SUV39H1-mediated DNMT3A is participated in the epigenetic regulation of Tim-3 and galectin-9 in cervical cancer

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Abstract

Background: Methylation of histone 3 at lysine 9 (H3K9) and DNA methylation are among the most highly conserved epigenetic marks that correlate well with gene silencing. The tumor microenvironment significantly influences therapeutic responses and clinical outcomes. The epigenetic-regulation mechanism of the costimulatory factors Tim-3 and galectin-9 in cervical cancer remains unknown.

Methods: The methylation status of HAVCR2 and LGALS9 was detected by MS-PCR in cervical cancer tissues and cell lines. The underlying molecular mechanisms of SUV39H1-DNMT3A-Tim-3/galectin-9 regulation was elucidated using cervical cancer cell lines containing siRNA or/and over-expression system. Confirmation of the regulation of DNMT3A by SUV39H1 used ChIP-qPCR.

Results: Here, we show that SUV39H1 up-regulates H3K9me3 expression in DNMT3A promoter region, which in turn induced expression of DNMT3A. In addition, our mechanistic studies indicate that DNMT3A mediates the epigenetic modulation of the HAVCR2 and LGALS9 genes by directly binding to their promoter regions in vitro. Moreover, in an in vivo assay, the expression profile of SUV39H1 up-regulates the level of H3K9me3 in the DNMT3A promoter region was found to correlate with Tim-3 and galectin-9 expression at the cellular level indicating that SUV39H1-H3K9me3-DNMT3A is a crucial regulatory axis in cervical cancer.

Conclusion: These results indicate that SUV39H1-DNMT3A is a crucial Tim-3 and galectin-9 regulatory axis in cervical cancer.

Background

Cervical cancer is the fourth common female malignancy worldwide [1]. In 2018, there was an estimated 569,847 new cases of cervical cancer and 311,365 deaths occurred worldwide [2]. High-risk subtypes of the human papillomavirus (HR-HPV) cause almost all cervix cancers [3]. Persistent HPV infection caused chronic microenvironment changes of cervix. During carcinogenesis, immune response is blocked such as cytotoxic tumor infiltrating lymphocytes can be eluded by over-adapted cancer cells in a scenario of immune-tolerance driven by T-regulatory cells [4]. T-cell immunoglobulin and mucin-domain containing-3 (Tim-3) is to negatively regulate Th1 immunity, once Tim-3 binds to its ligand galectin-9, Tim-3 inhibits Th1 and Th17 responses by hampering their expansion, its mediating immune exhaustion in tumor microenvironment [5-7]. Tim-3-expressing CD4+T cells in human cervical cancer could represent the functional regulatory T cells which contribute to the formation of the immune-suppressive tumor micromilieu [8].

The epigenetic regulation of genes, is critical for gene's transcription [9]. DNA methylation is often associated with gene expression changes that occur in cervical cancer [10]. Our previous study revealed that EZH2, H3K27me3 and DNMT3A mediate the epigenetic regulation of the negative stimulatory molecules, Tim-3 and galectin-9 in cervical cancer which is associated with HPV18 infection [11].
Trimethylation of histone 3 lysine 9 (H3K9me3) at gene promoters is a major epigenetic mechanism that silences gene expression [12, 13] and SUV39H1 is H3K9me3-specific histone methyltransferase [14].

In this study, we identified a critical role of histone and DNA methylation marks in regulating costimulatory factors Tim-3 and galectin-9 expression in cervical cancer. The underlying mechanism is mediated by the repression of Tim-3 and galectin-9 through recruitment of DNMT3A to their promoters. SUV39H1 targeted DNMT3A by increasing the level of H3K9me3 in DNMT3A promoter region so that regulated Tim-3 and galectin-9 by DNA methylation in cervical cancer. These results represent a significant step forward in understanding the contribution of SUV39H1 and DNMT3A to cancer progression and in providing a potential target for epigenetic-based cervical cancer therapy.

Materials And Methods

Patients and samples

24 cervical cancer tissues, accordingly matched peri-carcinomatous tissues and 16 normal cervical tissues were obtained from the First Affiliated Hospital of Xi’an Jiaotong University between January 2014 and December 2017. All patients were diagnosed by two senior pathologists and none had received chemotherapy or radiotherapy prior to surgery. The cervical cancer samples were collected as previously described [15]. After the tissues were dissected, each sample was washed with sterilized PBS and stored at -80°C. All procedures were performed on ice.

Data mining

Oncomine database (www.oncomine.org) was used to detect the HAVCR2 and LGALS9 mRNA expression levels in cervical cancer and normal cervix tissues. The correlation among HAVCR2 and LGALS9 expression were studied by the data obtained from the GEPIA (http://gepia.cancer-pku.cn/).

Cell lines and culture conditions

The cervical cancer cell lines SiHa, HeLa and C33A were obtained from Cell Bank, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai. All these cells were cultured in the high glucose Dulbecco's Modified Eagle's Medium (DMEM) (HyClone, USA) supplemented with 10% fetal bovine serum (FBS) (Biological Industries, Israel) at 37°C in an atmosphere of 5% CO₂.

Lentivirus vectors and stable expression cell lines construction
Lentiviral vector preparation of Plenti-CMV-puro-Dest vector containing SUV39H1 fragment. The SUV39H1 fragment was cloned from the genomic DNA of the cell line SiHa. DNA fragment treated with Kpn1 and Xho1 and then the target gene was linked to entry vector pENTR-MCS. Two-plasmid of Plenti-CMV-puro-Dest and pENTR-MCS recombination reactions were performed using LR Clonase II (Invitrogen, USA). Use Lip2000 (Invitrogen, USA) transfection plasmid into SiHa and HeLa cell lines. The transduced cells were then selected with puromycin. Stably transduced cells were maintained in culture in the presence of puromycin. The cell lines were named SiHa-SUV39H1, HeLa-SUV39H1, successively. The expression of SUV39H1 was determined by western blotting.

RNA interference

SiHa and HeLa were transfected with scramble and SUV39H1 and DNMT3A specific siRNA (GenePharma, Shanghai), the following siRNA oligos for SUV39H1 and DNMT3A are listed in table 1. The siRNAs respectively using X-tremeGENE siRNA Transfection Reagent (Roche) and analyzed for SUV39H1 and DNMT3A expression levels by western blotting. All cell lines were named SUV39H1-siRNA and DNMT3A-siRNA, successively.

5-Aza-2'-deoxycytidine Treatment

1.0×10^5/ well SiHa, HeLa and C33A cells were cultured in 6-well plates in DMEM with 10% FBS, and after 24 hours, the medium was replaced with fresh medium containing 0 µM, 2.5 µM or 5 µM 5-Aza-2'-deoxycytidine (Sigma, USA). The medium containing 5-Aza-CdR was replaced every 24 hours during a 72-hour period [15].

DNA extraction, bisulfite modification and methylation-specific PCR (MS-PCR)

Genomic DNA was isolated from cells and tissues using a TaKaRa Mini BEST Universal Genomic DNA Extraction Kit (TaKaRa, China) according to the manufacturer’s instructions. DNA modification was done as previous [15], in briefly, 500 ng of genomic DNA was bisulfite-modified by a EZ DNA Methylation-Gold™ Kit (Zymo Research, USA). The primers used in the MS-PCR are listed in table 1. The annealing temperature for the methylated primers of HAVCR2 and LGALS9 were 60°C and 60°C while that for the unmethylated primers were 55°C and 56.3°C. The MS-PCR products were separated on a 2% agarose gel, stained with Gelview and visualized under ultraviolet illumination (Bio-Rad, USA). Methylation level was calculated by the ratio of methylated and unmethylated levels. Grey value of each band represented its relative expression and was measured by Image J Software. Each reaction was performed in triplicate.
Western blotting analysis

Cells were harvested in RIPA Lysis Buffer which containing 1mM PIC and 1mM PMSF. Proteins were resolved by SDS-PAGE and electroblotted onto PVDF membrane (Millipore, Billerica, USA) was blocked 1 hour at room temperature in 5% skim milk and incubated with primary antibodies for overnight at 4°C followed by HRP conjugated secondary antibodies for 1 h at room temperature. Chemiluminescence signal was detected following incubation with enhanced chemiluminescence reagent (Millipore, Billerica, Mass). Grey value of each band was measured with Image J Software. The antibodies are listed in table 2.

Chromatin immunoprecipitation (ChIP) assay and ChIP-qPCR

ChIP assays were carried out using the Simple ChIP Enzymatic Chromatin IP Kit (Cell Signaling Technology, USA) according to the manufacturer’s instructions. The DNMT3A, HAVCR2 and LGALS9 promoters were detected by qPCR using promoter DNA-specific primers are listed in table 1. The qPCR with the former methods [16]. We used the cycle threshold (CT) as the representative point. The relative expression of genes in each group (fold-change compared with control) was calculated using the formula: RQ = 2^{-\Delta\Delta Ct}. Each reaction was performed in triplicate. The antibodies are listed in table 2.

Immunofluorescence staining

SiHa and HeLa cells were incubated with the primary antibody overnight at 4 °C. After thorough washing, the cells were incubated with Cy3-conjugated goat anti-rabbit IgG and FITC-conjugated Donkey anti-goat IgG for 1 h at room temperature. Finally, DAPI Fluoromount-G (SouthernBiotech) was used to counterstain the cell nuclei. The fluorescent was detected, and images were taken by Leica inverted fluorescence microscope. The antibodies are listed in table 2.

Immunohistochemical staining

Human cervical cancer specimens were fixed with neutral formalin, embedded in paraffin, and sectioned at a thickness of 4 μm. Sections were deparaffinized in xylene and rehydrated in a graded alcohol series. Antigen retrieval was performed using 0.01-M citrate buffer and 2 min of boiling. Hydrogen peroxide was applied to block endogenous peroxidase activity, and then sections were incubated with normal goat serum to block nonspecific protein binding. Sections stained with primary antibody for Tim-3 and galectin-9 were incubated overnight at 4 °C. Sections were stained in parallel with PBS as a negative control. Tim-3 and galectin-9 expression were then detected using DAB, and slides were counterstained with hematoxylin. Slides were view at 400× magnification. The antibodies are listed in table 2.
Xenograft mouse model

BALB/c nude mice (4-week-old) used in this study and were maintained in a specific-pathogen-free (SPF) condition facility. Mouse injected subcutaneously with $1\times10^7$ SiHa-SUV39H1/HeLa-SUV39H1 cells were randomly divided into four groups when tumor volumes were around $100\text{mm}^3$: (1/2) SiHa-mock/HeLa-mock control groups; (3/4) SiHa-SUV39H1/HeLa-SUV39H1 groups. Two diameters of the individual tumor were measured by electronic slide caliper every two days. Tumor volume was calculated using the following formula: tumor volume ($\text{mm}^3$) = $0.5 \times \text{length} \times \text{width}^2$. Mice were monitored for 21 days, at which time mice were euthanized and tumors and organs were extracted.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 7 software (GraphPad Software, USA). Paired t test and one-way ANOVA analysis were carried out on samples within groups. The $p$ value of <0.05 was considered statistically significant. The $p$ values are represented as **$p$<0.01, *$p$<0.05. The data are presented as mean ± standard error of the mean (SEM). All experiments were independently repeated at least thrice, with consistent results.

Results

Tim-3 and galectin-9 expression were increased due to genes methylation level decreased in cervical cancer

Using the Oncomine databases (https://www.oncomine.org/), we compared the mRNA expression of HAVCR2 and LGALS9 between cervical cancer and normal cervical samples. The results indicated that the expression levels of HAVCR2 and LGALS9 were all higher in cancer than in normal cervical samples (Fig. 1a, b). Furthermore, HAVCR2 was positively corrected with LGALS9 ($R = 0.26$, $p < 0.05$) based on GEPIA (Gene Expression Profiling Interactive Analysis) dataset (http://gepia.cancer-pku.cn/) (Fig. 1c). Hence HAVCR2 has a positive correlation with LGALS9 in cervical cancer. The expression of Tim-3 and galectin-9 protein in cancer tissues were higher than in normal cervix tissues (Fig. 1d, e). The detail data of patients’ clinicopathological is shown in table 3.

The online software “MethPrimer” (http://www.urogene.org/methprimer/) profiled CpG island in the region that was located from -2000 to -200 bp upstream from ATG, the transcription starts site (TSS) in the HAVCR2 and LGALS9 promoters respectively (Fig. 1f). One pair of primers was designed to amplify the genes promoter regions respectively. HAVCR2 and LGALS9 promoters in cervical cancer tissues displayed hypermethylation status in normal cervical tissues (Fig. 1g, h), possibly leading to the inhibition
of its gene expression in normal cervical tissues. Immunohistochemistry results revealed that Tim-3 and galectin-9 expressed in tumor cells of cervical cancer tissues (Fig. 1i).

Tim-3 and galectin-9 expression were reversed by altering the methylation status in the promoter regions of **HAVCR2** and **LGALS9** in cervical cancer cells

It showed that **HAVCR2** and **LGALS9** promoter regions from ATG in SiHa, HeLa and C33A cells were all partially methylated (Fig. 2a). The Tim-3 and galectin-9 expressed in SiHa, HeLa and C33A cells (Fig. 2b). The mRNA expression level of **HAVCR2** and **LGALS9** in SiHa, HeLa and C33A cell lines after treatment with DNA demethylation reagent 5-aza-2'-deoxycytidine (5-Aza-CdR) to identify whether the methylation status in the promoter regions regulate the expression of these genes at the transcriptional level. The results suggested that the expression of **HAVCR2** and **LGALS9** mRNA in SiHa, HeLa and C33A cells increased in dose-dependent manner after cellular DNA demethylation (Fig. 2c, d). It illustrated the Tim-3 and galectin-9 expression were reversed by 5-Aza-CdR, which promoted the expression of these genes at the transcriptional level. What's more, immunofluorescence assay showed that the SiHa and HeLa cells all staining cytoplasm and nuclear Tim-3 and galectin-9 (Fig. 2e).

Tim-3 and galectin-9 expression were repressed by DNMT3A through mediated DNA methylation

We found that knocking-down DNMT3A activated Tim-3 and galectin-9 expression (Fig. 3a, b), and was accompanied by decreased DNA methylation level of the gene's promoter in SiHa and HeLa cells (Fig. 3c, d). It was suggested that DNMT3A may play an important role in regulating Tim-3 and galectin-9 expression. The ChIP analysis revealed enhanced binding of DNMT3A (Fig. 3f, h) to the **HAVCR2** and **LGALS9** promoter regions (Fig. 3e, g) in SiHa and HeLa cells. Altogether, these results suggest that DNMT3A-mediated DNA methylation contributes to the transcriptional silencing of **HAVCR2** and **LGALS9**.

SUV39H1 mediated DNMT3A expression through up-regulating H3K9me3 in cervical cancer cells

SUV39H1 is the histone methyltransferase (HMTase) of histone H3 lysine 9 trimethylation (H3K9me3) [17]. SUV39H1 recognizes trimethylated H3K9 (H3K9me3) via its chromodomain (CD), and enriched H3K9me3 afterwards [18]. The H3K9me3 and DNMT3A expressed in SiHa, HeLa and C33A cells (Fig. 4e).

As shown in Fig. 4b, Overexpressed SUV39H1 in SiHa and HeLa cells (Fig. 4a) significantly increased H3K9me3 and DNMT3A expression. Fig. 4c showed that knocking-down SUV39H1 expression in SiHa and HeLa cells displayed dramatically down-regulation in H3K9me3 and DNMT3A protein expression (Fig. 4d). Collectively, these results indicated that SUV39H1 participate in regulation of DNMT3A through changing H3K9me3 expression in cervical cancer cells.
In a screening for epigenetic mechanisms that regulating DNMT3A expression, ChIP analysis revealed that the SiHa and HeLa cells exhibit the highest H3K9me3 level in a region upstream of the transcription initiation region (Fig. 4f). H3K9me3 regulated the expression of DNMT3A by acting on the -1000 to +1 region of the promoter region of $DNMT3A$ (Fig. 4g, h). Taken together, above results revealed that SUV39H1 regulated the expression of DNMT3A through elevating H3K9me3 level on the $DNMT3A$ promoter in cervical cancer cells.

**The methylation status of HAVCR2 and LGALS9 affected by SUV39H1 in cervical cancer cells**

We have determined the baseline levels of DNA methylation on $HAVCR2$ and $LGALS9$ promoters among cervical cancer cell lines and then evaluated whether SUV39H1 mediated DNA methylation through $DNMT3A$ is required for $HAVCR2$ and $LGALS9$ transcription. The results showed that SUV39H1 overexpression increased methylation levels at the $HAVCR2$ and $LGALS9$ promoters (Fig. 5a, b), these changed methylation level contributed to the decrease of Tim-3 and galectin-9 expression among overexpressed SUV39H1 cell lines (Fig. 5e). SUV39H1-knockdown cells showed the opposite results (Fig. 5c, d, f).

These results indicating that changes in histone modification precede the changes in DNA methylation level of $HAVCR2$ and $LGALS9$. Consistent with SUV39H1 affecting the expression of DNMT3A.

**SUV39H1 mediated Tim-3 and galectin-9 expression through DNA methylation in vivo**

For the purpose of investigating SUV39H1 mediated the costimulatory factors Tim-3 and galectin-9 expression through DNA methylation *in vivo*, we generated SiHa-SUV39H1 and HeLa-SUV39H1 tumor xenografts in nude mice. As shown in Fig. 6a, tumors formed from the SiHa-mock and HeLa-mock cells (Fig. 6b, c) grew faster than those formed from the SiHa-SUV39H1 and HeLa-SUV39H1 cells, respectively. All these data indicated that up-regulating SUV39H1 may inhibited the tumor growth in SiHa and HeLa cells.

To determine SUV39H1 mediated Tim-3 and galectin-9 expression through DNA methylation *in vivo*, the levels of H3K9me3, DNMT3A and Tim-3 and galectin-9 in the tumor xenograft tissues were examined by western blotting. As shown in Fig. 6d-f, the expression of DNMT3A increased significantly when SUV39H1 overexpressed, followed by the down-regulation of Tim-3 and galectin-9 in SiHa-SUV39H1 and HeLa-SUV39H1 cells derived tumors. SUV39H1 overexpression significantly up-regulated the methylation level of $HAVCR2$ and $LGALS9$ in tumor tissues (Fig. 6g). ChIP analysis revealed that H3K9me3 regulated the expression of DNMT3A by acting on the -1000 to +1 region of the promoter region of $DNMT3A$ (Fig. 6h, i) in tumor tissues. H3K9me3 directly regulate the expression of DNMT3A *in vivo*, these results indicating that SUV39H1 precede the changes in DNA methylation. All the results in xenograft tissues were
consistent with those in vitro, indicating that similar SUV39H1 mediated Tim-3 and galectin-9 expression through DNA methylation in vivo.

**H3K9me3 expression was independent from HR-HPV oncogenes**

As persistent HR-HPV infection contributes to almost all cervical cancer cases [19], we attempted to explore whether HR-HPV oncogenes E6 and E7 participated in SUV39H1 mediated DNA methylation in SiHa and HeLa cell lines. We found that there was no difference in the expression level of H3K9me3 in cervical cancer cells SiHa, HeLa and C33A (Fig. 4e). Expression of HPV16/18 E6 and E7 oncogenes were detected by western blotting in overexpression or knockdown SUV39H1 SiHa and HeLa cells respectively. As show in Fig. 7i and j, the expression level of HR-HPV oncogenes E6 and E7 were no difference between overexpressed or knocked-down SUV39H1 SiHa and HeLa cells with control. In the following studies we transiently overexpressed or knocked-down HPV16/18 E6 and E7 in cervical cancer cells for further illustration (Fig. 7a-d). The results showed that, the level of H3K9me3 was not changed in over-expressed or knocked-down HPV16/18 E6 and E7 cells compared with control (Fig. 7e-h).

Taken together, our data suggested that H3K9me3 expression was independent from HR-HPV oncogene E6 and E7 in cervical cancer.

**Discussion**

Aberrant DNA methylation is recognized as one of the most important events in cervical cancer carcinogenesis, which causes silencing of certain genes [20, 21]. Epigenetic modification plays an important role in regulating immune cell differentiation [22, 23]. The methylation status of immune genes influences the tumor immune response in the tumor microenvironment (TME) [24, 25]. The study showed that decreased activity of DNMTs in CD4+ Tregs was accompanied by demethylation of the forkhead box P3 (FOXP3) gene promoter and downregulation of immune responses in the TME [26]. Hypermethylation associated SMAD family member 3 (SMAD3) silencing in CAFs, which was associated with aberrant response to exogenous TGF-β1 [27]. Here, we identified a novel function of SUV39H1 regulates DNMT3A expression through elevating H3K9me3 level in DNMT3A promoter, which could mediate Tim-3 and galectin-9 expression through DNA methylation in cervical cancer. Tim-3 and galectin-9 are over-expressed in cervical cancer tissues, this biological effect is mediated through the aberrant epigenetic of Tim-3 and galectin-9, which is facilitated by the recruitment of DNMT3A to their promoter regions. Meanwhile, SUV39H1 contributed to Tim-3 and galectin-9 regulation by up-regulation H3K9me3 level in DNMT3A promoter which directly binding to the promoter of DNMT3A.

We found that Tim-3 and galectin-9 were over-expressed in cervical cancer tissues related to promoter regions of HAVCR2 and LGALS9 were hypo-methylated, and they were partial methylation in cervical cancer cells, indicating DNA methylation mediating costimulatory factors Tim-3 and galectin-9 in cervical cancer cells. DNA methylation-based gene silencing in cancer [28]. DNMT3A involved in the induction of
genes expression by directly binding to the HAVCR2 and LGALS9 promoters, suggesting that DNMT3A participate in the epigenetic regulation of HAVCR2 and LGALS9 in cervical cancer. Knocked-down DNMT3A in cervical cancer cells caused a decrease of HAVCR2 and LGALS9 methylation level, accompanied by the expression of Tim-3 and galectin-9 elevated.

We explored the role of histone methyltransferase and DNA methyltransferase in mediating the expression of Tim-3 and galectin-9 in cervical cancer. H3K9me3 and DNA methylation leading to depression of a collection of genes [29, 30]. The changes in histone modification precede the alterations in DNA methylation [31, 32]. Up-regulation of SUV39H1 could promote DNMT3A expression, ChiP results demonstrated H3K9me3 directly binding to the DNMT3A promoter to adjust its expression. These results suggesting that SUV39H1 facilitates the expression of DNMT3A in cervical cancer. Overexpression SUV39H1 also associated with increased methylation level of HAVCR2 and LGALS9 which in turn caused Tim-3 and galectin-9 expression decreased in cervical cancer. SUV39H1 may be a prerequisite for promoter DNA methylation by recruiting DNMT3A. H3K9me3 had direct action on DNMT3A promoter to regulate its expression, they cooperatively orchestrate epigenetic modification at the gene promoter of HAVCR2 and LGALS9. But HPV16 or 18 didn't affect the expression of H3K9me3 in cervical cancer cells.

The epigenetic regulation caused elevated expression of costimulatory factors Tim-3 and galectin-9 in cancer cells, and the abnormal secreted Tim-3 and galectin-9 by tumor cells caused tumor microenvironment immune imbalance, thereby promoting the development of cervical cancer. We provide evidence for SUV39H1 as a potential therapeutic target, which use an epigenetic agent or inhibitor aimed it, could decrease negative immune factors like Tim-3 and galectin-9. Restrain the expression of SUV39H1 may potentially be an effective approach to augment the efficacy of immune factors against cervical cancer.

**Conclusion**

In summary, our present study highlights the role of SUV39H1 and H3K9me3 in the DNA methylation regulation of Tim-3 and galectin-9 in cervical cancer microenvironment (Fig. 8). We provide a potential direction in exploring the relationship between SUV39H1 and DNMT3A. These findings add diverse roles and mechanistic insight into our understanding of crosstalk of SUV39H1 with DNMT3A.

**Declarations**

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**Authors’ contributions**
LZ and ST conducted the experiments. LZ, LS and XY participated in the data analysis. LZ, LS and XY designed the experiments. MZ, TY, SQ, QY collected samples from cervical cancer patients. LZ and XY wrote and edited the manuscript. All authors read and approved the final manuscript.

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**Data availability statement**

The dataset analyzed during the current study are publicly available from the online database: GEPIA database (http://gepia.cancer-pku.cn/) and Oncomine database (www.oncomine.org).

**Ethics approval and consent to participate**

The study was approved by the Ethics Committee of the First Affiliated Hospital of Xi’an Jiaotong University (G-272) in Shaanxi, China. Written informed consent was obtained from all patients to participate in this study. BALB/c nude mice used in this study were obtained from Experimental Animal Center of Xi’an Jiaotong University (License number SCXK (Shaanxi) 2006-001).

**Consent for publication**

Not applicable.

**Conflicts of interest**

The authors declare no potential conflicts of interest.

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Tables

Table 1 Primer sequences
| Name            | Application | Sequence                        |
|-----------------|-------------|---------------------------------|
| DNMT3A-ChIP-F1  | ChIP-qPCR   | ATCATCAGTAGGGCGGGGTGGCCAC       |
| DNMT3A-ChIP-R1  | ChIP-qPCR   | CTCCAATGCTTTCCAGGTCCCTCCTG     |
| DNMT3A-ChIP-F2  | ChIP-qPCR   | TTGGGAACCTCCCCGAGGAAACACC      |
| DNMT3A-ChIP-R2  | ChIP-qPCR   | GCCACCCCTTTTAGCTACAGAACC       |
| DNMT3A-ChIP-F3  | ChIP-qPCR   | CGTTGGGGGGGCGGGTGCTGGGCTG     |
| DNMT3A-ChIP-R3  | ChIP-qPCR   | TGAICTGGCAGCAGATGGCGTGCT      |
| DNMT3A-ChIP-F4  | ChIP-qPCR   | CATGGGGAAGGAGACAGCCTCCAC      |
| DNMT3A-ChIP-R4  | ChIP-qPCR   | GCACTGGGAAAGACTGAAAGATTTCAT   |
| HAVCR2-ChIP-F1  | ChIP-qPCR   | GTGGAAAAAATCTGTCACTTAGGG       |
| HAVCR2-ChIP-R1  | ChIP-qPCR   | ATTTTTAGTAGAGACGGGGTTTCTC     |
| HAVCR2-ChIP-F2  | ChIP-qPCR   | CCTGTAATCCCAGCTACTCAGGGAGG     |
| HAVCR2-ChIP-R2  | ChIP-qPCR   | CTTGTTCAATGTGTGTACTTCCCAT     |
| HAVCR2-ChIP-F3  | ChIP-qPCR   | CCCATGCACTTTAATGGCATAAGT       |
| HAVCR2-ChIP-R3  | ChIP-qPCR   | CAGCCACACTCCCATAACTGAGGTA     |
| HAVCR2-ChIP-F4  | ChIP-qPCR   | GGGAACCTCAACCTTTCTGATCTTTC    |
| HAVCR2-ChIP-R4  | ChIP-qPCR   | GACTTTTGACCTTAAACTTCCAACT     |
| LGALS9-ChIP-F1  | ChIP-qPCR   | GGTAGAGTAAATGTACAGTCCCTG      |
| LGALS9-ChIP-R1  | ChIP-qPCR   | GCGAGACCTTTGTCTCTACTAAAAT      |
| LGALS9-ChIP-F2  | ChIP-qPCR   | TCAGCCTCCCAATGTGCTGAATTAC     |
| Primer Name | Assay Type | Sequence |
|-------------|------------|----------|
| LGALS9-ChIP-R2 | ChIP-qPCR | CCAGATCCAAACCTTGACTTGAA GTG |
| LGALS9-ChIP-F3 | ChIP-qPCR | TCCTGTGGGCCTAGCTCCTTTTT ATT |
| LGALS9-ChIP-R3 | ChIP-qPCR | AGAAAAACTGCTTGGTAGTT GTAA |
| LGALS9-ChIP-F4 | ChIP-qPCR | CACATTGTGTCCCTTCTTTTGG |
| LGALS9-ChIP-R4 | ChIP-qPCR | ACACCTGTGGTCTCAGCTACAT GGG |
| HAVCR2-ML | MS-PCR | TATAAAAATGAGAAATTGGTCGG GCG |
| HAVCR2-MR | MS-PCR | TTACAAACATATACCACCACCC CGA |
| HAVCR2-UL | MS-PCR | GAAATTTGGTTGGTGTTGATT GGTT |
| HAVCR2-UR | MS-PCR | TATACCAACCACACAAATAATT TA |
| LGALS9-9-ML | MS-PCR | TTTTCGAGATAGGTTTGATT TTG |
| LGALS9-9-MR | MS-PCR | AATACCGACACCCTTCAATCAC CAC |
| LGALS9-9-UL | MS-PCR | GAGTTTTTGAGATAGGTTTGT GATT |
| LGALS9-9-UR | MS-PCR | ATACCAACCACCTTCAATCAC ACA |
| SUV39H1-sense | gene silencing | CUCUCCGUGUACAUAUGATT |
| SUV39H1-anti-sense | gene silencing | UCAUUGAUAGUACGAAGGTT |
| DNMT3A-sense | gene silencing | GCCAAGGUCAUUGCAGGAATT |
| DNMT3A-anti-sense | gene silencing | UUCUCGAAUGACCUUGGCTT |
| Negative control-sense | gene silencing | UUCUCGAACGUGUCACGUTT |
| Negative control-sense | gene silencing | ACUGACACGUUCGGAGAATT |

ChIP: chromatin immunoprecipitation, RT-PCR: Reverse Transcription-Polymerase chain reaction, MS-PCR: methylation-specific-Polymerase chain reaction; F: Forward primer, R: backward primer, ML/UL: methylation/unmethylation forward primer, MR/UR: methylation/unmethylation backward primer.
### Table 2 Antibodies

| Antibody   | Source                  | Dilution | Cat Number | Application |
|------------|-------------------------|----------|------------|-------------|
| H3K9me3    | Cell Signaling Technology | 1:1000   | 13969      | ChIP, WB    |
| SUV39H1    | Cell Signaling Technology | 1:1000   | 8729       | WB          |
| Tim-3      | Abcam                   | 1:50     | ab47997    | IF          |
| Tim-3      | Abcam                   | 1:250    | ab185703   | WB, IHC     |
| galectin-9 | Abcam                   | 1:200    | ab123712   | WB          |
| galectin-9 | Abcam                   | 1:250    | ab69630    | IF, IHC     |
| DNMT3A     | Abcam                   | 1:250    | ab13537    | ChIP, WB    |
| IgG        | Cell Signaling Technology | 1:500    | 2729       | ChIP        |
| Histone H3 | Cell Signaling Technology | 1:50    | 4620       | ChIP        |
| β-actin    | TransGen Biotech        | 1:500    | HC201-01   | WB          |

ChIP=chromatin immunoprecipitation; WB=western blotting; IF: Immunofluorescence; IHC: Immunohistochemical.

### Table 3 Patients’ clinicopathological details (n = 24)
| Item                       | No. |
|----------------------------|-----|
| Age                        |     |
| ≤44                        | 10  |
| >44                        | 14  |
| Clinical stages            |     |
| Ia                         | 0   |
| Ib                         | 8   |
| IIA                        | 11  |
| IIB                        | 5   |
| Pathological pattern       |     |
| Squamous cell carcinoma    | 21  |
| Adenocarcinoma             | 3   |
| Pathological grading       |     |
| I                          | 1   |
| II                         | 19  |
| III                        | 4   |
| Lymph nodes metastasis     |     |
| Yes                        | 3   |
| No                         | 21  |
| HPV infection              |     |
| Positive                   | 20  |
| Negative                   | 4   |

**Figures**
**Figure 1**

The expression of Tim-3 and galectin-9 in cervical cancer tissues and the genes methylation level. (a, b) An overview of mRNA levels of HAVCR2 and LGALS9 in cervical cancers based on Oncomine. (c) The correlation between HAVCR2 and LGALS9 in cervical cancer, analyzed by GEPIA. (d, e) The protein levels of Tim-3 and galectin-9 in cervical cancer (Ca), para-carcinoma (Cap) (n=24) and normal cervical tissues (NC) (n=16) detected by western blotting. Blot images of 4 representative samples are shown from each group. (f) Predicted CpG islands in the promoter region of HAVCR2 and LGALS9. Numbers indicate the positions in bp relative to the transcription start site. The blue region represents the CpG islands and the red vertical bars are the CpG loci in these input sequences. (g, h) Methylation level of HAVCR2 and LGALS9 promoter regions in cervical cancer (Ca) (n=9) and NC (n=9) detected by MS-PCR. (M: methylated; U: unmethylated); methylated and unmethylated levels were quantified as M/M+U% and
Methylation status in the promoter regions of HAVCR2 and LGALS9 in cervical cancer cell lines. (a) Detection of HAVCR2 and LGALS9 methylation status by MS-PCR in SiHa, Hela and C33A cell lines; (M: methylated; U: unmethylated). (b) Tim-3 and galectin-9 expressed in SiHa, HeLa and C33A cells detected by western blotting. (c, d) Relative expression of HAVCR2 and LGALS9 mRNA in SiHa, HeLa and C33A cells after treatment with different concentrations of 5-Aza-CdR. (e) Representative immunofluorescence staining of Tim-3 and galectin-9 expression in SiHa and HeLa cells. *P<0.05, **P <0.01
**Figure 3**

Tim-3 and galectin-9 were repressed by DNMT3A mediated DNA methylation. (a) Western blotting analysis of SiHa and HeLa cells knockdown DNMT3A against Tim-3, galectin-9 and DNMT3A. (b) Detected DNMT3A and Tim-3, galectin-9 in DNMT3A specific siRNA transfected overexpressed H3K9me3 SiHa, HeLa and C33A cells by western blotting. (c) The promoter methylation level of HAVCR2 and LGALS9 were monitored by MS-PCR in DNMT3A knockdown SiHa and HeLa cells. (d) Methylated and unmethylated levels were quantified as M/M+U% and U/M+U%, respectively. (e, g) Schematic representation of the 4 regions of the HAVCR2 and LGALS9 promoters amplified in the chromatin immunoprecipitation (ChIP)-quantitative PCR (qPCR) experiment. (f, h) Chromatin was cross-linked, fragmented and immunoprecipitated with either IgG (mock) or anti-DNMT3A ChIP-grade antibody and the purified DNA was used to amplify with respective primer pairs for indicated four regions in the HAVCR2/LGALS9 promoters in qPCR. The enrichment of DNMT3A on HAVCR2/LGALS9 promoters relative to IgG in SiHa and HeLa cells, and H3 against RPL30 was used as positive control. *P<0.05, **P<0.01
Figure 4

SUV39H1 increased DNMT3A expression in cervical cancer cells. (a, b) Detection of SUV39H1, H3K9me3 and and DNMT3A in SiHa, HeLa and C33A which overexpression SUV39H1 by western blotting. (c, d) Western blotting analysis of SUV39H1, H3K9me3 and DNMT3A in SiHa, HeLa and C33A cells 48 h after transfection with siRNAs against SUV39H1. (e) Detected H3K9me3 and DNMT3A expression in SiHa, HeLa and C33A cells by western blotting. (f) Schematic representation of the 4 regions of the DNMT3A promoter amplified in the ChIP-qPCR experiment. (g, h) Chromatin was cross-linked, fragmented and immunoprecipitated with either IgG (mock) or anti-H3K9me3 ChIP-grade antibody and the purified DNA was used to amplify with respective primer pairs for indicated four regions in the DNMT3A promoter in qPCR. The enrichment of H3K9me3 on DNMT3A promoter relative to IgG in SiHa and HeLa cells, and H3 against RPL30 was used as positive control. **P<0.01
Figure 5

SUV39H1 changed HAVCR2 and LGALS9 methylation level in cervical cancer cells. (a, c) The promoter methylation level of HAVCR2 and LGALS9 were monitored by MS-PCR in SUV39H1 overexpression and knockdown SiHa and HeLa cells, (M: methylated; U: unmethylated). (b, d) Methylated and unmethylated levels were quantified as M/M+U% and U/M+U%, respectively. (e, f) Western blotting analysis of SiHa and HeLa cells overexpression and knockdown SUV39H1 against Tim-3 and galectin-9. *P<0.05, **P<0.01
Figure 6

SUV39H1 mediated Tim-3 and galectin-9 expression through DNA methylation in vivo. (a) SiHa-SUV39H1 and HeLa-SUV39H1 tumor xenografts in nude mice. (b) Tumors formed from SiHa-SUV39H1 and HeLa-SUV39H1 cells as well as tumor growth curves. (c) The tumors weight formed from SiHa-SUV39H1 and HeLa-SUV39H1. (d-f) Western blotting results of SUV39H1, H3K9me3, DNMT3A, Tim-3 and galectin-9 in SiHa-SUV39H1 and HeLa-SUV39H1 cells formed tumors. (g) The promoter methylation level of HAVCR2 and LGALS9 were monitored by MS-PCR in tumor tissues. (h, i) Chromatin was cross-linked, fragmented and immunoprecipitated with either IgG (mock) or anti-H3K9me3 ChIP-grade antibody and the purified DNA was used to amplify with respective primer pairs for the indicated 4 regions in the DNMT3A promoter in qPCR. The enrichment of H3K9me3 on DNMT3A promoter relative to IgG in tumor tissues. *P<0.05, **P<0.01
Figure 7

HR-HPV E6/E7 wasn't participate in H3K9me3 mediated DNA methylation in cervical cancer. (a-d) mRNA levels in of HPV16 and 18 E6/E7-overexpressing C33A cells and of HPV16 and 18 E6/E7-knockdown SiHa and HeLa cells. (e, f) Detected H3K9me3 in HPV16 E6/E7 overexpressed C33A cells or knocked down SiHa cells by western blotting. (g, h) Detected H3K9me3 in HPV18 E6/E7 overexpressed C33A cells or knocked down HeLa cells by western blotting. (i) HPV16 E6/E7 expression in overexpression or knocked down SUV39H1 in SiHa cells. (j) HPV18 E6/E7 expression in overexpression or knocked down SUV39H1 in HeLa cells.
Figure 8

The pathway of SUV39H1 regulated Tim-3 and galectin-9 expression through DNA methylation. (a) SUV39H1 mediated H3K9 methylation. (b) H3K9me3 directly interacted with DNMT3A promoter so that up-regulated its expression. (c) DNMT3A directly bind to HAVCR2 and LGALS9 promoter regions respectively to increase their methylation level so that their expression level decreased.