



Reduction in immune cell number and loss of 5hmC are associated with lesion grade in cervical carcinogenesis

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Abstract

Tumor genome methylation is closely related to tumor immunosuppression. In the present study, we evaluated the fluctuations in DNA methylation levels, and the numbers of infiltrating T cells and their cytokines in different-grade cervical lesions. A total of 154 human cervical specimens that included LSIL (43 cases), HSIL (48 cases), and cervical squamous cancer (63 cases) were used for this study. Immunohistochemistry for 5-hydroxymethylcytosine (5hmC) and T-cell-attracting chemokines was performed, and multiplex immunofluorescence labeling was used to identify different T-cell subtypes. We found that the proportions of samples that immunostained weakly or negatively for 5hmC were increased commensurately with elevations in the severity of cervical lesions. The expression of T-cell-attracting chemokines—including CXCL9, CXCL10, and CXCL11—was positively associated with 5hmC levels, and CXCL9 was the cytokine that was most pronounced. With the progression of cervical lesions, the numbers of total T cells, CTL, and NK cells in the cervical tissues all gradually decreased. During the occurrence and development of cervical squamous carcinoma, 5hmC was gradually lost, and immunosuppression occurred in precancerous cervical lesions.

Keywords 5hmC · LSIL · HSIL · Cervical squamous carcinoma · T cell · Chemokines

Introduction

Cervical squamous carcinoma is a lethal malignancy with a high incidence in women, and it is the fourth leading cause of cancer-related deaths in women (Torre et al. 2017). The most essential pathogenesis of cervical squamous cancer is persistent infection with human papillomavirus (HPV) (Lee et al. 2016), and several genetic variants also contribute to the occurrence of cervical squamous cancer. Cervical squamous cancer generally develops from pre-existing, non-invasive, and squamous precursor lesions referred to as squamous intraepithelial lesions (SIL), which can be divided into low-grade SIL (LSIL) and high-grade SIL (HSIL) (El-Zein et al. 2016; Wu et al. 2019). Although the incidence of cancer increases commensurately with aggravation of the lesions, there are no definite boundaries across the grades, and not all lesions ultimately develop into cancer (Villa et al. 2018). Further study is needed to explore potential indicators and novel prediction methods for cervical squamous carcinoma and to ultimately elucidate the molecular events underlying the cancer process.

Methylation and associated demethylation are fundamental forms of epigenetic modification in the mammalian

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genome and are closely related to various pathogenic processes, including canceration (An et al. 2017). Active demethylation of DNA is derived from sequential oxidation of 5-methylcytosine (5mC) first to 5-hydroxymethylcytosine (5hmC), then to 5-formylcytosine (5fC), and finally to 5-carboxylcytosine (5caC), with the catalysis of ten–eleven translocations (TETs) (Bochtler et al. 2017). Pathologically, loss-of-function mutations in TET genes occur frequently in hematopoietic malignancy of both myeloid and lymphoid lineages (Quivoron et al. 2011; Delhommeau et al. 2009). However, mutations in TET genes are uncommon in solid tumors (Jin et al. 2011). Several mechanisms subserving the inhibition of TET activity have been reported, including deprivation of oxygen or an acquired metabolic disturbance due to a mutation in the isocitrate dehydrogenase (IDH) gene in gliomas (Xu et al. 2011).

TET activity has also been found to be significantly reduced across different types of solid tumors. A previous study show that immunostaining for 5hmC (an intermediate product of DNA demethylation) was weak or negative in carcinomas of the liver, lung, breast, and prostate (Yang et al. 2013), and other investigators reported that loss of 5hmC occurred in melanoma, glioblastoma, and colon carcinoma (Orr et al. 2012; Lian et al. 2012; Haffner et al. 2011). Whether the global eradication of 5hmC occurs in the progressive cancer process from a precancerous lesion to carcinoma in the cervix remains unclear.

Extensive clinical data on cancer of the colorectum, ovary, and pancreatic duct suggest that carcinoma cases with fewer immune effector T cells tend to have a worse prognosis (Shankaran et al. 2001). Mature T cells can be identified by the expression of specific proteins on their cell membranes, such as CD3+/CD8+ cytotoxic T lymphocytes (CTL) and CD3-/CD56+ NK cells (Santin et al. 2001). Both in vitro and clinical studies have shown that tumor-related NK cells and CTLs working together possess a cooperative inhibitory effect on the development of cervical squamous carcinoma (Caserta et al. 2010; Santin et al. 1999). In mouse glioma models, an IDH1 mutation—which can inhibit TET activity and reduce 5hmC production—has been found to reduce T-cell-attracting chemokines, including CXCL9, CXCL10, and CXCL11, and can subsequently lead to the inhibition of T-cell migration (Kohanbash et al. 2017). Reduced TET activity in human colon cancer was associated with decreased T-cell-attracting chemokines and tumor-infiltrating lymphocytes (Xu et al. 2019). The T-cell-attracting chemokine pathways also a significant participant in anti-tumor immune regulation and play is an important role in the occurrence and development of cervical squamous carcinoma (Nagarsheth et al. 2017). Changes in cellular immunomodulation in the progression of cervical lesions and their relationship with 5hmC levels; however, remain unclear.

In the present study, the expression of 5hmC was determined in human cervical samples taken from precancerous lesions progressing to cervical squamous cell carcinomas. Then the levels of infiltrating T-cell subtypes were evaluated and compared to 5hmC levels in samples, revealing a positive relationship between lymphocytic populations and tumor 5hmC levels. In the progression of cervical squamous carcinoma, 5hmC levels were downregulated, and the numbers of total infiltrating T cells, CTLs, and NK cells were also reduced. The findings indicated that weakly or negatively immunostaining 5hmC may be used as a marker for indicating immunosuppression in cervical lesions, which might contribute to malignant transformation and tumor progression.

Methods

Histopathologic evaluation and grouping of human cervical tissue specimens

One hundred and fifty-four formalin-fixed, paraffin-embedded human cervical tissue specimens were obtained from patients at the First Affiliated Hospital of Zhengzhou University from January 2016 to June 2017. No patients had undergone radiotherapy, chemotherapy, or other targeted treatments prior to surgery. The average age of the patients was 65.57 years (range, 25–85 years). Informed consent allowing specimens to be used for scientific research was obtained from all patients, and this study was approved by the Institutional Review Board of Fudan University (Y2018-06), following the Declaration of Helsinki (2013).

Two pathologists independently reviewed hematoxylin and eosin (H&E)-stained and p16 IHC (p16 antibody—Ventana, Roche Diagnostics) slides from all cases. Specimen classification results indicated there were 43 LSIL samples, 48 HSIL samples, and 63 cervical squamous carcinoma samples. All specimens were fixed in 10% formalin for 24 h at room temperature, embedded in paraffin, cut into continuous 4- μ m sections, and used for further analysis. According to their 5hmC staining results (the methods are described in the next section), all samples of the three pathologic lesion grades (LSIL, HSIL, and cervical squamous carcinoma) were subdivided into two groups (a high 5hmC group and a low 5hmC group), resulting in a total of six groups.

Immunohistochemical (IHC) staining

Cervical tissue specimens were cut at 4 μ m, placed onto microscope slides, deparaffinized in xylene, rehydrated through a graded series of ethanol, and incubated in a 3% hydrogen peroxide/methanol solution at room temperature for 30 min to inactivate endogenous peroxidases.

Heat-induced antigen retrieval was performed using ethylenediaminetetra acetic acid (EDTA) buffer (pH 8) for CXCL9/CXCL10 and citrate-buffered saline (CBS) (pH 6.0) for CXCL11 and 5hmC at 95 °C. For 5hmC staining, the slides were treated with 2 N HCl for 15 min at room temperature, and sections were neutralized with 100 mM of Tris–HCl (pH 8.5) for 10 min and washed three times with phosphate-buffered saline (PBS). Then, primary antibodies to 5hmC (Active Motif, 1:1,000 dilution), CXCL9 (Abcam, 1:100 dilution), CXCL10 (Abcam, 1:100 dilution), and CXCL11 (Abcam, 1:100 dilution) were applied to the sections, and slides were incubated at 4 °C overnight. The slides were incubated with REAL EnVision horseradish peroxidase (HRP), substrate buffer, and 3'-3-diaminobenzidine (DAB) Chromogen (Dako North America, Carpinteria, CA, USA). Cells showing either cytoplasmic or nuclear signals (brown) were counted as positive. Finally, the slides were counter-stained with hematoxylin, dehydrated with graded ethanol, and mounted using Entellan mounting solution (Sigma-Aldrich, Germany). Positive and negative controls were processed at the same time and under the same conditions as the experimental samples.

Multiplex immunofluorescence staining

Multiplex immunofluorescence staining was performed with an OPAL IHC kit from PerkinElmer (PerkinElmer Inc., Woburn, MA, USA). Briefly, slides were first deparaffinized in xylene, rehydrated with a graded series of ethanol. The following five steps of multiplex immunohistochemistry were followed consecutively for each marker: blocking was performed with antibody diluent (ARD1001EA, PerkinElmer, Waltham, MA, USA), followed by incubation with primary antibody for 1 h, detection using Opal Polymer HRP anti-mouse and rabbit secondary antibody (ARH1001EA, PerkinElmer, Waltham, MA, USA), and visualization using Opal tyramide signal amplification (TSA) plus agent, after which the section was placed in citrate buffer (pH 6.0) and heated using microwave treatment. Antibodies against CD3 (R&D Systems, 1:300 dilution), CD4 (SHJiehao, 1:100 dilution), CD8 (R&D Systems, 1:100 dilution), CD56 (R&D Systems, 1:100 dilution), and CXCR4 (Cell Signaling Technology, 1:800 dilution) were used as primary antibodies to determine T-cell subtypes. All antibodies were used in order and marked with a specific color. In the first set of assays, CD8+ T cells and CD56+ CTLs were identified, with anti-CD8 marked with red (Cy5) and anti-CD56 marked with green (FITC), while anti-CD3 was marked with aqua blue (Cy3) to reveal all infiltrating T cells. Similarly, in the other set of experiments, CD4+ T cells were identified, with anti-CD4 marked with red (Cy5) and anti-CXCR4 marked with green (FITC), while anti-CD3 was marked with aqua blue (Cy3) to reveal all infiltrating T cells. After staining, the specimens were washed and then sealed

with glycerine. All of the above experimental procedures were conducted at room temperature, and slides were maintained in the dark. Slides in which primary antibodies was omitted were used as negative controls.

Image acquisition and processing

A detailed description of 5hmC staining intensity and distribution was recorded for each case, and the patterns were categorized the patterns into two groups: strongly positive group and patchy, weakly positive group.

To obtain and process the images after CXCL9, CXCL10, and CXCL11 IHC staining, five visual fields (172 μm^2 /field) from the intraepithelial lesions were selected for each section. Images were captured using a charge-coupled device (CCD) camera and analyzed using Motic Images Advanced software (version 3.2, Motic, China Group Co. Ltd.). The integrated optical density (IOD) of each image was calculated by i-Solution software (IMT i-Solution Inc., BC, Canada), and the average IOD of each section was calculated as mean \pm SD for further statistical analysis.

To determine the results of multispectral immunofluorescence staining, The Vectra 3.0 Automated Quantitative Pathology Imaging System (PerkinElmer, Waltham, MA, USA) was used to obtain spectral information. The image files created by Vectra were analyzed using Inform 2.2 image analysis software (PerkinElmer, Waltham, MA, USA). Five visual fields were selected from the intraepithelial lesions and captured under a microscope by a CCD camera. Nuance™ software was then used to divide the cells into positive and negative intensity based on the threshold for each fluorescence referenced to the spectral library set up by a typical stained section and photomicrographs into red, green, and blue (RGB)-formed images were gotten. The numbers per mm^2 of CD3, CD56, CD4, CD8, and FoxP3 positive cells were counted using inForm™ software.

Statistical analysis

GraphPad Prism7.0 was used to conduct statistical analysis. Multiple comparisons were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test, and data from each pathologic grade were analyzed using an unpaired, two-tailed Student's *t* test. The ratios of 5hmC-positive samples in different malignant grades were tested by the chi-squared (χ^2) test followed by the Fisher's exact test. For all tests, a two-sided $P < 0.05$ was defined as statistically significant.

Results

Expression level of 5hmC is diminished during cervical tumorigenesis

Although 5hmC was strongly positive in the cellular nuclei of normal cervical squamous epithelium (Fig. 1A), of the 43 LSIL cases, six (14%) showed weak or negative staining; of the 48 HSIL cases, 28(58.3%) showed weak or negative 5hmC staining; and of the 63 carcinoma cases, 50 (79.3%)

Table 1 The cases (proportion) of weak or negative 5hmC expression among different grades of lesions

Lesion grade	Strong	Weak or negative	Total cases
LSIL	37 (86%)	6 (14%)	43 (100%)
HSIL	20 (41.7%)	28 (58.3%)	48 (100%)
Carcinoma	13 (20.6%)	50 (79.3%)	63 (100%)

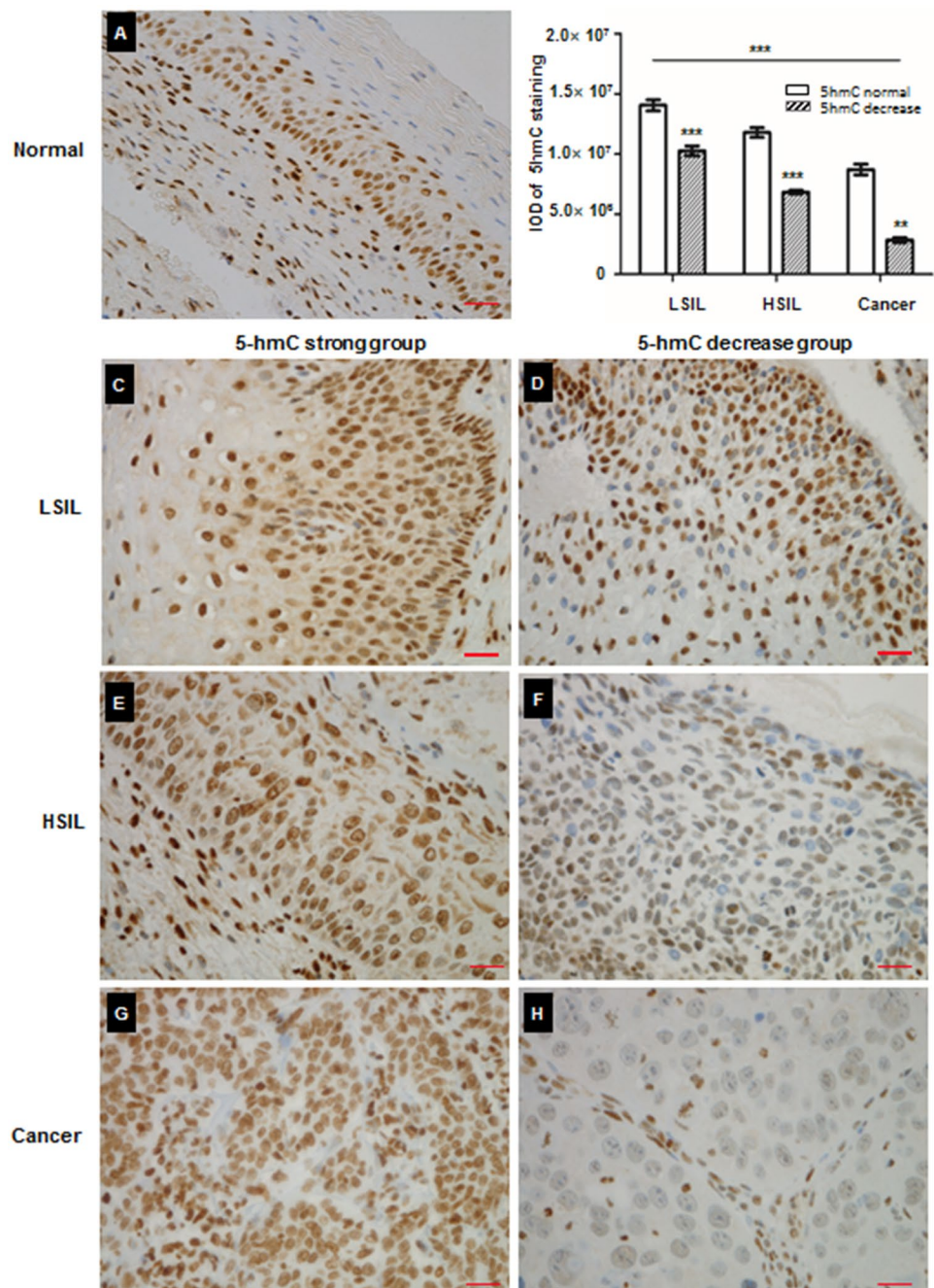
Statistical analysis (2-tailed Fisher exact test):

LSIL vs. HSIL: $***P < 0.001$

LSIL vs. Cancer: $***P < 0.0001$

HSIL vs. Cancer: $*P = 0.0213$

Fig. 1 The expression of 5hmC in cervical tissues is gradually diminished, commensurate with the progression of the cervical lesion. **A** IHC staining of 5hmC in normal cervical squamous cells. **B** IOD values of 5hmC-positive cells in cervical tissue samples stained with IHC. With the progression of the lesion, the levels of 5hmC gradually diminished. Comparison among lesion grade were conducted was by one-way ANOVA. $**P < 0.01$, $***P < 0.001$, compared with samples at the same lesion grade by unpaired Student's *t* test. Data are represented as mean \pm SD. Scale bar is 50 μ m. **C–H** Representative photomicrographs of normal 5hmC expression or weak/negative staining in LSIL, HSIL, and cervical squamous carcinoma, as indicated.



manifested a loss of 5hmC staining, as indicated in Table 1 (representative images are shown in Fig. 1B–G). Statistical analysis showed that 5hmC was markedly decreased during cervical tumorigenesis (Fig. 1C–H). IOD measurements of 5hmC IHC staining also revealed dynamically reduced 5hmC levels commensurate with elevated malignant grade (Fig. 1B). The above results depicted a dramatic diminution in the expression of 5hmC with the progression from LSIL to HSIL to cervical squamous carcinoma.

Expression levels of T-cell-attracting chemokines in cervical tissues are attenuated with the loss of 5hmC

Since impaired TET activity suppresses STAT1 protein (an important regulator of T-cell-attracting chemokine genes that further modulate T-cell infiltration) (Kohanbashi et al. 2017), it was necessary to verify whether changes in 5hmC affect T-cell-attracting chemokine expression in cervical lesions. The expressions of CXCL9, CXCL10, and CXCL11 in cervical samples were evaluated by IHC staining (Fig. 2A), indicating that with aggravation of cervical lesions, the expressions of all three chemokines were significantly reduced ($P < 0.01$). Other observations included a lack of 5hmC staining, staining in patchy cell clusters was

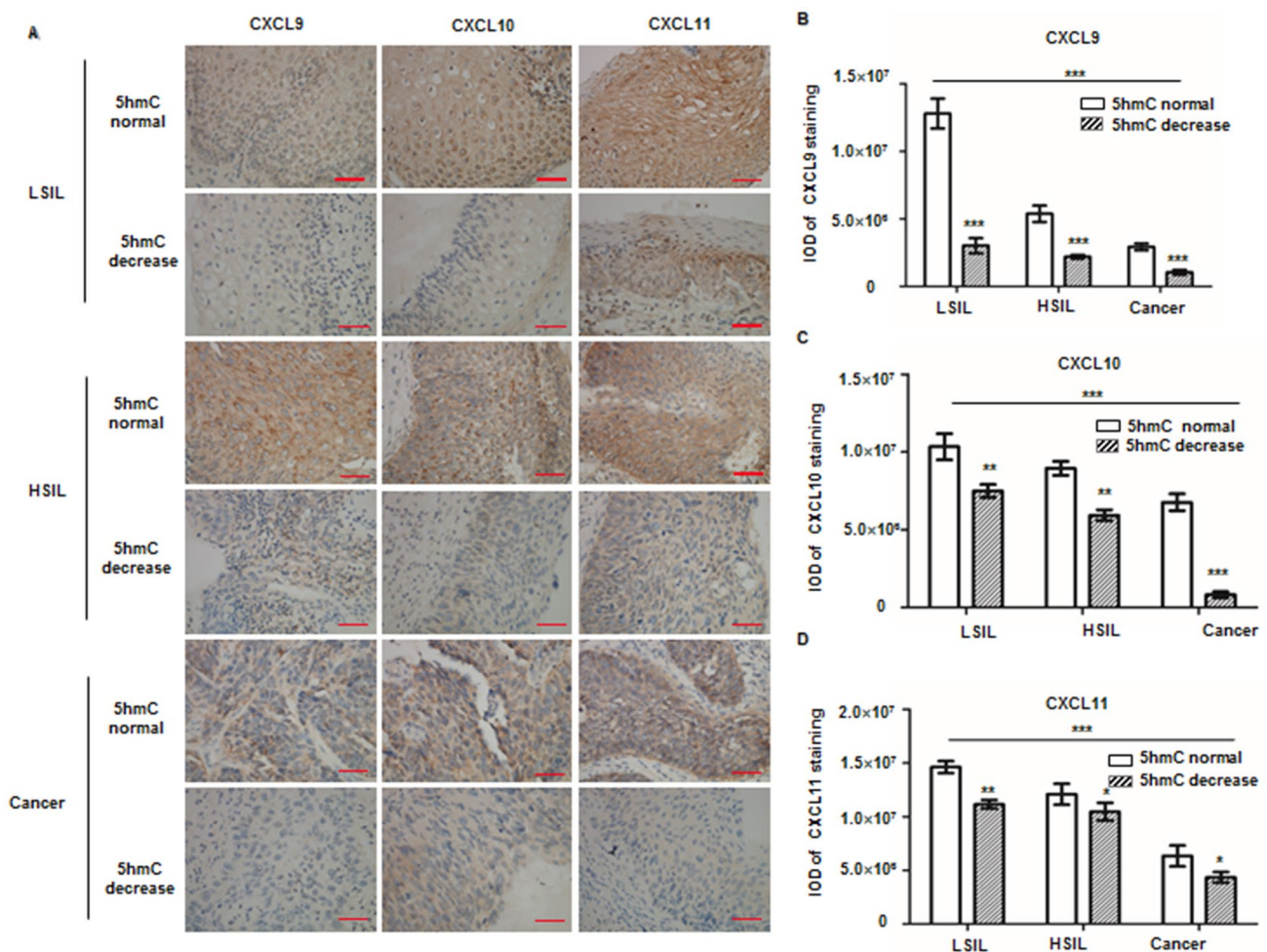


Fig. 2 Intratumoral CXCL9, CXCL10, and CXCL11 levels are correlated with 5hmC levels in cervix lesions. **A** Representative photomicrographs show the expression levels of CXCL9, CXCL10, and CXCL11 in LSIL, HSIL, and cervical squamous carcinoma specimens for samples with high 5hmC levels, and samples with low 5hmC levels. Scale bars are 50 μ m. **B–D** Quantification of **B** CXCL9, **C** CXCL10, and **D** CXCL11 expression classified by high and low

5hmC staining. For each case, five fields were randomly selected to calculate the integrated staining density by i-Solution image analysis software. Comparisons among lesion grades were conducted by one-way ANOVA. ** $P < 0.01$, *** $P < 0.001$, compared with samples at the same lesion grade by unpaired Student's *t* test. Data are represent as mean \pm SD. Scale bar is 50 μ m

subdivided as the low 5hmC group, whereas continuous, diffuse nuclear staining in all cell layers was noted as the high 5hmC group. CXCL9, CXCL10, and CXCL11 chemokines in the low 5hmC group were all lower than in the high 5hmC group, and of these, CXCL9 changed most dramatically. With the decline in 5hmC expression, there was a sustained loss of CXCL9 for all pathologic grades (Fig. 2B–D).

Total numbers of infiltrating T cells, CTL and NK cells are positively correlated with 5hmC levels in cervical lesions

CXCL9, CXCL10, and CXCL11 (which are often referred to as T cell-attracting chemokines, and are recognized by the CXC chemokine receptor 3 [CXCR3]), are expressed

in several types of anti-tumor effector T cells, including cytotoxic CD8+ T cells, IFN- γ -expressing Th1 cells, NK cells, and NKT cells (Nagarsheth et al. 2017). Thus, the immune cells in cervical tissues were analyzed using multiplex immunofluorescence staining, and total CD3+ cells were registered as T cells. CD3+/CD8+ cells were counted as CTL, while CD3-/CD56+ cells were counted as NK cells (Fig. 3A). With lesion progression, the numbers of T cells, CTLs, and NK cells in the cervical tissues all gradually decreased. In samples with the same pathologic grade, the high 5hmC groups exhibited a greater number of infiltrating immune cells relative to the low 5hmC group. These results indicated that the expression of 5hmC affected the infiltration of immune cells into the extracellular tumor microenvironment (Fig. 3B–D).

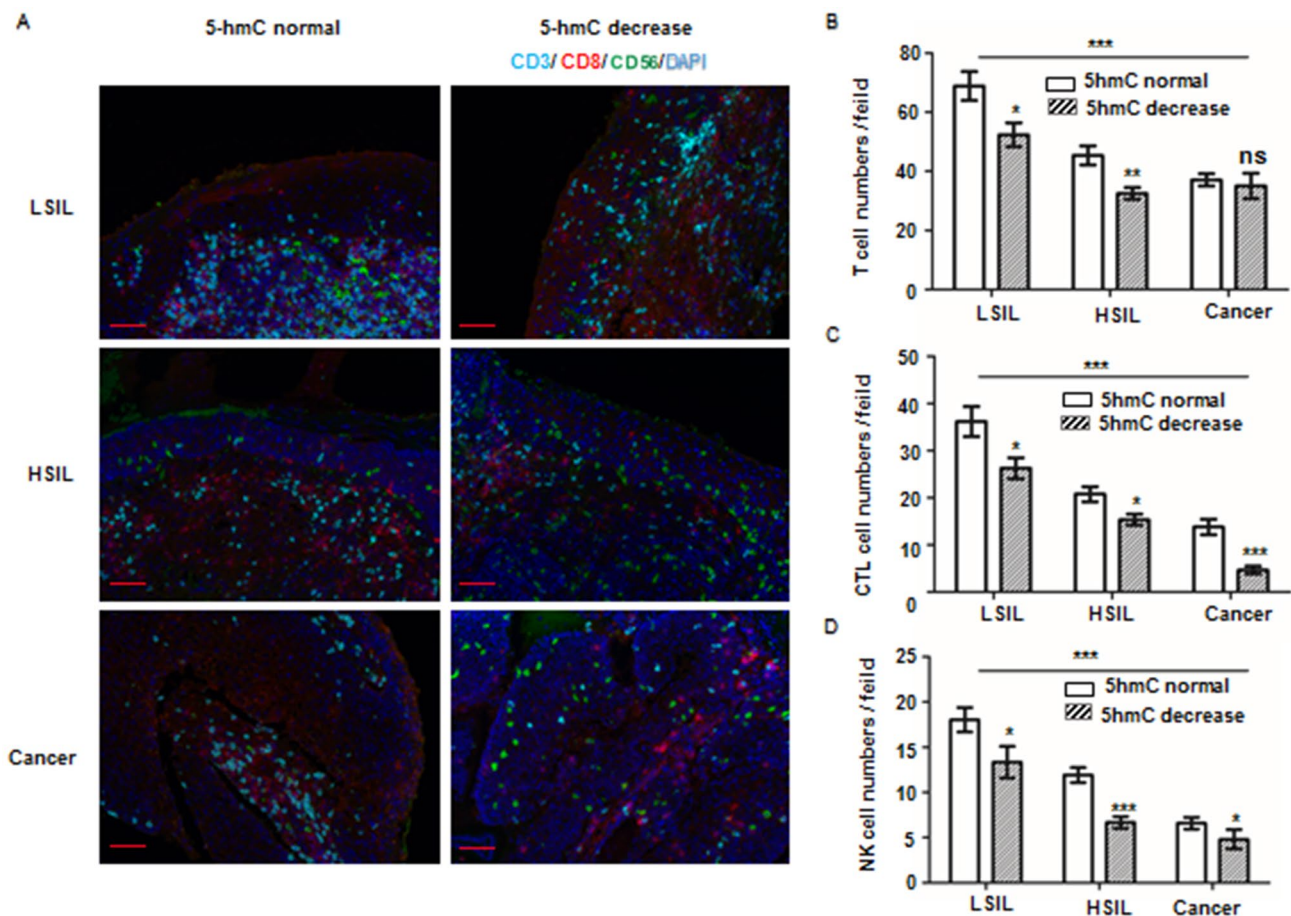


Fig. 3 Numbers of infiltrating lymphocytes—including CD3+ T cells, CD8+ cytotoxic T lymphocytes (CTLs), and CD56+ NK cells—decline commensurately with loss of 5hmC in human cervical lesions. **A** Representative photomicrographs show multicolor, fluorescently labeled inflammatory cells in different lesions as indicated. CD3, aqua blue; CD8, red; CD56, green; nuclear, blue. Scale bars=50 μ m. **B–D** Quantification of CD3+ T cells, CD8+ CTLs, and

CD56+ NK cells in cervical lesions classified by high and low 5hmC staining. Comparisons among lesion grades are conducted by one-way ANOVA. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with samples at the same lesion grade by unpaired Student's t test; ns, not statistically significant. Data are represent as mean \pm SD. Scale bar is 50 μ m.

Numbers of Th1 cells exhibit no relationship with pathologic malignancy or 5hmC levels in cervical lesions

Th1 cells in the cervical tissues were represented by CXCR4-, CD3-, and CD4-positive cells (CXCR4+/CD4+/CD3+) (Fig. 4A). The infiltration of Th1 cells showed no consistent relationship with sample malignancy or tumor cell methylation level (Fig. 4B).

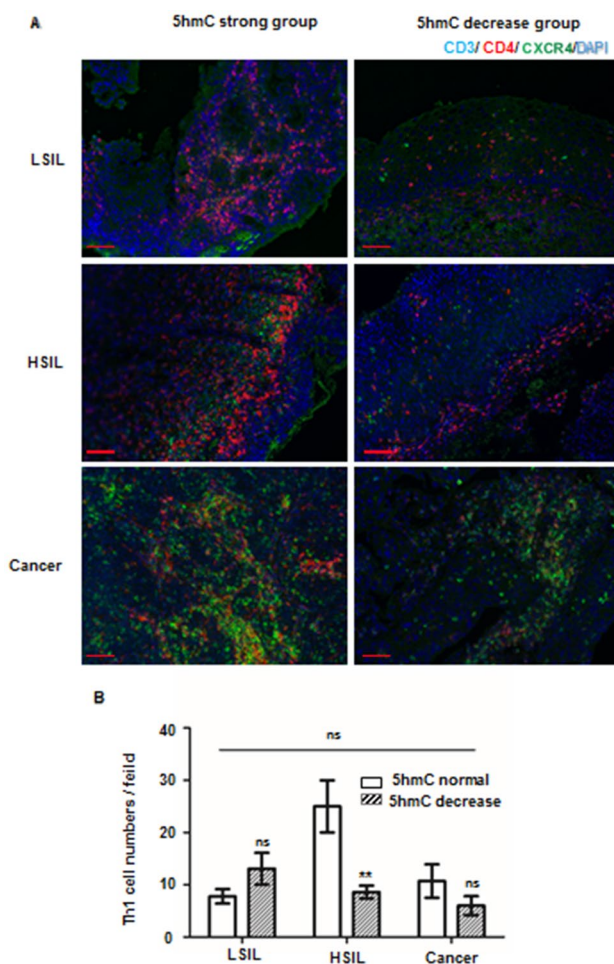


Fig. 4 The numbers of CD3+/CD4+/CXCR4+ Th1 cells show no consistent relationship with 5hmC expression in cervical lesions. **A** Representative photomicrographs show multicolor, fluorescently labeled inflammatory cells in different lesions as indicated. CD3, aqua blue; CD, red; CXCR4, green; nuclear, blue. **B** The numbers of total Th1 cells in HSIL cervical lesions with normal 5hmC were significantly higher than in other groups. * $P < 0.05$, ** $P < 0.01$, compared with samples at the same lesion grade by unpaired Student's t test. Data are represented as mean \pm SD; ns not statistically significant

Discussion

A previous study reported that a reduction in 5hmC indicated malignant cell transformation and poor tumor prognosis,²⁵ but few investigators have monitored fluctuations in 5hmC levels during the process of cancer pathogenesis from precancerous lesions onward. The present study found that in LSIL lesions, 14% of cases showed weak or negative 5hmC staining; in HSIL lesions, 58.3% of cases showed weak or negative 5hmC staining; and in carcinoma cases, 79.3% of cases showed weak or negative 5hmC staining. The decline in 5hmC was commensurate with increasing severity of lesions.

It has been proven that chemokines and tumor-infiltrating T cells play an important role in anti-tumor immunity, and that immunosuppression is involved in cancer development and progression (Nagarsheth et al. 2017). TET acts as an important mediator of the IFN- γ /JAK/STAT-signaling pathway, a major regulator of T-cell-attracting cytokines, and a recruit or of infiltrating immunocytes (Xu et al. 2019). Therefore, in the present study, it was speculated that the reduction in 5hmC expression due to attenuation of TET activity was related to the immunosuppression during the cervical cancerous process. Through analyzing the expression of T-cell-attracting chemokines and infiltrating T cells in the microenvironment of cervical lesions, it was found that in the process of cervical squamous carcinoma progression from precancerous lesions, intratumoral expression levels of CXCL9, CXCL10, and CXCL11 were attenuated and positively correlated with 5hmC levels. Furthermore, at the time of diagnosis of cervical squamous carcinoma, the total numbers of infiltrating T cells, CTLs and NK cells were reduce, which indicated that immunosuppression was inhibited during lesion progression.

Other studies have provided more insights into the effects of methylation levels on cervical squamous carcinoma. For example, one study showed that different HPV genotypes exhibited different reactions to host gene methylation (Hsu et al. 2017). The cervical tissue samples selected in the present study were all HPV-infected, but their HPV genotypes were not assessed. Therefore, exploring the influence of HPV subtypes on the methylation level of host cells may require further investigation.

Overall, this study suggests that the change in genomic methylation level as characterized by the decline in the 5hmC level may promote the occurrence and development of cervical squamous carcinoma through immunosuppression. Nuclear positivity or negativity for 5hmC was easily detected by routine immunohistochemical staining, and thus might be a good indicator for evaluating the potential malignant transformation of cervical precancerous lesions.

However, confirmation of 5hmC as an appropriate cervical cancer indicator will require further investigation.

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Author contributions XY and ZL performed the research. XS contributed essential reagents. YL and WL designed the research work. XY, ZL, and YL wrote the paper.

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Declarations

Ethical approval Concerning the use of human material, this experiment was approved by the Institutional Review Board by Fudan University (Y2018-06). All work has been carried out in compliance with the Helsinki Declaration.

Availability of data and materials All data generated or analysed during this study are included in this published article.

Conflict of interest The authors declare that they have no conflicts of interest.

Consent for publication Not applicable.

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