squamous cell carcinoma showed SIRT1 overexpression. The authors thus suggested that SIRT1 may serve as a potential biomarker for distinguishing between CIN and invasive cervical cancer [27]. In our study, 165 cervical cancer samples had a higher positive nuclear SIRT1 expression (96.9%, 128/165) than 88 cancer precursors (79.5%, 70/88). Moreover, when we used a SIRT1 siRNA treatment to suppress the SIRT1 gene we found that siRNA treatment reduced CC cell invasion/migration and enhanced E-cadherin expression. Higher SIRT1 is thus associated with lower patient survival.

Conclusion

We show here that H3K9me2 suppresses E-cadherin expression. The binding of MPP8 and SIRT1 with H3K9me2 may mediate E-cadherin gene silencing, promote ITF formation with cancer invasiveness and lower patient survival. Using BIX01294 to epigenetically target H3K9me2 may represent a novel strategy for the treatment of invasive cervical cancer, particularly for those cases with a squamous cell type.

Conflict of interest

The authors have no conflicts of interest to declare.

Grant support

This work is supported by research grants NTUH 112-S0130, MS-164 and ME-109 from the National Taiwan University Hospital, and grant NSC 102-2314-B-002-110-MY2 from the Ministry of Science and Technology, Taiwan.

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