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Piwil1 causes epigenetic alteration of PTEN gene via upregulation of DNA methyltransferase in type I endometrial cancer

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ABSTRACT

Piwil1, a member of the Piwi family, has been well demonstrated to mediate tumorigenesis associated with DNA hypermethylation. It has been reported that Piwil1 is overexpressed in various types of cancer, including endometrial cancer. However, the underlying mechanism of Piwil1 in endometrial cancer remains largely unclear. PTEN exerts an important tumor suppressor role in endometrial carcinogenesis. The present study aimed to investigate whether Piwil1 could regulate the expression of PTEN. Herein, we found that Piwil1 could promote the loss of PTEN expression and increase aberrant hypermethylation of PTEN gene promoter in Ishikawa cells. We also found that Piwil1 could regulate the expression of DNA methyltransferase 1 (DNMT1). Silencing DNMT1 gene could upregulate the PTEN gene expression and change the methylation status of PTEN gene promoter in Ishikawa cells. These results suggested that Piwil1 caused the loss of PTEN expression through DNMT1-mediated PTEN hypermethylation. Taken together, these data provide a novel regulatory mechanism of Piwil1 in endometrial cancer.

1. Introduction

Endometrial cancer is a common malignancy of the female genital tract. According to their etiological and pathological features, endometrial cancer could be broadly categorized into two different clinicopathologic types: type I and Type II [1]. Most patients present with Type I and recent studies have indicated that epigenetic alterations may play a significant role in this type of cancer [2]. The best known epigenetic event is aberrant DNA methylation. As we known, DNA methylation is mediated by a family of proteins called DNA methyltransferases (DNMTs), including DNMT1, DNMT3A and DNMT3B [3]. Compared to normal endometrium, expression levels of both DNMT1 were significantly increased in type I cancer but downregulated in type II cancer [4,5].

Promoter hypermethylation of tumor suppressor genes is a major event in the origin of many tumors, including endometrial cancer. A number of studies establish a list of tumor suppressor genes frequently hypermethylated in endometrial cancer [5–8]. It is well known that phosphatase and tension homolog deleted on chromosome ten (PTEN) which controls proliferation and apoptosis acts as a tumor suppressor gene in endometrial carcinomas. DNA methylation-mediated silencing of PTEN gene predicts poor prognosis in endometrial cancer [9].

Piwil1 belongs to the Piwi family. Piwi is first identified in Drosophila as an essential factor for the self-renewal of germline stem cells (GSC) [10]. piRNA is defined as a class of small RNAs binding mammalian Piwi proteins [11,12]. Previous studies have shown that Piwi-piRNA pathway is epigenetically regulated in Drosophila and mice. piRNAs have been identified at heterochromatic regions and heterochromatin protein 1a (HP1a) is found to bind to piRNA and Piwis [13,14]. Upon knock out of Piwis, de novo methylation of IAP and Line1 transposon region is reduced and transposon expression is activated. These data implicate de novo methylation as an important epigenetic mechanism of the piRNA-Piwi pathway to maintain transposon silencing [15–17]. Piwil1 has been reported to be widely expressed in various types of human cancer [18–20]. Thus, Piwil1 may play an important role in tumor development [21–23]. It is reported that Piwil1 could upregulate the expression of DNA methyltransferase 1 and then mediate DNA methylation-associated silencing of tumor suppressor gene which potentially accounting for Piwil1-mediated tumorigenesis [24].

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Here, we show that Piwil1 can regulate the expression of PTEN via DNMT1-mediated DNA methylation of PTEN prompter in type I endometrial cancer. Our results suggest that the Piwil1/DNMT1/PTEN pathway may play an important role in endometrial cancer.

2. Materials and methods

2.1. Ethics statement

The study was approved by the Human Investigation Ethics Committee of Shanghai First People's Hospital Affiliated to Shanghai Jiao Tong University. The samples of endometrial carcinoma were collected after written informed consent from the patients.

2.2. Tissue specimens

Tissue samples for immunohistochemistry were obtained at Shanghai First People's Hospital Affiliated to Shanghai Jiao Tong University from 2012 to 2014. The stages and histological grades of these tumors were established according to the criteria of the Federation International of Gynecology and Obstetrics (FIGO) surgical staging system (2009) [25]. None of the patients underwent hormone therapy, radiotherapy, or chemotherapy prior to surgery.

2.3. Cell culture

Ishikawa cell line is a human endometrial adenocarcinoma cell line which contains estrogen and progesterone receptors [26]. Ishikawa cells were obtained from the Chinese Academy of Sciences Committee Type Culture Collection (Shanghai, China). According to the provider's instructions, cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM)/F12 (Gibco) supplemented with 10% fetal bovine serum (Gibco).

2.4. Assessment of DNA methylation levels

Genomic DNA was isolated by TIANamp Genomic DNA Kit (TIANGEN). Global DNA methylation levels were assessed by Methylflash Methylated DNA Quantification Kit (Colorimetric, Epigentek) and read on a plate reader at 490 nm, according to manufacturer's protocol.

2.5. Total RNA extraction and real-time RT-PCR

Total RNA from Ishikawa was isolated by Trizol (Invitrogen) and cDNA was prepared using the reverse transcriptase kit (TaKaRa). The cDNA was analyzed by real-time PCR using SYBR Premix Ex Taq (TaKaRa) in an Eppendorf Mastercycler realplex. Data was calculated using the $2^{-\triangle\triangle Ct}$ formula. The Primers for real-time RT-PCR see supplementary table 1.

2.6. Western blot

Cells were lysed in lysis buffer (Beyotime) for 30 min at 4 °C. Total proteins were fractionated by SDS–PAGE and transferred onto PVDF membranes (Millipore). The membranes were then incubated with primary antibodies against Piwil1 (1:1000, ab85125, Abcam), PTEN (1:1000, #9188, CST), DNMT1 (1:500, ab13537, Abcam) and GAPDH (1:2000, #5174, CST) at 4 °C overnight, followed by incubation with peroxidase-linked secondary antibody (1:10000, 112-005-003, Jackson ImmunoResearch). The probed proteins were detected by enhanced chemiluminescent reagents (Thermo). GAPDH was used as an internal control.

2.7. Methylation analysis by methylation-specific PCR

The DNA was modified using ZymoTaq TM PreMix (ZYMO Research). Methylation-specific PCR was carried out in a volume of 20 μl containing 100 ng of modified DNA. Primer sequences used to amplify a 173 bp unmethylated product were 5′-TGGGTTTT GGAGGTTGTTGGT-3′ (sense)

and 5′-ACTTAACTCTAAACCACAACCA-3′ (antisense), which amplify a 173 bp product, and primer sequences for the methylated reaction were 5′-GGTTTCG-GAGGTCGTCGGC-3' (sense)

and 5′-CAACCGAATAATAACTACTACGACG-3′ (antisense), generating a 155 bp product [27]. The PCR conditions were: 40 cycles at 95 °C for 45 s, 57 °C for 30 s, 72 °C for 30 s after 95 °C for 5 min, and finally 72 °C for 10 min. The amplified DNA was analyzed on 2% agarose gels by standard electrophoresis.

2.8. RNA interference (RNAi) analysis

RNAi experiments in Ishikawa cells were performed using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's protocol. Small interfering RNA (siRNA) oligonucleotides against Piwil1 or DNMT1 genes or scrambled sequences were synthesized by the Shanghai GenePharma Corporation. The following siRNA sequences were used:

siPiwil1, 5′-GUGGGCCUUAUAUCAGUAUTT-3′ (sense)
and 5′-AUACUGAUAUAAGCCCACTT-3′ (antisense).
siDNMT1, 5′- CCAUGAGCACCGUUCUCCUUTT-3′ (sense)
and 5′-AAGGAGAACGGUGCUCAUGGTT-3′ (antisense).

The following scrambled sequence was used as a control (NC), 5′-UUCUCCGAACGUGUCACGUTT-3′ (sense)
and 5′-ACGUGACACGUUCGGAGAATT-3′ (antisense).

2.9. Immunohistochemistry

Tissue immunohistochemistry was performed by the 3,3'-diaminobenzidine (DAB) method with a heat-induced antigen retrieval step. Briefly, slides were incubated with rabbit polyclonal anti-Piwil1 (1:200, ab85125, Abcam), rabbit monoclonal anti-PTEN (1:125, #9188, CST) and mouse monoclonal anti-DNMT1 (1:1000, ab13537, Abcam) overnight at 4 °C and then incubated with horseradish peroxidase (HRP)-linked anti-rabbit or anti-mouse secondary antibody (Boster) at room temperature for 30 min followed by chromagen detection with DAB (Boster) and hematoxylin (Boster) counterstaining.

Two independent pathologists, who were blinded to the clinical and pathological data, evaluated the specimens. Sections were evaluated according to semi quantitative immunoreactivity scores. We separately scored for the percentage of positive staining (0 = negative, 1 = 25%, 2 = 25–50%, 3 = 50–75% and 4 = 75%) and the staining intensity (0 = none, 1 = weak, 2 = moderate, and 3 = strong). For each specimen, the summation of the two above gave the final score (−: 0–2; +: 3–4; ++: 5–6; +++: 7) [28].

2.10. Statistical analysis

Statistical analyses were made using the software package SPSS v. 18 (SPSS Inc.). Values were expressed as mean ± the standard deviation and analyzed with the Student's *t*-test. Fisher's Exact Test for 2 × 2 tables was used to compare the categorical data. Significant differences were indicated for P values <0.05. All experiments were performed at least three times.

3. Results

3.1. Piwil1 expression correlated with DNA methylation

Since Piwil1 have been implicated in gene silencing via DNA methylation [24], we also examined global DNA methylation levels in Ishikawa cells transfected with siRNA against Piwil1. Global DNA methylation detects cytosine methylation at both CpG sites and non-CpG sites using an antibody against 5-Methyl Cytosine. We found that global DNA methylation was significantly decreased (approximately 60%, $P < 0.05$, Fig. 1A) in Ishikawa cells transfected with siRNA against Piwil1 as compared to control cells, suggesting that Piwil1 expression led to an increase in DNA methylation in Ishikawa cells.

3.2. PTEN expression and PTEN methylation status in Ishikawa cells

As shown in Fig. 1B and C, the expression of PTEN mRNA and protein was upregulated when Piwil1 was silenced in Ishikawa cells. To investigate the mechanism underlying the increased PTEN expression, we analyzed whether PTEN promoter region hypomethylation was responsible for the downregulation of PTEN expression. Methylation-specific PCR analysis indicated that the methylation level of promoter region of PTEN gene from Ishikawa cells transfected with siRNA against Piwil1 was decreased as compared to control cells (Fig. 1D).

3.3. DNMT1 was involved in Piwil1-induced PTEN hypermethylation

To gain insights into the possible involvement of DNMT1 in Piwil1-induced PTEN hypermethylation, we examined the expression of DNMT1 in Ishikawa cells transfected with siRNA against Piwil1. As illustrated in Fig. 2A and B, we found that expression of DNMT1 mRNA and protein were also markedly decreased in Ishikawa cells transfected with siRNA against Piwil1 compared with control cells.

To determine the function of DNMT1, the expression of DNMT1 gene was inhibited by siRNA against DNMT1. As illustrated in Fig. 2C, transfection with siRNA against DNMT1 but not scrambled siRNA increased the expression of PTEN in Ishikawa cells. We further used methylation-specific PCR to examine the methylation status of PTEN promoter after DNMT1 knockdown. As shown in Fig. 2D, the methylation level of promoter region of PTEN gene from Ishikawa cells transfected with siRNA against DNMT1 was decreased as compared to control cells.

3.4. Piwil1 repressed PTEN in type I endometrial cancer tissues

We further analyzed the expression of Piwil1, PTEN and DNMT1 in 37 type I endometrial carcinoma samples used immunohistochemistry. The expression rates of Piwil1, PTEN and DNMT1 were 81.08% (30/37), 24.32% (9/37) and 75.7% (28/37) respectively in type I endometrial carcinoma tissues (Fig. 3A, B and C).

The pattern of PTEN and DNMT1 expression was well correlated with Piwil1 expression in endometrial cancer tissues. Statistical analysis found a strong correlation between Piwil1 expression and PTEN expression and we also found a strong correlation between Piwil1 expression and DNMT1 expression in type I endometrial carcinomas ($P < 0.01$, Table 1), indicating that DNMT1 and PTEN may be downstream targets of Piwil1 in endometrial cancer.

4. Discussion

Although Piwil1 is overexpressed in various types of tumor and involved in the development of tumor including endometrial

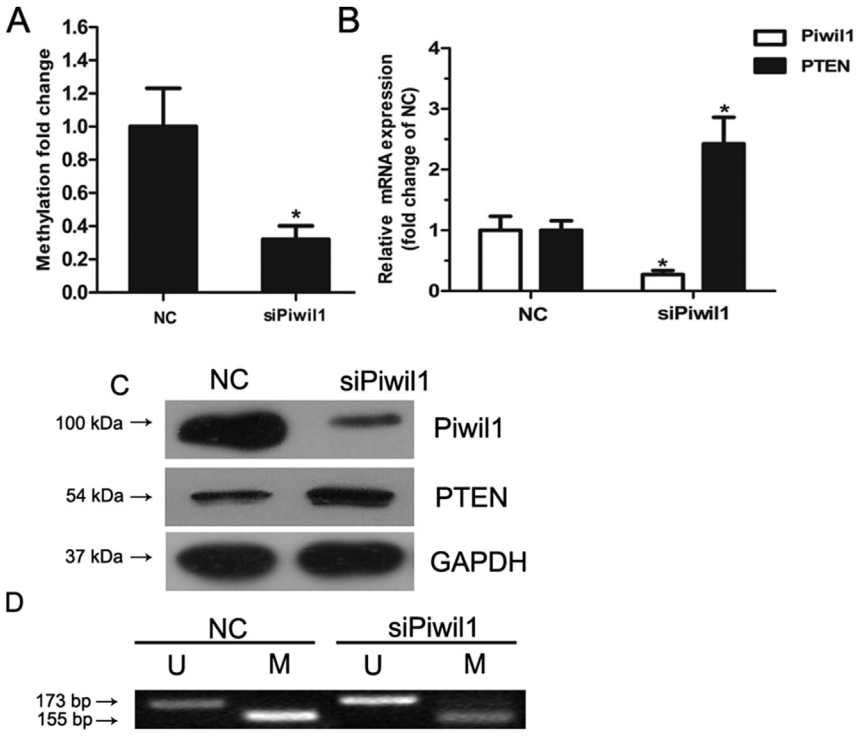

Fig. 1. Methylation status of PTEN gene promoter and expression of PTEN in Ishikawa cells. (A) Global DNA methylation was decreased in Ishikawa cells transfected with siRNA against Piwil1 as compared to control cells. Error bars represent standard error. * = $p < 0.05$. (B) The expression of PTEN mRNA was assessed by real-time RT-PCR. Results represent mean ± SD. * = $p < 0.05$ vs. control cells. (C) Western blot analysis to measure PTEN protein levels in Ishikawa cells transfected with siRNA against Piwil1 as compared to control cells. (D) Results of MSP analysis of PTEN gene promoter in Ishikawa cells transfected with siRNA against Piwil1 as compared to control cells. Bisulfite-treated DNA was amplified with methylated and unmethylated specific PTEN primers. The 173 bp product was indicative of an unmethylated PTEN allele, whereas the 155 bp product indicated a methylated PTEN allele.

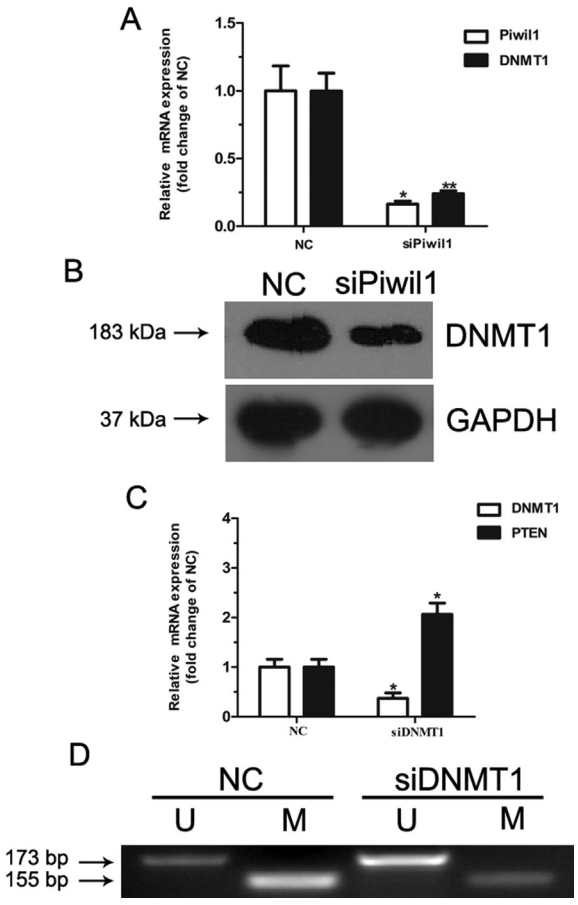

Fig. 2. DNMT1 was involved in Piwil1-induced PTEN hypermethylation. (A) Real time RT-PCR was performed to determine the relative change in DNMT1 mRNA expression in Ishikawa cells transfected with siRNA against Piwil1 as compared to control cells. Results represent mean ± SD. * = $p < 0.05$, ** = $p < 0.01$. (B) Western blot was used to measure DNMT1 protein levels in Ishikawa cells transfected with siRNA against Piwil1 as compared to control cells. (C) Real time RT-PCR demonstrated expression level of PTEN mRNA in Ishikawa cells transfected with siRNA against DNMT1. Results represent mean ± SD. * = $p < 0.05$. (D) Results of MSP analysis of PTEN gene promoter in Ishikawa cells transfected with siRNA against DNMT1 as compared to control cells. Bisulfite-treated DNA was amplified with methylated and unmethylated specific PTEN primers. The 173 bp product was indicative of an unmethylated PTEN allele, whereas the 155 bp product indicated a methylated PTEN allele.

Table 1
The correlation of PTEN and DNMT1 expression with Piwil1 expression in type I endometrial cancer.

Piwil1	PTEN		*P*	DNMT1		*P*
	−	+		−	+	
−	2	5	0.0049	7	5	0.0020
+	26	4		2	23	

cancer, the mechanism of Piwil1 still has to be demonstrated. Herein, for the first time, we provide a potentially novel mechanism of Piwil1 in endometrial cancer.

As a common molecular alteration in human tumors, epigenetics is defined as heritable changes in gene expression without alteration of the nucleotide sequence, including DNA methylation [29]. Previous study showed that Piwil1-mediated tumorigenesis was associated with DNA hypermethylation [24]. In our study, we found that global DNA methylation correlated directly with Piwil1 levels in Ishikawa cells, indicating that Piwil1 may be involved in epigenetic regulation in endometrial cancer.

Several reports suggest that PTEN exerts an important tumor suppressor role in endometrial carcinogenesis [30,31]. Inactivation of PTEN has been observed in a number of human tumors, including endometrial cancer. PTEN mutations occur in 30–50% of endometrial cancer, making it the most commonly known genetic alteration associated in this disease [32,33]. As the alternative mechanism of PTEN inactivation, PTEN promoter hypermethylation was found in about 20% of sporadic type I endometrial carcinoma and was significantly associated with metastatic disease and with MSI phenotype [9]. In the present study, we observed a marked shift of PTEN promoter methylation status and upregulation of PTEN expression after knockdown of Piwil1 in Ishikawa cells. We also found that PTEN expression was negatively correlated with the expression of Piwil1 in endometrial cancer tissues. These results suggested that the loss of PTEN expression was likely attributed, at least in part, to epigenetic regulation through Piwil1-mediated hypermethylation of PTEN promoter in endometrial cancer cells.

A crucial step in DNA methylation involves DNA methyltransferases (DNMTs) that catalyze methylation of CpG dinucleotides in genomic DNA [34]. DNMT1 maintains attachment of methyl groups to hemimethylated DNA during replication and is overexpressed in type I endometrial cancer [4,35,36]. In our study, we found that DNMT1 was downregulated in Ishikawa cells in which Piwil1 was knockdown. The enhanced expression of DNMT1

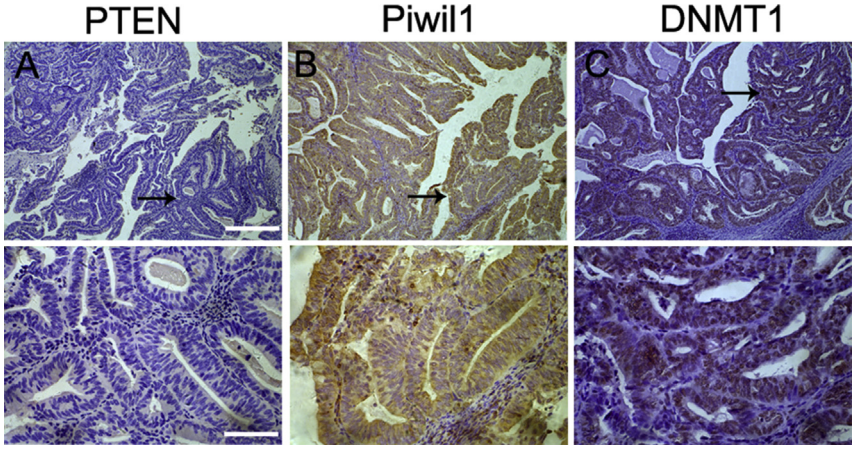

Fig. 3. Piwil1 repressed PTEN in type I endometrial cancer tissues. (A, B, C) Immunohistochemical analysis of PTEN, Piwil1 and DNMT1 expression in the same type I endometrial cancer tissues. No or weak expression of PTEN, strong cytoplasmic and nuclear expression of Piwil1 and strong nuclear expression of DNMT1 were observed in the majority of tumor tissues. Magnification: × 200, bar = 50 μm (above) and ×400 (down).

is responsible for abnormal methylation patterns of PTEN gene in hepatocellular carcinoma and breast cancer [37,38]. In this study, knockdown of DNMT1 by RNAi inhibited PTEN gene expression, suggesting that the downregulation of DNMT1 contributed to hypomethylation of the PTEN gene promoter. Thus, we hypothesized that Piwil1 inhibits PTEN expression through DNMT1-mediated DNA methylation of PTEN gene promoter in type I endometrial cancer. As the lower expression of DNMT1 in type II endometrial cancer [4], more studies are needed to identify different mechanisms of Piwil1 in this type of endometrial cancer.

In summary, we provide the first demonstration that Piwil1 regulates PTEN expression through DNMT1-mediated DNA methylation of PTEN gene promoter in type I endometrial cancer. We suggest that the Piwil1/DNMT1/PTEN signaling axis may play an important role in the progression of type I endometrial cancer.

Conflict of interests

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.06.028.

Transparency document

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