Am J Cancer Res 2015;5(1):180-190
www.ajcr.us /ISSN:2156-6976/ajcr0003031

Original Article

Frequent *SOCS3* and *3OST2* promoter methylation and their epigenetic regulation in endometrial carcinoma

Haiyan Chen[1,2*], Cuijuan Zhang[1*], Yan Sheng[1], Shuzhe Yao[1], Zhiyan Liu[1], Cheng Zhang[1], Tingguo Zhang[1]

[1]*Institute of Pathology and Pathophysiology, Shandong University School of Medicine, Jinan 250012, P R China;* [2]*Department of Pathology, Shandong Provincial Chest Hospital, Jinan 250012, P R China.* [*]*Equal contributors.*

Received October 8, 2014; Accepted November 28, 2014; Epub December 15, 2014; Published January 1, 2015

Abstract: DNA methylation has been considered as an important means of early diagnosis of cancer, which cooperates with histone modifications, playing a crucial role in silencing tumor suppressor genes (TSGs). However, how TSGs are regulated by these epigenetic mechanisms in cancer remains unknown. In this study, we first evaluated 7 TSGs methylation in the early diagnosis of endometrial carcinoma (EC), and then explored the epigenetic mechanisms of their transcriptional regulation. The results showed that *SOCS3* and *3OST2* were the most frequently methylated genes in EC (88.3% and 78.3%, respectively), and *3OST2* was correlated with younger patients (< 57 years, P = 0.030) and well-differentiated EC (P = 0.026). Unlike *3OST2*, *SOCS3* methylation occurred even in complex hyperplasia (53.3%) and atypical hyperplasia (54.2%). 5-aza-2'-deoxycytidine (5-Aza-CdR) or trichostatin A (TSA) alone could partially reverse *SOCS3* and *3OST2* methylation, and their combination completely reversed the methylation of both genes. In addition, UHRF1 and methylated H3R8 were enriched on both hypermethylated *SOCS3* and *3OST2* promoters, but after 5-Aza-CdR or TSA treatment, the UHRF1 and H3R8me2s enrichment was decreased while H3R8me2a enrichment was increased. In conclusion, we demonstrate for the first time that *SOCS3* and *3OST2* methylation plays an important role in endometrial carcinogenesis, and could be directly regulated by UHRF1. Moreover, H3R8me2s acts as a repressive mark, while H3R8me2a was correlated with transcriptional activity in EC.

Keywords: Endometrial carcinoma, DNA methylation, histone modifications, tumor suppressor genes, gene expression

Introduction

Endometrial carcinoma (EC) is one of the most common malignancies of the female reproductive tract worldwide. The accumulation of genetic and epigenetic alterations contributes to the transformation of normal endometrial epithelium to EC via the precursor, atypical endometrial hyperplasia. Promoter methylation, which silences the tumor suppressor genes (TSGs), has emerged as a key mechanism in the origin of many cancers [1-3]. Indeed, aberrant DNA methylation appears to be more frequent than genetic alterations in EC. A growing number of studies have demonstrated that tumors of almost any type involve methylation of a group of TSGs, but normal cells do not. Despite the methylation of multiple TSGs being reported in EC, including that of *PTEN*, *p16*, *APC*, *RASSF1A*, *GSTP1*, and *E-cadherin*, and so on [4-7], far fewer methylated TSGs are found in EC than in other tumors such as hepatocellular carcinoma, breast cancer, and the like. To identify more new methylation biomarkers of EC, the present study investigated the methylation status of 7 TSGs (*SOCS1, SOCS3, 3OST2, DLC1, hMLH1, hMSH2,* and *RARβ2*) in EC, and then explored the relationship between DNA methylation and transcriptional regulation.

Covalent modification of histone proteins is another transcription-regulating epigenetic phenomenon during disease processes. It is interestingly clear now that DNA methylation is often colocalized with histone marks in gene repression. Recently, a new protein, ubiquitinlike with PHD and ring finger domain 1 (UHRF1), also known as ICBP90/Np95, was reported to recognize both DNA methylation and histone modifications, and links them [8]. In 2008, three different groups simultaneously reported that the SRA domain of UHRF1 can recognize hemi-

Table 1. PCR primer sequences of multiple tumor suppressor genes (TSGs) for Methylation-specific PCR analysis

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Annealing temperature
SOCS1	M: TGAAGATGGTTTCGGGATTTACGA	M: ACAACTCCTACAACGACCGCACG	54°C
	U: TGAAGATGGTTTTGGGATTTATGA	U: CACAACTCCTACAACAACCACACAC	54°C
SOCS3	M: GTAGTGCGTAAGTTGTAGGAGAGC	M: GTAAAAAAATAACGCTAATCCGAA	55°C
	U: TAGTGTGTAAGTTGTAGGAGAGTGG	U: CTAAACATAAAAAAATAACACTAATCCAAA	55°C
DLC1	M: TTTAAAGATCGAAACGAGGGAGCG	M: CCCAACGAAAAAACCCGACTAACG	50°C
	U: TTTTTTAAAGATTGAAATGAGGGAGTG	U: AAACCCAACAAAAAAACCCAACTAACA	54°C
3OST2	M: CGGTTGTTCGGAGTTTTATC	M: GTAACGCTACCACGACCACG	58°C
	U: TGGAGTTTTATTGTTTAGGATT	U: AAAACTCACATAACACTACCACA	58°C
hMLH1	M: ACGTAGACGTTTTATTAGGGTCGC	M: CCTCATCGTAAC-TACCCGCG	58°C
	U: TTTGATGTAGATGTTTTATTAGGGTTGT	U: ACCACCTCATCATAACTACCCACA	60°C
hMSH2	M: TCGTGGTCGGACGTCGTTC	M: CAACGTCTCCTTCGACTACACCG	56°C
	U: GGTTGTTGTGGTTGGATGTTGTTT	U: CAACTACAACATCTCCTTCAACTACACCA	56°C
RARβ2	M: TCGAGAACGCGAGCGATTCG	M: GACCAATCCAACCGAAACGA	59°C
	U: TTGAGAATGTGAGTGATTTGA	U: AACCAATCCAACCAAAACAA	59°C

M, methylation; U, unmethylation.

methylated DNA by a base-flipping mechanism [9-11]. Subsequently, it was revealed that UHRF1 also recognizes histone H3K9 trimethylation through its tandem Tudor domain. Therefore, UHRF1 is considered one of several critical molecules linking DNA methylation and histone modifications [12].

Among the diverse covalent modifications of histone N-terminal tails, histone methylation that which can interact with DNA methylation plays an important role in tumor development [13, 14]. Histone methylation occurs on both lysine and arginine residues, and it has been identified as an important modification for both transcriptional activation and transcriptional repression. However, in contrast to lysine methylation, the function of arginine methylation in chromatin structure and transcription is unexplored so far. Arginine methylation exists in three different states: monomethylated (me1), symmetrically dimethylated (me2s), or asymmetrically dimethylated (me2a). More recently, H3R2 methylation has been shown to inhibit the binding of UHRF1 to the histone H3 tail (amino acids 1-21), while H3R8 dimethylation by and large did not significantly affect the binding [15], suggesting that H3R8 dimethylation may bind to UHRF1 and work together to regulate gene expression. In this study, to determine the epigenetic transcriptional mechanisms of hypermethylated TSGs in EC and understand the interactions between DNA methylation and histone modifications, we aimed first to explore whether UHRF1 was involved in endometrial carcinogenesis and whether it could directly regulate the expression of the hypermethylated TSGs on their promoters. Next, we investigated whether H3R8 could be monomethylated or dimethylated (me2s or me2a) in transcriptional regulation of the TSGs, and whether the three methylation states would exert similar or opposite effects. In addition, the effects of a DNA methyltransferase (DNMT) inhibitor, 5-aza-2'-deoxycytidine (5-Aza-CdR), and a histone deacetylase (HDAC) inhibitor, trichostatin A (TSA), alone or in combination on UHRF1 and H3R8 methylation as well as TSG methylation and expression were observed.

Materials and methods

Patients and samples

Paraffin-embedded tissue blocks of normal endometrium (n = 27), simple hyperplasia (n = 25), complex hyperplasia (n = 30), atypical hyperplasia (n = 24), and endometrial adenocarcinoma (n = 60) were obtained from Shandong University Qilu Hospital between 2009 and 2012. We collected the clinical parameters of each patient, namely age, tumor differentiation, depth of myometrial invasion, and lymph node metastasis. All lesions were diagnosed according to the diagnostic criteria

defined by the World Health Organization (WHO, 2008). Ethics approval was obtained from the Committee of Ethical Research at Shandong University.

Cell cines and drugs treatment

The human endometrial cancer cell line Ishikawa (the European Collection of Cell Cultures, Sigma, UK) was maintained in RPMI-1640 with 10% fetal bovine serum. The Ishikawa cells were treated with 5-Aza-CdR and TSA either alone or in combination. The cells were exposed continuously to 8 μmol/L 5-Aza-CdR for four days or to 300 ng/ml TSA for two days (Sigma, St. Louis, MO, USA). For combined treatment, the cells were initially treated with 8 μmol/L 5-Aza-CdR for two days and subsequently co-treated with 300 ng/mL TSA for an additional two days.

Methylation-specific PCR (MSP)

Genomic DNA was extracted from the paraffin blocks and the Ishikawa cells with a Genomic DNA Purification Kit (Qiagen, Hilden, Germany). The extracted DNA (1 μg) underwent bisulfite conversion using a CpGenome DNA Modification kit (Intergen Co., Purchase, NY, USA), according to the manufacturer's protocol. In brief, 2 μL of modified DNA was PCR-amplified in a total volume of 50 μL. The primer sequences and annealing temperatures are listed in **Table 1**. The PCR products were separated on 2% agarose gels and visualized with ethidium bromide staining.

Reverse transcription real-time quantitative PCR (qRT-PCR)

Total RNA was extracted from the Ishikawa cells treated or untreated with the inhibitors using TriZol (Life Technologies, Rockville, MD, USA). First-strand cDNA was synthesized from 1 μg total RNA with Primer Mix and RT Enzyme Mix (Toyobo, Japan) according to the manufacturer's instructions. The qRT-PCR was performed in a 20-μL reaction, which included 3 μL cDNA template, 5 uM each of forward and reverse primer, and 10 μL SYBR Green I (Bio-Rad, Hercules, CA, USA). The following primer pairs were used: *SOCS3* (forward, 5'-TTCTACTGGAGCGCAGTGAC-3'; reverse, 5'-ACTGGGTCTTGACGCTGAG-3'), *3OST2* (forward, 5'-CGGCGAGATGGGGCGAGTCC-3'; reverse, 5'-TCGGAGCTGGTCTATCACTT-3'), and UHRF1 (forward, 5'-CCAGCAGAGCAGCCTCATC-3'; reverse, 5'-TCCTTGAGTGACGCCAGGA-3').

Immunohistochemistry

The Ishikawa cells were seeded in a six-well plate that contained slides. The inhibitors were added after the cells were adhered. To ensure that the antibody could enter the nucleus, the slides were incubated with 0.5% TritonX-100 for 30 min. The slides were incubated with anti-UHRF1 antibody (1:500; Abcam, Cambridge, UK) at 4°C overnight, followed by incubation with secondary antibody at room temperature for 30 min and visualized using diaminobenzidine. Only distinct nuclear staining was considered as positive. The protein levels of all groups were evaluated by a staining score system based on the percentage of positive cells and staining intensity. At least 1000 cells were counted for each slide. Four semi-quantitative classes were used to describe staining intensity (intensity score: absent, 0; weak, 1; moderate, 2; strong, 3) and the percentage of positive cells was graded as follows: extent score: absent, 0; < 10%, 1; 10-50%, 2; > 50%, 3. By multiplying the intensity score with extent score, a score index was derived, ranging from 0 to 9.

Western blot analysis

Acid extraction of histones from cells treated or untreated with inhibitors was performed according to the instructions provided by Upstate Biotechnology. Cellular nuclear protein (50 μg/lane) was separated by 12% SDS-PAGE and hybridized separately with antibodies specific for UHRF1 (1:500; Active Motif), H3R8me1 (1:200; Active Motif), H3R8me2s (1:200; Novus Biologicals) and H3R8me2a (1:500; Novus Biologicals) at 4°C overnight, followed by incubation with secondary antibody for 1 h at room temperature. After extensive washes, the membranes were visualized with ECL plus™ Western Blot Detection System (Amersham Biosciences, Buckingham, UK). To determine the relative expression level of each sample, the β-actin expression level was measured as the internal control.

Chromatin immunoprecipitation (ChIP)-qPCR

In brief, proteins were cross-linked to DNA by adding 1% formaldehyde, and the lysates were

Table 2. Multiple tumor suppressor genes (TSGs) methylation frequencies in normal endometrium, simple hyperplasia, complex hyperplasia, atypical hyperplasia, and endometrial carcinoma

Gene	Normal endometrium	Simple hyperplasia	*P*	Complex hyperplasia	*P*	Atypical hyperplasia	*P*	Endometrial carcinoma	*P*
SOCS1	0%	0%	1.000	3.3%	1.000	0%	1.000	13.3%	0.054*
SOCS3	14.8%	24%	0.492	53.3%	0.005**	54.2%	0.006**	88.3%	0.000**
3OST2	11.1%	16%	0.698	33.3%	0.061	37.5%	0.160	78.3%	0.000**
DLC1	0%	0%	1.000	6.7%	0.492	4.2%	0.471	21.7%	0.008**
hMLH1	3.7%	4.0%	1.000	3.3%	1.000	8.3%	1.000	18.3%	0.094
hMSH2	0%	0%	1.000	0%	1.000	0%	1.000	5.0%	1.000
RARβ2	14.8%	28.0%	0.317	13.3%	1.000	25.0%	0.276	33.3%	0.118

p value is relative to normal endometrium (χ^2 tests). *$p < 0.05$; **$p < 0.01$.

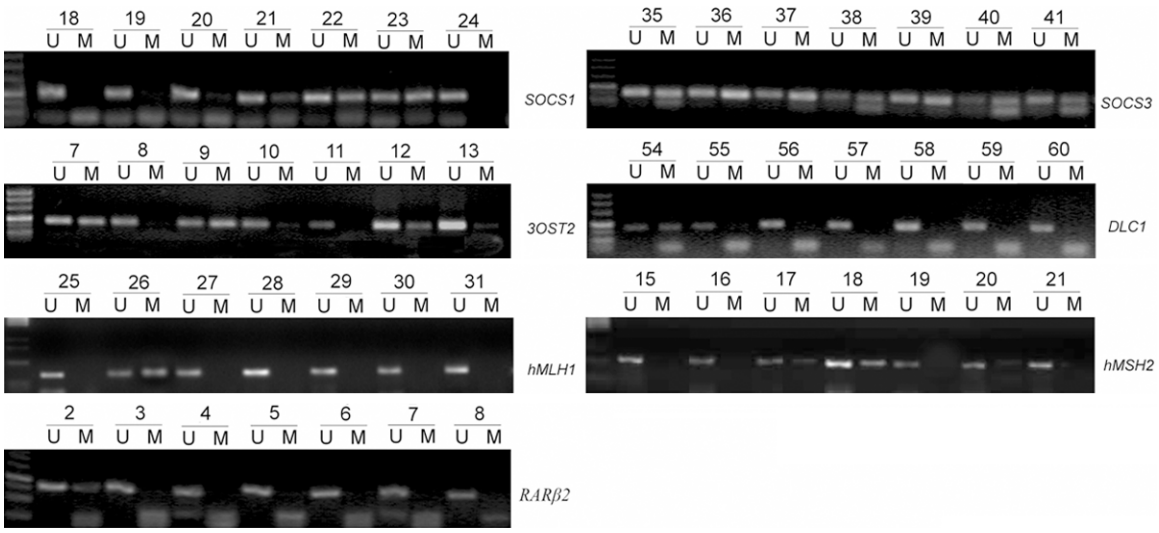

Figure 1. Methylation analysis of the promoters of 7 tumor suppressor genes (*SOCS1, SOCS3, 3OST2, DLC1, hMLH1, hMSH2,* and *RARβ2*) in endometrial carcinoma. The MSP products in the M lanes indicate the presence of methylated alleles, and those in the U lanes indicate the presence of unmethylated alleles.

sonicated to shear DNA to average lengths of approximately 200-1000bp. To reduce nonspecific background, the DNA-protein complexes were pre-cleared by incubation with 80 μL of Protein A agarose beads. Anti-symmetrical dimethyl-H3R8 antibody (5 μg; Novus Biologicals), anti-asymmetrical dimethyl-H3R8 antibody (5 μg; Novus Biologicals), or normal rabbit serum was added and incubated overnight at 4°C. The primers used for the ChIP-qPCR were: *SOCS3*: (forward, 5'-GGTCACCCACAGCAAGTTTCC-3'; reverse, 5'-TCGCGGATCAGAAAGGTGC-3') and *3OST2*: (forward, 5'-GTTCCAACCACTCCGGCTCA-3'; reverse, 5'-CGTCCGGGTGTACTCGGATAA-3').

Statistical analysis

Statistical analysis was performed using SPSS 16.0 for windows (SPSS Inc, Chicago, IL). Differences of protein expression among groups were tested by one-way analysis of variance (ANOVA). Frequencies of methylation were compared using Chi-square test and Fisher exact test. A value of $P < 0.05$ was considered statistically significant.

Results

Promoter methylation status of TSGs in a series of primary endometrial lesions

Table 2 lists the methylation rates of the 7 genes in a series of endometrial lesions. Representative examples of the MSP are illustrated in **Figure 1**. Among the TSGs studied, the most frequently methylated genes in EC were *SOCS3* (88.3%) and *3OST2* (78.3%), which were selected for further analysis. Interestingly, high frequencies of *SOCS3* methylation were

Table 3. Association of the *SOCS3* and *3OST2* gene promoter methylation with the clinicopathological data of endometrial carcinoma

Clinical Feature	*SOCS3*		*P*	*3OST2*		*P*
Age, y	M (U)	(%)	0.436	M (U)	(%)	0.030*
< 57 (35)	32 (3)	91.4		31 (4)	88.6	
≥ 57 (25)	21 (4)	84.0		16 (9)	64	
Differentiation			0.235			0.026*
G_1 (32)	30 (2)	93.8		29 (3)	90.6	
$G_{2\sim3}$ (28)	23 (5)	82.1		18 (10)	64.3	
Depth of myometrial invasion			1.000			1.000
< 1/2 (42)	37 (5)	88.1		33 (9)	78.6	
≥ 1/2 (18)	16 (2)	88.9		14 (4)	77.8	
Lymph node metastasis			N/A			0.668
Yes (9)	9 (0)	100		8 (1)	88.9	
No (51)	44 (7)	86.3		39 (12)	76.4	

Fisher's exact test was conducted. M, methylation; U, unmethylation; G_1, Well differentiation; $G_{2\sim3}$, moderate differentiation and poor differentiation; N/A, not available. *$p < 0.05$.

also found in complex hyperplasia (53.3%) and atypical hyperplasia (54.2%) as compared with normal endometrium (14.8%) and simple hyperplasia (24%), and there was no difference between the two groups. Significant differences of *SOCS3* methylation between complex hyperplasia/atypical hyperplasia and EC were also found (P < 0.001 and P = 0.002, respectively). Unlike *SOCS3*, *3OST2* was frequently methylated in only the EC group (78.3%) but much less so in the other groups (from 11.1% to 37.5%).

The association between gene methylation and clinicopathological parameters such as age, tumor differentiation, depth of myometrial invasion, and lymph node metastasis was also analyzed (**Table 3**). *3OST2* methylation was correlated with younger patients (< 57 years, P = 0.030) and well-differentiated EC (P = 0.026), while no correlation between *SOCS3* methylation and any clinicopathological parameters was found.

SOCS3 and 3OST2 promoter methylation and mRNA expression in EC cells before and after treatment with 5-Aza-CdR and/or TSA

To demonstrate whether the methylation of the TSGs could directly control their transcription, we investigated the changes in *SOCS3* and *3OST2* promoter methylation status and mRNA expression in the Ishikawa cells after treatment with 5-Aza-CdR with MSP and qRT-PCR, respectively. To further elucidate the relationship between DNA methylation and histone modifications, we also treated the Ishikawa cells with TSA alone or initially treated cells with 5-Aza-CdR and then in combination with TSA.

The MSP showed that both *SOCS3* and *3OST2* promoters were completely methylated in Ishikawa cells and could be partially reversed by 5-Aza-CdR. Interestingly, TSA alone also could alter the methylation status of the two genes. Moreover, the promoter methylation of both genes was completely reversed by the two inhibitors in combination (**Figure 2A**, **2B**). The qRT-PCR revealed significant elevated mRNA expression of both genes after treatment with TSA (**Figure 2C**, **2D**). Although the fold increase was relatively small (< 2 fold) by 5-Aza-CdR, it was a statistically significant change. Combining 5-Aza-CdR with TSA synergistically increased *SOCS3* and *3OST2* mRNA expression in the Ishikawa cells.

UHRF1 expression changes in EC cells after treatment with 5-Aza-CdR and/or TSA

UHRF1, which possesses several domains that can read both DNA methylation and histone methylation, thus, physically linking these two epigenetic marks, is overexpressed in numerous cancers. However, its role in EC has not been investigated. We first performed immunohistochemistry to detect the protein expression of UHRF1 in the Ishikawa cells. The results showed that UHRF1 was highly expressed in the cells (**Figure 3**) and moderately downregulated by 5-Aza-CdR alone (P = 0.029). TSA exhibited more efficient than 5-Aza-CdR (P = 0.001). After treatment with 5-Aza-CdR combined with TSA, the UHRF1 expression was significantly decreased (P < 0.001).

Next, to quantitatively measure the levels of UHRF1 expression changes after treatment with 5-Aza-CdR and/or TSA, we performed qRT-PCR and western blot assays. The results demonstrated that the mRNA and protein levels of UHRF1 in the Ishikawa cells were very high

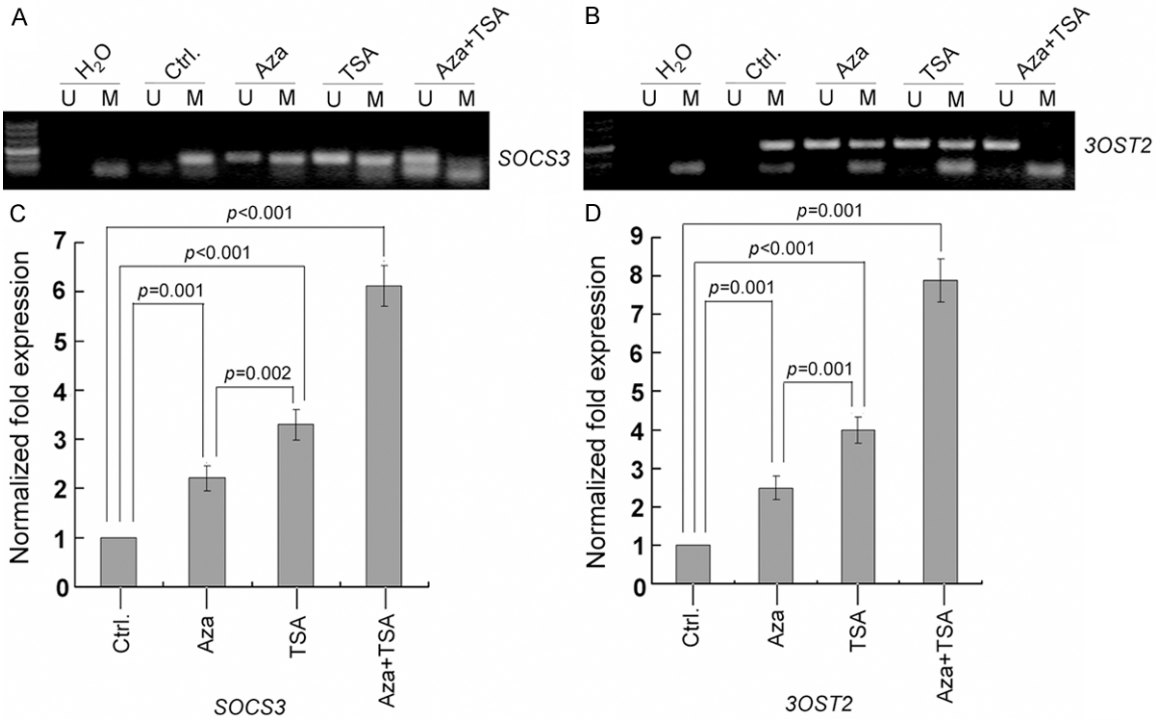

Figure 2. A, B. MSP analysis of the methylation status of *SOCS3* and *3OST2* promoters in Ishikawa cells before and after 5-Aza-CdR and/or TSA treatment. C, D. Real-time RT-PCR analysis of *SOCS3* and *3OST2* mRNA expression in Ishikawa cells before and after 5-Aza-CdR and/or TSA treatment. Expression of the two genes was normalized using β-actin as an internal control.

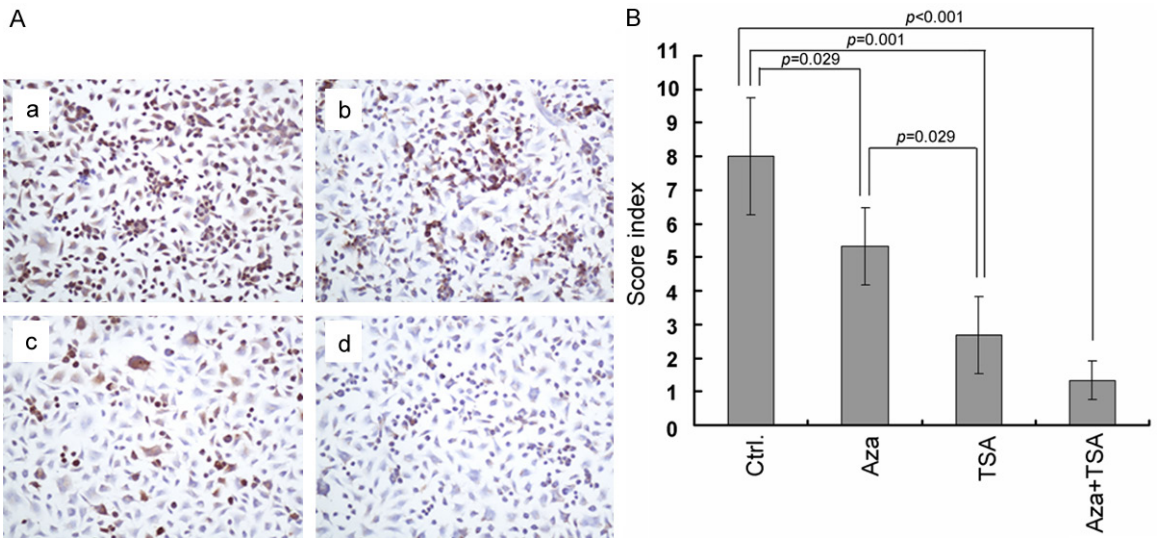

Figure 3. A, B. Immunohistochemical analysis of UHRF1 protein expression in Ishikawa cells. (a) Untreated Ishikawa cells. (b) After 5-Aza-CdR treatment (c) After TSA treatment (d) After 5-Aza-CdR and TSA combined treatment. Score index = intensity score × extent score.

(**Figure 4A-C**), and were sharply reduced by treatment with 5-Aza-CdR or TSA ($P < 0.001$). The combination treatment was more effective than either inhibitor alone, which was consistent with the result of immunohistochemistry.

Functions of three methylation states of histone H3R8 in EC cells

Multiple arginine residues in histone tails, including R2, R8, R17 and R26 in H3 and R3 in

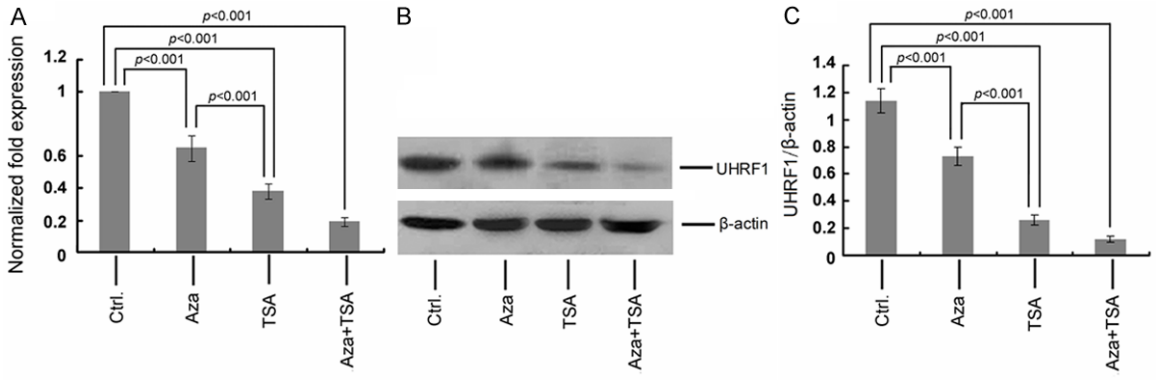

Figure 4. UHRF1 protein and mRNA expression in Ishikawa cells before and after 5-Aza-CdR and/or TSA treatment. (A) Real-time RT-PCR analysis of UHRF1 mRNA expression, which was normalized using β-actin as an internal control. (B) Western blot analysis of UHRF1 protein expression. (C) The ratio of UHRF1 to β-actin is shown on the y-axis.

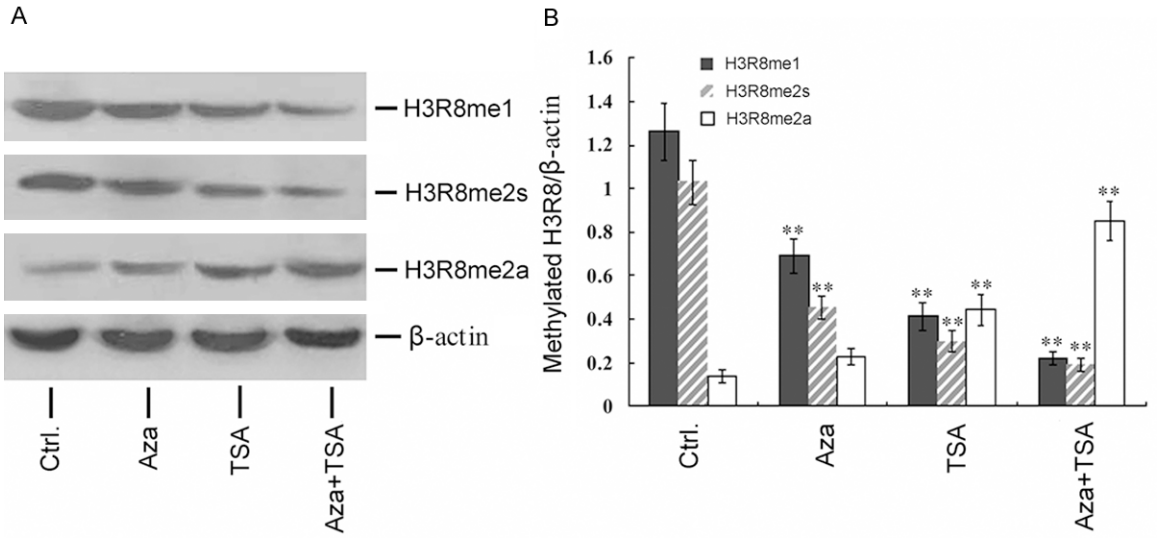

Figure 5. A. Western blot analysis of three methylation states of H3R8 (H3R8me1, H3R8me2s and H3R8me2a) in Ishikawa cells before and after 5-Aza-CdR and/or TSA treatment. B. The ratio of UHRF1 to β-actin is shown on the y-axis. *P < 0.05 versus controls; **P < 0.01 versus controls.

H4 have been shown to be monomethylated, or symmetrically or asymmetrically dimethylated [16, 17]. In order to understand whether H3R8 could be methylated in Ishikawa cells, we first performed western blot analysis in the whole-cell extract treated or untreated with the inhibitors. The results showed that H3R8me1 and H3R8me2s were present prominently in the untreated cells and were decreased after treatment with 5-Aza-CdR or TSA (**Figure 5**). On the contrary, the levels of H3R8me2a in the untreated cells were very low and significantly increased only after TSA treatment. There was slight elevation after 5-Aza-CdR treatment, but no statistical significance was found. When combined with the two inhibitors, H3R8me1 and H3R8me2s levels were significantly reduced; concurrently, H3R8me2a levels were significantly increased.

ChIP-qPCR assays

To investigate whether UHRF1 and H3R8 methylation were directly involved in the transcriptional regulation of the hypermethylated TSGs, and whether they could be altered by 5-Aza-CdR and/or TSA treatment, we further conducted ChIP assays, followed by ChIP-qPCR. We determined that UHRF1 was recruited to both the *SOCS3* and *3OST2* promoters in the Ishikawa cells, and the recruitments were decreased moderately by the 5-Aza-CdR treatment, while the TSA treatment decreased

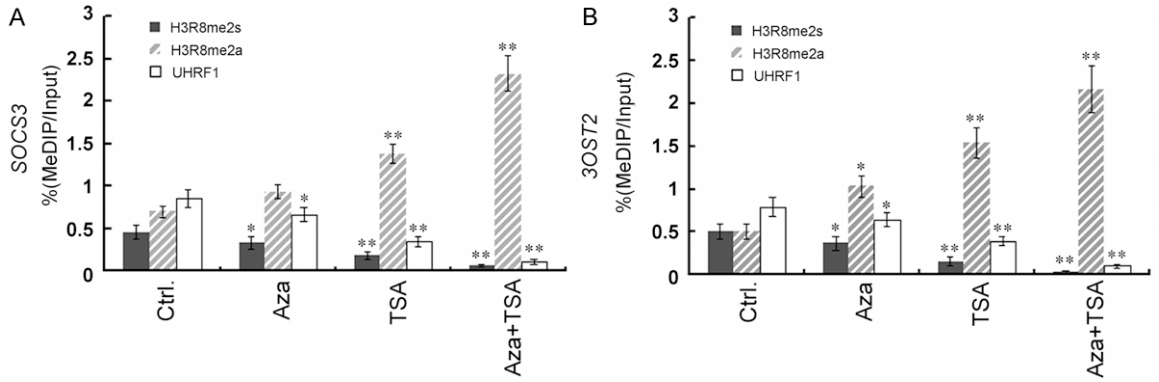

Figure 6. ChIP-qPCR assays for UHRF1, H3R8me2s and H3R8me2a on (A) *SOCS3* and (B) *3OST2* promoters. Cross-linked chromatin from Ishikawa cells treated or untreated with 5-Aza-CdR and/or TSA underwent immunoprecipitation using control IgG, anti-UHRF1, anti-H3R8me2s or anti-H3R8me2a antibody, and the immunoprecipitated DNA was analyzed by qPCR using primers specific for *3OST2* and *SOCS3*. Shown is the enrichment, relative to input obtained in each ChIP sample. $^{*}P < 0.05$ versus controls; $^{**}P < 0.01$ versus controls.

recruitments significantly (**Figure 6**), consistent with the results of the qRT-PCR and western blot, indicating that *SOCS3* and *3OST2* expression is directly regulated by UHRF1. Furthermore, the ChIP-qPCR assays also showed that there was enrichment of both H3R8me2s and H3R8me2a binding to the *SOCS3* and *3OST2* promoters, but the H3R8me2s enrichment was significantly decreased after exposure to TSA, while that of H3R8me2a was increased accordingly. There was only slight or moderate change of the two marks after 5-Aza-CdR treatment. The 5-Aza-CdR and TSA combination had synergistic effects on the reduction of UHRF1 or H3R8me2s recruitment to the promoters as well as the binding of H3R8me2a to the promoters. As no ChIP-grade H3R8me1 antibody was available, the role of H3R8me1 in regulation of the *SOCS3* and *3OST2* genes is yet to be studied.

Discussion

EC is subcategorized into type I and II according to pathological characteristics. The molecular mechanisms involved in type I EC appear to be distinct from type II [18]. Type I EC frequently demonstrates microsatellite instability, and genetic (e.g., mutations, gene amplification/deletion) or epigenetic alterations (primarily promoter DNA methylation). By contrast, type II EC exhibits less promoter hypermethylation and more frequent DNA mutations [19, 20]. Based on this literature, 60 type I EC samples were included in this study. The results provided the specific TSG methylation profile for EC and demonstrated that *SOCS3* and *3OST2* are the most frequently methylated genes in EC but the frequency is very low in normal endometrial samples.

Suppressor of cytokine signaling (*SOCS*) proteins are inhibitors of cytokine signaling that function via the JAK/STAT pathway [21]. Among the eight *SOCS* family members, *SOCS1* and *3* are best characterized for their regulation and functions, which are downregulated in several human cancers. Cooper et al. has reported that in leukemia cells, the *SOCS1* gene is silenced by DNA hypermethylation, while the loss of *SOCS3* expression occurs through a mechanism independent of epigenetic silencing by DNA methylation [22]. However, in our study, *SOCS3* but not *SOCS1* hypermethylation played an important role in endometrial carcinogenesis. The methylation rate of *SOCS3* increased as the lesion grade increased, and although there was a statistically significant difference between the EC group and the other groups, frequent methylation of the *SOCS3* promoter were also demonstrated in the complex hyperplasia and atypical hyperplasia samples (53.3% and 54.2%, respectively). This suggested that the DNA methylation process starts in this gene before it expands to involve EC, which is consistent with the findings of Nieminen et al., who pointed out that, contrary to the traditional view, complex hyperplasia both without and with atypia are equally important as precursor lesions of EC [23]. The *3OST2* gene, which encodes an O-sulfotransferase responsible for the final modification step of heparin sulfate

proteoglycans (HSPGs) that are important in the field of migration, cell growth and adhesion [24], also undergoes frequent promoter methylation in EC (78.3%). However, different from *SOCS3*, *3OST2* was frequently methylated in only the EC group but much less so in the other groups, including the complex hyperplasia group and atypical hyperplasia group.

Taken together, our data indicate that *SOCS3* methylation is a very early event in EC development, even during the precancerous stage, just as we and others have demonstrated the presence of some TSGs methylation in morphologically normal cells adjacent to cancer tissues [25-27]. During endometrial carcinogenesis, certain TSGs such as *SOCS3* are methylated at the early stage, while others such as *3OST2* may be methylated at the later stage, a reflection that each TSG may function differently and that the accumulation of these TSG silencing will promote the multistep process of cancer. As for the early diagnostic value of the studied TSGs in differentiating EC from atypical hyperplasia, we consider *3OST2* methylation better than *SOCS3* methylation, which occurred even in complex hyperplasia. Although there was a significant difference in *DLC1* methylation between the EC group and the other groups, its low methylation frequency limits its diagnostic value in clinics. In addition, the present study assessed the association between *SOCS3* or *3OST2* methylation and clinical characteristics. *3OST2* methylation was found to be associated with well-differentiated EC and younger patients. It should be noted that as well-differentiated EC often occurs in younger patients, the correlation between *3OST2* methylation and younger patients may be an accompanied phenomenon.

Unlike genetic alterations, epigenetic changes are reversible. It was generally believed that the expression of methylated genes can be restored only by DNMT inhibitors, and that HDAC inhibitors only activate the transcription of unmethylated genes [28-30]. In the present study, we used an endometrial cell line (another cell line, a hepatocellular carcinoma cell line was also used, data not shown) to observe the effects of these inhibitors. However, our data show that either 5-Aza-CdR or TSA alone was able to partially reverse the TSG promoter methylation, and after treatment with 5-Aza-CdR in combination with TSA, both *SOCS3* and *3OST2* promoter methylation were completely reversed, and their expression was synergistically increased, demonstrating the cross-talk between DNA methylation and histone modifications. Recently, Arzenani et al. reported that TSA treatment could reduce global DNA methylation and the DNMT1 protein level and could alter DNMT1 nuclear dynamics and interactions with chromatin [31]; this may explain why TSA could reverse the TSG methylations.

A growing body of evidence has revealed that UHRF1, characterized by reading both DNA methylation and histone modifications, interacts with many histone modulators, including HDAC1, G9a, DNMT3a, DNMT3b, and Tip60 [32-35], and plays a fundamental role in DNA methylation, histone methylation, histone acetylation, cell proliferation, and apoptosis [36-39]. Our results showed that UHRF1 was highly expressed in EC cells, and was downregulated by 5-Aza-CdR or TSA alone. UHRF1 binds to the methylated promoter regions of various TSGs, including *p16INK4A* and *p14ARF*, via its SRA domain [35], which is found only in the UHRF family [40]. The present study showed that UHRF1 could bind to both *SOCS3* and *3OST2* promoters to directly repress their expression. In addition, H3R8 methylation was involved in the suppression of *SOCS3* and *3OST2* genes in EC. Pal et al. reported that H3R8me2s correlated with the repression of the suppressor ST7 in lymphoid cancer cells [41]. However, our results revealed that H3R8me2s was a repressive mark, while H3R8me2a was linked to TSG activation, opposite to the function of H3R8me2s. Although the western blotting results showed that H3R8me1 was present in EC and was inhibited by 5-Aza-CdR and/or TSA, its regulation role of *SOCS3* and *3OST2* remains unexplored. In our data, all three methylation states of H3R8 could be changed by 5-Aza-CdR and/or TSA, suggesting that the two inhibitors may affect not only DNMT1 but also protein arginine methyltransferases (PRMTs), although which PRMT is involved needs to be further studied.

In conclusion, we have identified two new methylation biomarkers to warn of EC, and gain valuable insight into not only the role of epigenetic changes in regulation of the methylated TSGs but also the mechanisms of interaction between DNA methylation and histone modifications. We also describe for the first time that H3R8me2s acts as a repressive mark, while

H3R8me2a was correlated with transcriptional activity in EC.

Acknowledgements

This work is supported by the National Natural Science Foundation of China (No. 81272277, 81372810), the Independent Innovation Foundation of Shandong University (No. 2012JC028), and the Science and Technology Program of Shandong, China (No. 2011GSF11802).

Disclosure of conflict of interest

None.

Address correspondence to: Tingguo Zhang or Cuijuan Zhang, Institute of Pathology and Pathophysiology, Shandong University School of Medicine, Jinan 250012, P. R. China. Tel: 86-531-88382574; Fax: 86-531-88383168; E-mail: ztguo@sdu.edu.cn (TGZ); E-mail: cuijuanzhang@sdu.edu.cn (CJZ)

References

[1] Zhang MY, Fung TK, Chen FY and Chim CS. Methylation profiling of SOCS1, SOCS2, SOCS3, CISH and SHP1 in Philadelphia-negative myeloproliferative neoplasm. J Cell Mol Med 2013; 17: 1282-1290.

[2] Nishida N, Kudo M, Nagasaka T, Ikai I and Goel A. Characteristic patterns of altered DNA methylation predict emergence of human hepatocellular carcinoma. Hepatology 2012; 56: 994-1003.

[3] Ling Q, Shi W, Huang C, Zheng J, Cheng Q, Yu K, Chen S, Zhang H, Li N and Chen M. Epigenetic silencing of dual oxidase 1 by promoter hypermethylation in human hepatocellular carcinoma. Am J Cancer Res 2014; 4: 508-517.

[4] Kang S, Kim JW, Kang GH, Lee S, Park NH, Song YS, Park SY, Kang SB and Lee HP. Comparison of DNA hypermethylation patterns in different types of uterine cancer: cervical squamous cell carcinoma, cervical adenocarcinoma and endometrial adenocarcinoma. Int J Cancer 2006; 118: 2168-2171.

[5] O'Hara AJ and Bell DW. The genomics and genetics of endometrial cancer. Adv Genomics Genet 2012; 2012: 33-47.

[6] Liao X, Siu MK, Chan KY, Wong ES, Ngan HY, Chan QK, Li AS, Khoo US and Cheung AN. Hypermethylation of RAS effector related genes and DNA methyltransferase 1 expression in endometrial carcinogenesis. Int J Cancer 2008; 123: 296-302.

[7] Joensuu EI, Abdel-Rahman WM, Ollikainen M, Ruosaari S, Knuutila S and Peltomaki P. Epigenetic signatures of familial cancer are characteristic of tumor type and family category. Cancer Res 2008; 68: 4597-4605.

[8] Unoki M. Current and potential anticancer drugs targeting members of the UHRF1 complex including epigenetic modifiers. Recent Pat Anticancer Drug Discov 2011; 6: 116-130.

[9] Arita K, Ariyoshi M, Tochio H, Nakamura Y and Shirakawa M. Recognition of hemi-methylated DNA by the SRA protein UHRF1 by a base-flipping mechanism. Nature 2008; 455: 818-821.

[10] Hashimoto H, Horton JR, Zhang X, Bostick M, Jacobsen SE and Cheng X. The SRA domain of UHRF1 flips 5-methylcytosine out of the DNA helix. Nature 2008; 455: 826-829.

[11] Avvakumov GV, Walker JR, Xue S, Li Y, Duan S, Bronner C, Arrowsmith CH and Dhe-Paganon S. Structural basis for recognition of hemi-methylated DNA by the SRA domain of human UHRF1. Nature 2008; 455: 822-825.

[12] Karagianni P, Amazit L, Qin J and Wong J. ICBP90, a novel methyl K9 H3 binding protein linking protein ubiquitination with heterochromatin formation. Mol Cell Biol 2008; 28: 705-717.

[13] Jorgensen S, Schotta G and Sorensen CS. Histone H4 lysine 20 methylation: key player in epigenetic regulation of genomic integrity. Nucleic Acids Res 2013; 41: 2797-2806.

[14] Amente S, Lania L and Majello B. Epigenetic reprogramming of Myc target genes. Am J Cancer Res 2011; 1: 413-418.

[15] Rajakumara E, Wang Z, Ma H, Hu L, Chen H, Lin Y, Guo R, Wu F, Li H, Lan F, Shi YG, Xu Y, Patel DJ and Shi Y. PHD finger recognition of unmodified histone H3R2 links UHRF1 to regulation of euchromatic gene expression. Mol Cell 2011; 43: 275-284.

[16] Wysocka J, Allis CD and Coonrod S. Histone arginine methylation and its dynamic regulation. Front Biosci 2006; 11: 344-355.

[17] Bedford MT and Richard S. Arginine methylation an emerging regulator of protein function. Mol Cell 2005; 18: 263-272.

[18] Balch C, Matei DE, Huang TH and Nephew KP. Role of epigenomics in ovarian and endometrial cancers. Epigenomics 2010; 2: 419-447.

[19] Hecht JL and Mutter GL. Molecular and pathologic aspects of endometrial carcinogenesis. J Clin Oncol 2006; 24: 4783-4791.

[20] Zhou XC, Dowdy SC, Podratz KC and Jiang SW. Epigenetic considerations for endometrial cancer prevention, diagnosis and treatment. Gynecol Oncol 2007; 107: 143-153.

[21] Lee TL, Yeh J, Van Waes C and Chen Z. Epigenetic modification of SOCS-1 differentially regulates STAT3 activation in response to interleukin-6 receptor and epidermal growth factor receptor signaling through JAK and/or MEK in head and neck squamous cell carcinomas. Mol Cancer Ther 2006; 5: 8-19.

[22] Cooper JC, Shi M, Chueh FY, Venkitachalam S and Yu CL. Enforced SOCS1 and SOCS3 expression attenuates Lck-mediated cellular transformation. Int J Oncol 2010; 36: 1201-1208.
[23] Nieminen TT, Gylling A, Abdel-Rahman WM, Nuorva K, Aarnio M, Renkonen-Sinisalo L, Jarvinen HJ, Mecklin JP, Butzow R and Peltomaki P. Molecular analysis of endometrial tumorigenesis: importance of complex hyperplasia regardless of atypia. Clin Cancer Res 2009; 15: 5772-5783.
[24] Liu J, Shworak NW, Sinay P, Schwartz JJ, Zhang L, Fritze LM and Rosenberg RD. Expression of heparan sulfate D-glucosaminyl 3-O-sulfotransferase isoforms reveals novel substrate specificities. J Biol Chem 1999; 274: 5185-5192.
[25] Zhang X, Li HM, Liu Z, Zhou G, Zhang Q, Zhang T, Zhang J and Zhang C. Loss of heterozygosity and methylation of multiple tumor suppressor genes on chromosome 3 in hepatocellular carcinoma. J Gastroenterol 2013; 48: 132-143.
[26] Guo M, House MG, Hooker C, Han Y, Heath E, Gabrielson E, Yang SC, Baylin SB, Herman JG and Brock MV. Promoter hypermethylation of resected bronchial margins: a field defect of changes? Clin Cancer Res 2004; 10: 5131-5136.
[27] Svrcek M, Buhard O, Colas C, Coulet F, Dumont S, Massaoudi I, Lamri A, Hamelin R, Cosnes J, Oliveira C, Seruca R, Gaub MP, Legrain M, Collura A, Lascols O, Tiret E, Flejou JF and Duval A. Methylation tolerance due to an O6-methylguanine DNA methyltransferase (MGMT) field defect in the colonic mucosa: an initiating step in the development of mismatch repair-deficient colorectal cancers. Gut 2010; 59: 1516-1526.
[28] Jiemjit A, Fandy TE, Carraway H, Bailey KA, Baylin S, Herman JG and Gore SD. p21(WAF1/CIP1) induction by 5-azacytosine nucleosides requires DNA damage. Oncogene 2008; 27: 3615-3623.
[29] Bhakat KK and Mitra S. CpG methylation-dependent repression of the human O6-methylguanine-DNA methyltransferase gene linked to chromatin structure alteration. Carcinogenesis 2003; 24: 1337-1345.
[30] Xu L and Jain RK. Down-regulation of placenta growth factor by promoter hypermethylation in human lung and colon carcinoma. Mol Cancer Res 2007; 5: 873-880.
[31] Arzenani MK, Zade AE, Ming Y, Vijverberg SJ, Zhang Z, Khan Z, Sadique S, Kallenbach L, Hu L, Vukojevic V and Ekstrom TJ. Genomic DNA hypomethylation by histone deacetylase inhibition implicates DNMT1 nuclear dynamics. Mol Cell Biol 2011; 31: 4119-4128.
[32] Kim JK, Esteve PO, Jacobsen SE and Pradhan S. UHRF1 binds G9a and participates in p21 transcriptional regulation in mammalian cells. Nucleic Acids Res 2009; 37: 493-505.
[33] Meilinger D, Fellinger K, Bultmann S, Rothbauer U, Bonapace IM, Klinkert WE, Spada F and Leonhardt H. Np95 interacts with de novo DNA methyltransferases, Dnmt3a and Dnmt3b, and mediates epigenetic silencing of the viral CMV promoter in embryonic stem cells. EMBO Rep 2009; 10: 1259-1264.
[34] Achour M, Fuhrmann G, Alhosin M, Ronde P, Chataigneau T, Mousli M, Schini-Kerth VB and Bronner C. UHRF1 recruits the histone acetyltransferase Tip60 and controls its expression and activity. Biochem Biophys Res Commun 2009; 390: 523-528.
[35] Unoki M, Nishidate T and Nakamura Y. ICBP90, an E2F-1 target, recruits HDAC1 and binds to methyl-CpG through its SRA domain. Oncogene 2004; 23: 7601-7610.
[36] Cheng J, Yang Y, Fang J, Xiao J, Zhu T, Chen F, Wang P, Li Z, Yang H and Xu Y. Structural insight into coordinated recognition of trimethylated histone H3 lysine 9 (H3K9me3) by the plant homeodomain (PHD) and tandem tudor domain (TTD) of UHRF1 (ubiquitin-like, containing PHD and RING finger domains, 1) protein. J Biol Chem 2013; 288: 1329-1339.
[37] Kofunato Y, Kumamoto K, Saitou K, Hayase S, Okayama H, Miyamoto K, Sato Y, Katakura K, Nakamura I, Ohki S, Koyama Y, Unoki M and Takenoshita S. UHRF1 expression is upregulated and associated with cellular proliferation in colorectal cancer. Oncol Rep 2012; 28: 1997-2002.
[38] Arita K, Isogai S, Oda T, Unoki M, Sugita K, Sekiyama N, Kuwata K, Hamamoto R, Tochio H, Sato M, Ariyoshi M and Shirakawa M. Recognition of modification status on a histone H3 tail by linked histone reader modules of the epigenetic regulator UHRF1. Proc Natl Acad Sci U S A 2012; 109: 12950-12955.
[39] Jin W, Liu Y, Xu SG, Yin WJ, Li JJ, Yang JM and Shao ZM. UHRF1 inhibits MDR1 gene transcription and sensitizes breast cancer cells to anticancer drugs. Breast Cancer Res Treat 2010; 124: 39-48.
[40] Bronner C, Achour M, Arima Y, Chataigneau T, Saya H and Schini-Kerth VB. The UHRF family: oncogenes that are drugable targets for cancer therapy in the near future? Pharmacol Ther 2007; 115: 419-434.
[41] Pal S, Vishwanath SN, Erdjument-Bromage H, Tempst P and Sif S. Human SWI/SNF-associated PRMT5 methylates histone H3 arginine 8 and negatively regulates expression of ST7 and NM23 tumor suppressor genes. Mol Cell Biol 2004; 24: 9630-9645.