Promoter hypermethylation of SOX11 promotes the progression of cervical cancer *in vitro* and *in vivo*

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Abstract. The development of cervical cancer (CC) is a multi-gene, multi-step carcinogenic process that involves complex genetic and epigenetic mechanisms. SRY-related HMG-box gene 11 (SOX11) is a member of the SOX family of transcription factors with an emerging crucial role in the development of various tumor types. To elucidate the function of SOX11 in cervical carcinogenesis, the expression level of SOX11 during the development of human CC was analyzed by immunohistochemistry and western blot analysis. Additionally, the methylation status of the *SOX11* was examined using bisulfite sequencing and methylation-specific polymerase chain reaction. The *SOX11* expression and promoter methylation in human CC cell lines were also determined. The effect of *SOX11* expression restoration after 5-aza-2'-deoxycytidine (5-Aza-dC) treatment on the CC cell proliferation ability was evaluated in CC cell lines. *SOX11* was highly expressed in normal cervix (NC) and precancerous low-grade squamous intraepithelial lesions, but weakly expressed or virtually absent in precancerous high-grade squamous intraepithelial lesions and CC, which is consistent with the result of the western blot analysis. Hypermethylation of the *SOX11* promoter was detected in CC, which was significantly higher than that in NC samples at each CpG site. The expression level of *SOX11* in the CC cell lines was downregulated compared with the positive control, Tera-1human teratoma cell line. Upon 5-Aza-dC treatment, *SOX11* expression was significantly upregulated in the CC cell lines at the mRNA and protein levels, and cell proliferation was inhibited. The results indicated that the downregulation of *SOX11* in CC is due to the hypermethylation of the *SOX11* promoter region. Thus, *SOX11* methylation may have a role in the growth of CC cells and cervical carcinogenesis.

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Introduction

Cervical cancer (CC) is the fourth most common cancer in women worldwide, ~85% of which occur in low-income and middle-income countries (1,2). There are various histologic types of CC, with the majority classified as squamous cell cervical carcinomas. Squamous cell CC is considered to be the result of a multi-step process, involving a transition from precancerous low-grade squamous intraepithelial lesions (LSIL) to high-grade squamous intraepithelial lesion (HSIL) to invasive CC (3,4). Multiple factors are associated with CC development, including persistent infection with certain major subtypes of oncogenic human papillomavirus (HPV) (5,6), smoking (7,8) and immunosuppression (9). Persistent infection with certain types of oncogenic HPV is central to the etiology of CC (5), and vaccines against HPV have been approved for use and progressively introduced since 2006 (10). Based on cytologic screening for high-risk types of HPV, tests have been widely used for screening precancerous lesions and CC, and also direct the subsequent follow-up investigations, which has significantly decreased the incidence and mortality of CC (2,10). However, the specificity of CC screening methods is not high enough, and implementation is still limited and unsuiTable in many poor regions (1,6,11). Furthermore, the application of cervical biopsy under a colposcope, which is the gold standard for diagnosis of CC and depends on the results of the screening, is costly and has sometimes produced unnecessary tests in previous years (4,12). Accordingly, increased understanding of cervical carcinogenesis, and early and easy-access diagnostic methods for CC remain important. In past decades, research into cancer epigenetic modifications, particularly DNA methylation, and their contribution to tumor carcinogenesis has been continuously increasing (13-15); however, the effects of epigenetic factors on CC remain largely unknown and provide new research directions for investigating cervical carcinogenesis and CC development.

Studies have established that proteins encoded by the stem cell-related SOX family of genes are important transcription factors (TF) containing high-mobility-group (HMG) domain (16,17) that have important regulatory roles in the development, differentiation and metabolism (18,19). Abnormalities in these TFs may result in abnormal cell protein expression. SRY-related HMG-box gene 11 (SOX11), which is involved in embryonic development, cell proliferation, differentiation and apoptosis, has been reported to influence the survival, growth and transformation of tumor cells in certain solid malignancies (20-22). In recent years, several studies have reported that SOX11 acts as a tumor suppressor gene (TSG) (23-26). For instance, SOX11 was downregulated (23) and improved disease-free survival (24) in ovarian cancer. Additionally, hypermethylation-induced silencing of *SOX11* was detected in ovarian epithelial cell carcinoma, B cell lymphoma (25) and nasopharyngeal carcinoma (26), suggesting that DNA methylation of *SOX11* may have a significant role in the development of malignant tumors. The aforementioned studies demonstrated that dysfunctional SOX11 is associated with tumorigenesis in several types of cancer. However, the expression and function of the *SOX11* gene in CC has, to the best of our knowledge, not been investigated previously.

In the present study, it was demonstrated that *SOX11* was downregulated at the transcriptional and translational levels, while the promoter region of the *SOX11* gene was hypermethylated in CC tissues and cell lines compared with normal tissue or Tara-1 cells, respectively. The association between the methylation status of the *SOX11* promoter and the development of CC suggested that SOX11 may, at least in part, function as a tumor suppressor in CC and contribute to the carcinogenesis of CC.

Materials and methods

Study subjects and ethics statement. Samples from patients with CC (n=54) admitted to the Department of Gynecology at the Second Affiliated Hospital of Xi'an Jiaotong University (Xi'an) from December 2016-November 2017 were used in the present study. Biopsy samples were obtained by colposcopy prior to surgery, chemotherapy or radiotherapy. Normal cervix (NC) samples were collected from 30 hospitalized age-matched patients with uterine fibroids during hysterectomy. LSIL and HSIL samples were collected from 20 and 24 patients, respectively, undergoing colposcopy and cervical biopsies or after cervical loop electrosurgical excision procedure during the same period. All hematoxylin and eosin (H&E) sections of the specimens were reviewed and confirmed by two pathologists.

The design and implementation of the study were approved by the Ethics Committee of Medical School of Xi'an Jiaotong University (Xi'an, China; no. 2017-266). During the study, a written informed consent was obtained from all participants.

Cell culture. The human CC cell lines HeLa, SiHa, C33A and CaSki were purchased from American Type Culture Collection (Manassas, VA, USA) and Tera-1, which served as a positive control, was a gift from Dr Yue Li (Xi'an Jiaotong University Health Science Center, Xi'an, China). As SOX11 is a stem cell-associated gene, Tera-1 is a teratoma cell line that abundantly expresses stem cell-associated genes. Tera-1 was selected as the positive control. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), except for the CaSki cells which were maintained in McCoy's 5A medium (Sigma-Aldrich; Merck KGaA) supplemented with 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in a cell culture incubator at 37˚C with 5% CO_2.

5-Aza-deoxycytidine (5-Aza-dC) treatment. Cells were trypsinized, counted and seeded in triplicate in 6-wells plate. After 24 h, the medium was replaced with fresh medium containing 0 (PBS), 5, or 10 μM 5-Aza-dC (Sigma-Aldrich; Merck KGaA). The medium was replaced every 24 h. After 72 h, the total cellular RNA and protein were extracted for subsequent experiments.

Bisulfite sequencing (BSQ) and methylation-specific polymerase chain reaction (PCR). Genomic DNA was extracted using the Universal Genomic DNA Extraction kit ver. 3.0 (cat. no. DV811A; Takara Biotechnology Co., Ltd., Dalian, China). Genomic DNA (2 μg/sample) was bisulfite-modified, and the bisulfite-modified DNA was purified according to the manufacturer's protocol (EpiTect BisuLfite kit; Qiagen GmbH, Hilden, Germany). Then, the purified bisulfite-modified DNA was amplified using the following primers: *SOX11* forward, 5'-AGAGAGATTTTAATTTTTTGTAGAAGGA-3' and reverse, 5'-CCCCCTTCCAAACTACACAC-3'. The modified DNA was amplified by PCR using the following conditions: 95˚C for 3 min, and then 35 cycles of 95˚C for 30 sec, 54˚C for 30 sec and 72˚C for 30 sec, followed by a 10-min incubation at 72˚C. The PCR products were analyzed by gel electrophoresis in 2.5% agarose to confirm that a single band had been produced. Then, TA clones were established according to the instructions of the protocol for the pEASY-T1 Cloning kit (Beijing Transgen Biotech Co., Ltd., Beijing, China). The 10-15 positive monoclonal bacteria solution were sequenced by Xi'an Qing Biological Co., Ltd. (Xi'an, China). The sequencing primers were M13 forward, 5'-GTTTTCCCAGTCACGAC-3' and reverse, 5'-CAGGAAACAGCTATGAC-3'. The sequencing results were analyzed using the BiQ Analyzer 2.0 (Max Planck Institute for Informatics, Saarbrücken, Germany) and were output as circle graphs.

Reverse transcription-quantitative PCR (RT-PCR). Total RNA was extracted using the RLT reagent with 1% β-mercaptoethanol in the RNeasy Mini Kit (cat. no. 74106; Qiagen GmbH) according to the manufacturer's protocol. Total RNA (500 ng) was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (cat. no. 4368814; Applied Biosystems; Thermo Fisher Scientific, Inc.) with incubation for 10 min at 25˚C, 120 min at 37˚C and 5 min at 85˚C. qPCR was performed on an Applied Biosystems 7700 Prism RT-PCR machine (Applied Biosystems; Thermo Fisher Scientific, Inc.) and conditions were as follows: Enzyme activation for 10 min at 95˚C, PCR cycle denaturation for 15 sec at 95˚C and annealing/elongation 1 min at 60˚C. The sequences of the SOX11 primers were as follows: Forward, 5'-GGTGGATAAGGATTTGGATTCG-3' and reverse, 5'-GCTCCGGCGTGCAGTAGT-3'. Expression was normalized to the expression of

18S and transformed using the relative standard curve method and comparative quantification cycle (Ct) method (ΔΔCq) as described previously (27).

Immunoblotting. Cells and CC cell line samples were washed twice with cold PBS, followed by lysis buffer (cat. no. P0013D; Beyotime Institute of Biotechnology, Haimen, China). Protein concentration was quantified using a bicinchoninic acid kit (cat. no. P0009; Beyotime Institute of Biotechnology) according to the manufacturer's instructions. Protein lysates (25 μg) were separated at 80 V on 10% acrylamide gel for ~2 h. Transfer to Immobilon-FL (EMD Millipore, Billerica, MA, USA) membrane was performed at 20 V for 1.5 h. Following blocking in the Odyssey blocking buffer (cat. no. 927-40000; LI-COR Biosciences, Lincoln, NE, USA) for 50 min at room temperature, a primary antibody rabbit polyclonal anti-human SOX11 (1:500 dilution; cat. no. sc-20096; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), GAPDH (1:10,000 dilution; cat. no. G8795; Sigma-Aldrich; Merck KGaA) and β-tubulin (1:5,000 dilution; cat. no. sc-80011; Santa Cruz Biotechnology) were incubated at 4˚C overnight. Secondary antibody conjugated to Alexa Fluor® 680 dye (cat. no. A32734; Invitrogen; Thermo Fisher Scientific, Inc.) or IRdye800 (cat. no. 610-731-002; Rockland Immunochemicals Inc., Limerick, Pennsylvania, USA) was subsequently incubated with the membrane for 1 h at room temperature to visualize the proteins at 700 or 800 nm using a LI-COR Odyssey imaging system (LI-COR Biosciences). The results were quantitatively analyzed using the ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Immunohistochemistry. Human specimens were fixed with 4% paraformaldehyde overnight at room temperature, then embedded in paraffin and sectioned into 4 μm slices. The slides were baked at 65˚C overnight prior to deparaffinization in xylene twice for 20 min and hydrated in a series of graded ethanol (100, 95, 90 and 80% ethanol; 5 min each). The sections were boiled in citrate buffer (pH 6.0) by heating in a pressure cooker for 1-2 Alexa Fluor® 680 min for antigen retrieval. Endogenous peroxidases were blocked for 10 min with freshly prepared 3% H_2O_2 at room temperature. Following blocking with goat serum for 30 min at room temperature, the SOX11 primary antibody (1:200 diluted in 1% bovine serum albumin PBS solution; cat. no. sc-20096; Santa Cruz Biotechnology, Inc.) was incubated with the slides at 4˚C overnight. The following day, the slides were incubated with a secondary antibody labeled with horseradish peroxidase (1:50 dilution, cat. no. GAR-HRP; Pierce; Thermo Fisher Scientific, Inc.) for 40 min at room temperature. Diaminobenzidine was used to color the slides for ~20 min at room temperature. The sections were observed under the microscope to control the reaction time when the sections were incubated with the DAB reagent for signal amplification. Then the sections were stained with H&E. Histological analysis was performed by two blinded pathologists. At least 10 high-power fields at x1,000 magnification were examined for each sample. The staining score was classified into four grades based on the staining intensity as follows: 1, no cell staining; 2, weak yellow; 3, moderate yellow; and 4, strong brown yellow. The proportion of positively stained cells was classified into four levels as follows: 1 (0-25%), 2 (26-50%), 3 (51-75%) and 4 (76-100%). The immunoreactivity score (IRS) was obtained by multiplying the intensity and proportion values, and samples were grouped into three levels as follows: Negative (-, score 1-4), positive (+, score 5-9), strongly positive (++, score 10-16). The mean score was used for comparison between groups.

Proliferation assay. Cell viability was measured using the PrestoBlue kit (Invitrogen; Thermo Fisher Scientific, Inc.). A total of 2,000 cells in the logarithmic growth phase were seeded into a 96-well plate in triplicate and allowed to adhere overnight. Throughout the 6-day period, the medium was replaced with fresh medium every day, and the same concentration of 5-Aza-dC was added. PrestoBlue reagent was used to assess the proliferative ability according to the standard manufacturer's protocol. The fluorescence was read at a wavelength of 570 nm using a FLUOstar Optima microplate spectrophotometer (BMG Labtech GmbH, Ortenberg, Germany).

Statistical analysis. Statistical analyses were performed using the Prism 7 software (GraphPad Software Inc., La Jolla, CA, USA) and SPSS version 22.0 for Windows (IBM Corp., Armonk, NY, USA). The unpaired t-test with Welch's correction was used to analyze the difference between two groups. The two-tailed Mann Whitney U test with Bonferroni's correction for post hoc comparisons was performed following the Kruskal-Wallis test and Tukey's post hoc test was used following the one-way analysis of variance test to evaluate the statistical comparison of multiple groups. The Pearson χ^2 analysis was used to analyze the association between SOX11 expression and the clinical features, when the number was <5, Fisher's exact test was used. Pearson's correlation test was used to analyze the correlation between the SOX11 mRNA expression level and its promoter methylation level. Error bars represent ± standard error. $P<0.05$ was considered to indicate a statistically significant difference.

Results

SOX11 expression is downregulated during the development of CC. SOX11 has been demonstrated to have different properties in different human cancers. It is upregulated in gastric (28) and prostate cancer (29), and other types of cancers, whereas it is downregulated in medulloblastoma (30) and malignant gliomas (20). To determine the expression of SOX11 during the development of CC, SOX11 protein expression level was analyzed by immunohistochemistry and western blot analysis in NC, LSIL, HSIL and CC tissues. SOX11 was localized in the nuclei of all positive cells with different levels in different specimens. SOX11 was highly expressed in NC and LSIL epithelial basal cells, and weakly expressed or virtually absent in tumor parenchymal cells of HSIL and CC. The IRS of SOX11 was as follows: NC, 9.933±0.948; LSIL, 7.200±0.766; HSIL, 4.917±0.492; and CC, 3.074±0.301 (Fig. 1B). With the progression of cervical lesions, the expression level of SOX11 gradually decreased, and there were statistical differences in IRS levels of SOX11 in the four groups (NC vs. LSIL, P=1.000; NC vs. HSIL, P=0.022; NC vs. CC, P<0.0001; LSIL vs. HSIL, P=0.625; LSIL vs. CC, P<0.0001; HSIL vs. CC, P=0.057; P-value are adjusted). In addition, the

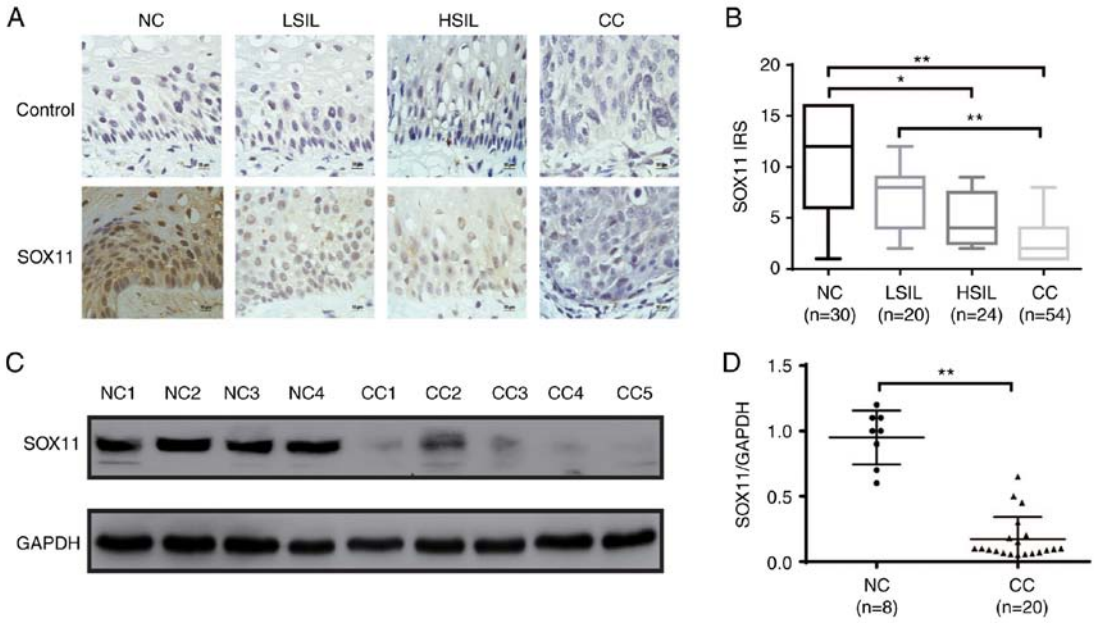

Figure 1. SOX11 expression during the development of CC. (A) Expression of SOX11 in normal cervix, LSIL, HSIL and CC was evaluated by immunohistochemistry. PBS was used instead of SOX11 antibody as control group. (B) IRS presented as box plots and significance calculated by the Mann Whitney U test with Bonferroni's correction (new P<0.0083 was required for significance, the adjusted P-values are shown as *P≤0.05, **P≤0.01). (C) SOX11 protein expression level was determined by western blot analysis in randomly selected tissues (8 NC and 20 CC). (D) Relative expression of the SOX11/GAPDH was estimated by densitometry. Bars represent standard error and data was analyzed by unpaired t tests. *P≤0.05, **P≤0.01. NC, normal cervix; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion; CC, cervical cancer; SOX11, SRY-related HMG-box gene 11.

expression level of SOX11 was determined by western blot analysis in randomly selected tissues including 8 NC and 20 CC (Fig. 1C). The relative expression of the SOX11protein is presented as mean SOX11 expression of 0.95±0.07 in NC, and 0.17±0.04 in CC (Fig. 1D). Thus, the expression of SOX11 protein in NC was 5.59 fold higher than that in CC specimens (P<0.001). The association between SOX11 expression based on the immunohistochemistry staining results and the clinicopathological characteristics in the patients with CC are summarized in Table I. The Pearson χ^2 analysis revealed a significant association between SOX11 expression and the tumor grade (P=0.005) and HPV status (P<0.001); however, there was no significant association between SOX11 expression and other clinical features, including age, International Federation of Gynecology and Obstetrics stage, lymph node, myometrial invasion depth and histologic type. The association between SOX11 expression and the HPV status in the patients with LSIL and HSIL in Tables II and III reveal a significant association between SOX11 expression and HPV status (P=0.032 and 0.035, respectively). These findings suggest that the expression of SOX11 decreases with the development of CC malignancy, which strongly suggested that the loss of SOX11 function, which may act as a tumor suppressor, promotes the progression of CC.

Promoter region of SOX11 is hypermethylated in CC. The hypermethylation of promoter CpG islands is one of the essential mechanisms of transcriptional silencing of TSGs, and also the most important early, common event in the process of cervical disease progression to cancer (13,31-34). To further investigate the mechanism of the downregulation of SOX11 during the development of CC, 10 NC and 10 CC specimens were randomly selected to determine the methylation status of the SOX11 promoter. Four CpG islands of SOX11 were identified with a GC content >50% and observed/expected CpG ratio >0.6 when to promoter region (2,000 bp upstream of the transcription start site) of SOX11 was analyzed using MethPrimer 2.0 (urogene.org/methprimer2/; Fig. 2A). Subsequent sequencing experiments were performed on the fourth CpG island, which had previously been reported to be determinative for SOX11 expression in multiple cell lines (25) and includes 28 CpG sites (Fig. 2A). The results are presented in Fig. 2B, with the CpG sites are indicated with circles (solid circle indicate methylation and unshaded circle indicates no methylation). The SOX11 promoter was hypermethylated in CC (total mean level was 85.71%), which was significantly higher than that in the NC samples (total mean level was 12.68%) at each CpG site (Fig. 2C). The mRNA expression level of SOX11 in CC was significantly lower than that in NC tissues (P<0.001; Fig. 2D). Pearson's correlation analysis of the SOX11 mRNA expression level and its promoter methylation level revealed that the SOX11 mRNA expression level in CC was negatively correlated with hypermethylation in the promoter region (r=-0.8080; P<0.001; Fig. 2E). The expression of SOX11 protein was downregulated, with its promoter region hypermethylated in CC. These results suggest that hypermethylation of the SOX11 promoter may be involved in the mechanism of downregulation of SOX11 in CC.

Methylation status of SOX11 promoter in CC cell lines. The transcriptional and translational level of SOX11 and its promoter methylation status was examined in CC cell lines. A marked difference was observed between the SOX11 expression and promoter methylation level. The Tera-1 cell line as

Table I. Association between SOX11 expression and clinicopathological characteristics in patients with cervical cancer.

Factor	n	SOX11 expression		P-value
		Negative	Positive	
Age, years				0.483
<45	8	7	1	
≥45	46	35	11	
Grade				0.005
I	20	11	9	
II-III	34	31	3	
International federation of gynecology and obstetrics stage				0.591
I	6	4	2	
II	42	34	8	
III-IV	6	4	2	
Lymph node				0.855
N0	10	8	2	
N1	44	34	10	
Myometrial invasion depth				0.562
<1/2	22	18	4	
≥1/2	32	24	8	
Histologic type				0.595
Squamous carcinoma	40	37	3	
Adenocarcinoma	14	12	2	
HPV infection				<0.001
Positive	43	40	3	
Negative	11	4	7	
Total	54			

SOX11, SRY-related HMG-box gene 11.

Table II. Association between SOX11 expression and clinicopathological characteristics in patients with low-grade squamous intraepithelial lesion.

HPV infection	n	Sox11 expression		P-value
		Negative	Positive	
Positive	16	14	2	0.032
Negative	4	1	3	

SOX11, SRY-related HMG-box gene 11.

Table III. Association between SOX11 expression and clinicopathological characteristics in patients with high-grade squamous intraepithelial lesion.

HPV infection	n	Sox11 expression		P-value
		Negative	Positive	
Positive	20	17	3	0.035
Negative	4	1	3	

SOX11, SRY-related HMG-box gene 11.

a positive control. The results revealed high levels of *SOX11* promoter methylation in HeLa, C33A, CaSki and SiHa (90.71, 77.14, 99.64 and 98.21%, respectively) cell lines, consistent with a lack of *SOX11* mRNA and protein expression. By contrast, *SOX11* promoter methylation was low in the Tera-1 (10.36%), and its mRNA and protein expression was significantly higher than those in the four CC cell lines (Fig. 3). These findings suggest that SOX11 expression in the CC cell lines is negatively associated with the methylation level of the promoter, which is consistent with the results in human tissue specimens.

SOX11 expression is enhanced by 5-Aza-dC and increased SOX11 expression inhibits the proliferation of CC cells. As the silencing of TSGs by aberrant methylation of the gene

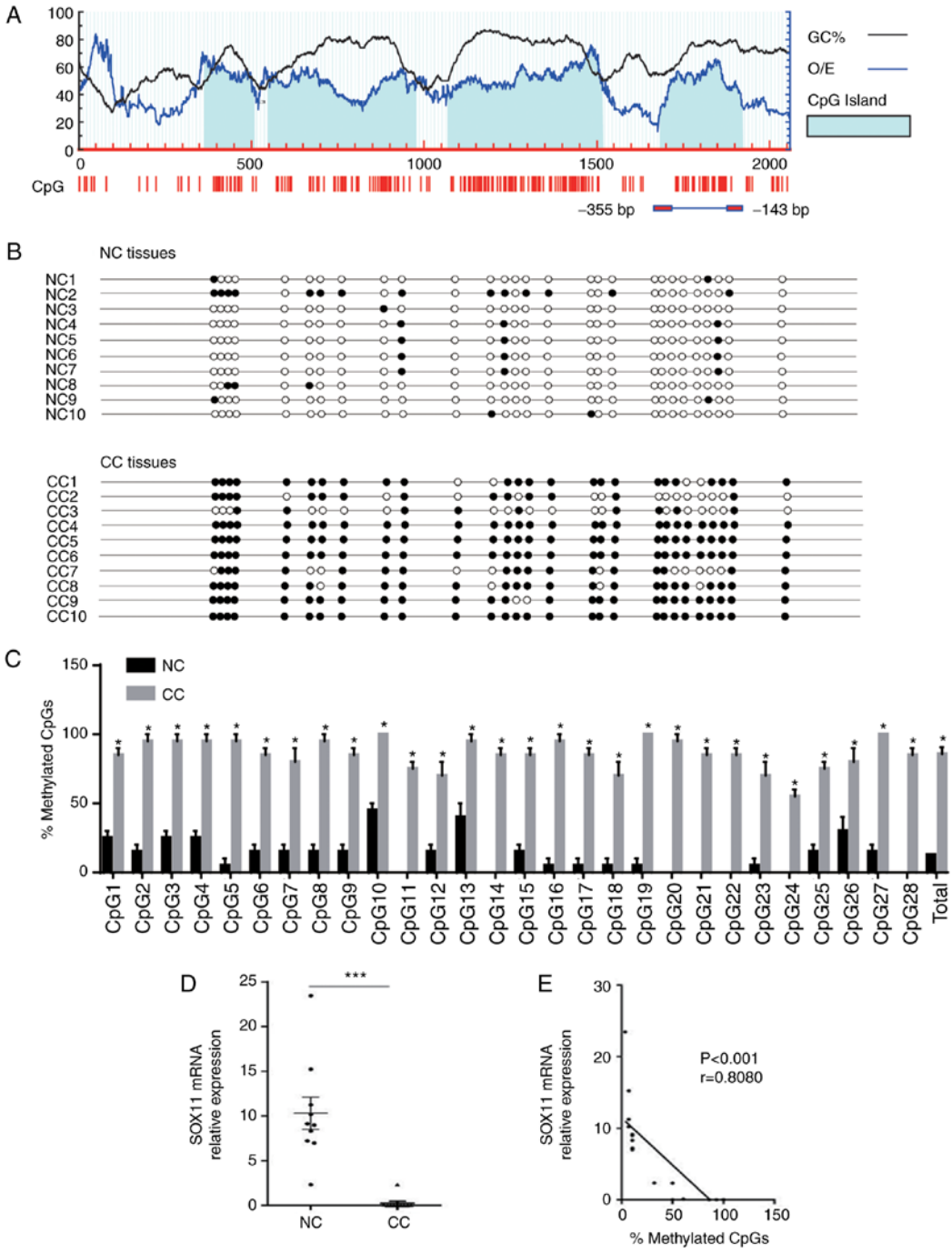

Figure 2. SOX11 promoter region is hypermethylated in CC patients. (A) A schematic representation of the CpG islands in the transcription start site of the SOX11 genomic locus. Four CpG islands on the *SOX11* gene promoter region were identified with a GC content >50% and O/E CpG ratio above 0.6 by MethPrimer 2.0 (http://www.urogene.org/methprimer2/). The fourth CpG island regions marked in red were bisulfite sequenced. (B) Bisulfite-converted DNAs from 10 randomly selected normal tissues and 10 randomly selected CC specimens were amplified at the SOX11 promoter, and the fragments were sequenced. A total of 28 CpG sites are represented as shown. The solid circles are methylated CpG sites, and the hollow circles are unmethylated. (C) The methylation level ratio of each CpG sites of the sequenced *SOX11* promoter region in CC and NC. (D) The mRNA expression level of *SOX11* in CC and NC tissues. (E) Pearson linear regression analysis of the *SOX11* mRNA expression level and its promoter methylation level. Bars represent standard error. *P<0.05, ***P≤0.001. O/E, observed/expected; NC, normal cervix; CC, cervical cancer; SOX11, SRY-related HMG-box gene 11.

promoter is reversible (35), to further investigate the role of hypermethylated promoter level in the expression regulation of SOX11, HeLa, C33A, CaSki and SiHa cell lines were treated with different doses of the demethylating agent 5-Aza-dC. The mRNA and protein expression levels of *SOX11* under different conditions were measured by RT-qPCR and western blot analysis. As shown in Fig. 4A, when DNA of cervical cells was demethylated with different doses of 5-Aza-dC, the *SOX11* mRNA level was increased from 0.95±0.05 to 15.65±0.65 in HeLa, 0.95±0.07 to 34.30±0.70 in C33A, 1.12±0.11 to 57.85±1.05 in CaSki and 1.00±0.12 to 31.2±0.80 in SiHa cells treated with 10 μM compared with no 5-Aza-dC treatment. Similarly, the SOX11 protein expression level was gradually increased from 0.78 to 1.42 in HeLa, 0.52 to 1.28 in C33A,

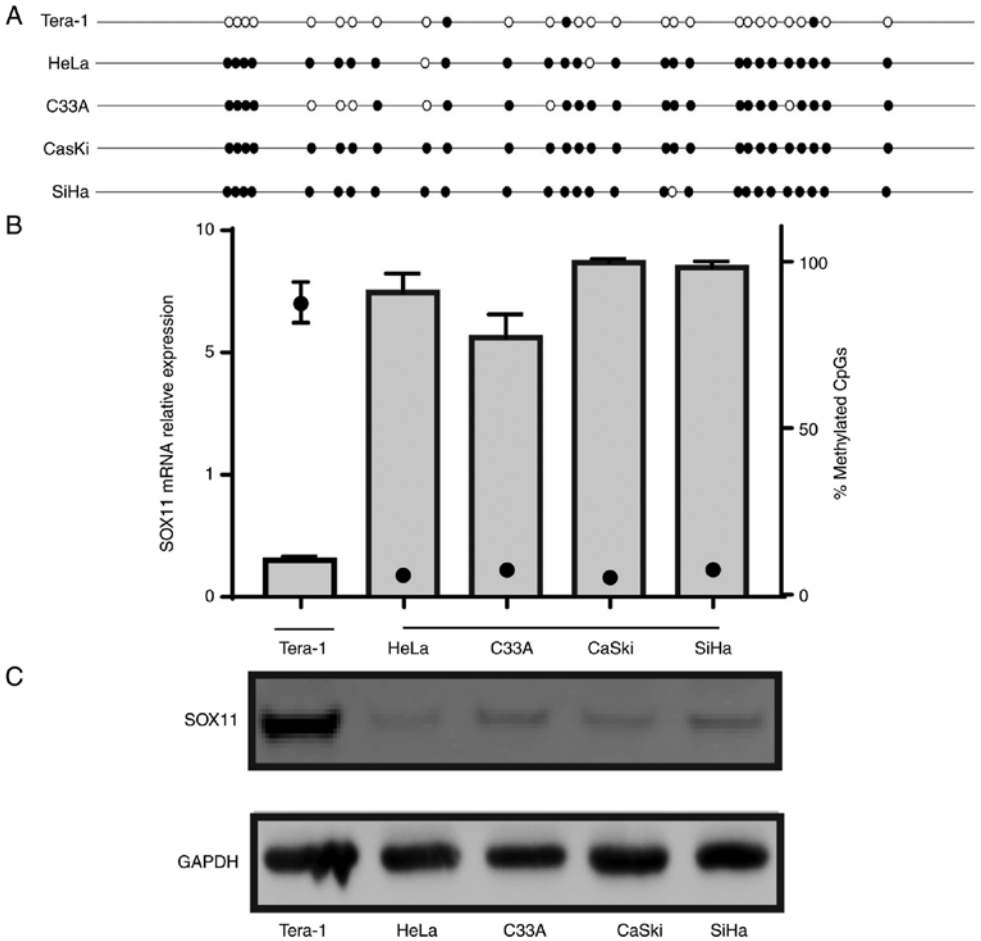

Figure 3. Methylation status of SOX11 promoter region correlated to SOX11 expression in cervical cancer cell lines. (A) Bisulfite sequencing of the *SOX11* promoter in the Tera-1 human teratoma 1 cell line and the cervical cancer cell lines, HeLa, C33A, CaSki and SiHa. (B) Methylation status of the *SOX11* promoter is indicated by columns (right y-axis) and corresponding mRNA expression level of SOX11 is indicated by dots (left y-axis), and (C) the SOX11 protein expression detected by western blot analysis. SOX11, SRY-related HMG-box gene 11.

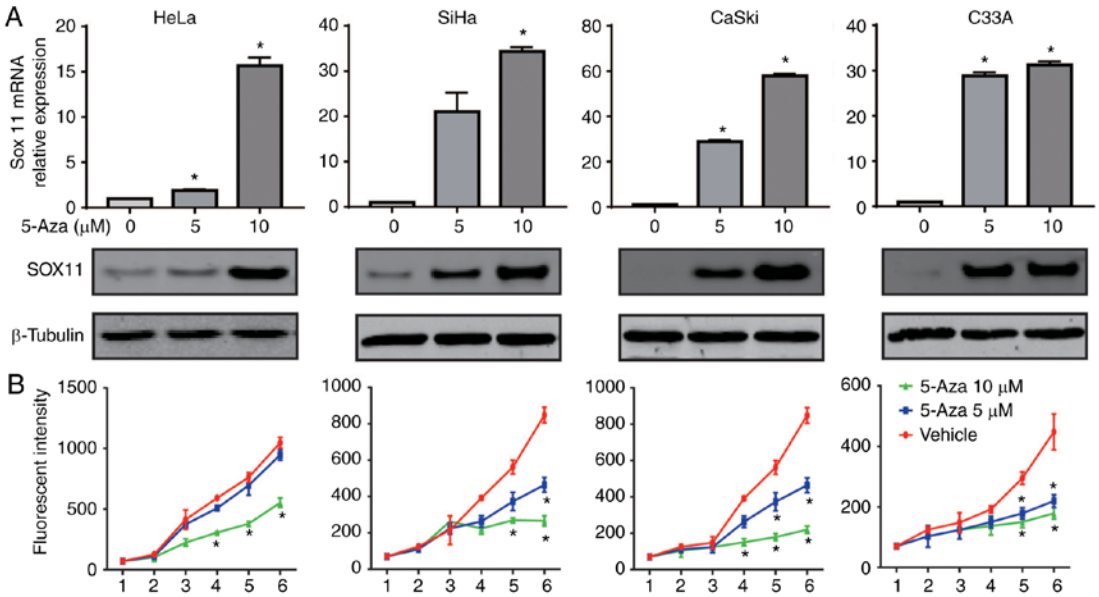

Figure 4. SOX11 expression and effect on cell proliferation upon treatment with the demethylating agent 5-Aza in cervical cancer cells. (A) SOX11 mRNA levels were quantified by reverse transcription-quantitative polymerase chain reaction analysis for three independent RNA samples in cervical cancer cell lines following treatment with different doses of 5-Aza (0, 5 and 10 μM) for 72 h. Data presented averaged from three independent experiments in biological triplicate, relative to non-treated cells (± standard error and significance calculated by analysis of variance followed by Tukey's multiple comparison test). *P≤0.05 vs. 0 μM. Corresponding protein expression is shown below. (B) Cell proliferation ability was determined using the PrestoBlue kit after treatment with different doses of 5-Aza (0, 5 and 10 μM). The fluorescence intensity, which represents the relative viable cells, is shown in the linear diagram. Data are averaged from three independent experiments in biological triplicate, relative to non-treated cells (±standard error and significance calculated by analysis of variance followed by Tukey's multiple comparison test). *P≤0.05 vs. 0 μM. SOX11, SRY-related HMG-box gene 11; 5-Aza, 5-aza-2'-deoxycytidine.

0.38 to 1.36 in CaSki and 0.70 to 1.38 in SiHa cells comparing no 5-Aza-dC treatment to 10 μM. Cell viability was also to determine the contribution of the SOX11 expression level to CC cell growth. The proliferation ability of cervical cells was significantly suppressed by 5-Aza-dC (Fig. 4B). These results suggest that the hypermethylation of the promoter reduces *SOX11* expression in the four CC cell lines and activation of *SOX11* by demethylation of the promoter significantly inhibited the proliferation of cervical cells.

Discussion

The development of CC is a multi-gene, multi-step carcinogenesis process that involves complex genetic and epigenetic mechanisms. DNA methylation is a common form of epigenetic modification and silencing of TSGs via hypermethylation of their promoter, particularly CpG islands, is associated with the genesis of multiple tumors (23,36). Paz *et al* (37) reported that at least one hypermethylated gene exists in each of 70 tumor cell lines analyzed. In addition, studies have demonstrated that cancer stemness genes in the tumor are downregulated due to methylation, such as SOX9 in gastric cancer (38), and Krüppel-like factor 4 in multiple tumors including bladder (39) and colorectal cancer (40), and CC (41). Accordingly, changes in gene methylation status are one of the key factors involved in carcinogenesis.

Currently, the role of SOX11 in tumor development is controversial, as it has been associated with both improved and worsened survival (23-25,42,43). In recent years, researchers have focused on the relevance of the SOX11 expression in carcinogenesis (26,28). Xu *et al* (28) reported that the silencing of *SOX11* as TSG in gastric cancer cell lines and primary tissues was due to the hypermethylation of the *SOX11* promoter region, which could be a novel target for the treatment of GC. Sernbo *et al* (23) reported that the re-expression of *SOX11* using the demethylating drug 5-Aza-dC in epithelial ovarian cancer inhibited the growth of ovarian cancer cell lines. Additionally, hypermethylation of *SOX11* contributed to the downregulation of *SOX11* and promoted cell growth and invasion in nasopharyngeal carcinoma (26). These findings suggest that aberrant DNA methylation of *SOX11* has a major role in the development of certain malignant tumors.

However, the regulatory mechanism of SOX11 expression is not precisely the same, or even be opposite, in different malignancies. Histone acetylation can affect the corresponding chromosome structure, change the level of gene transcription, and subsequently, affect the cell cycle, differentiation and apoptosis regulation, which can ultimately lead to tumor formation. A highly acetylated state usually leads to transcriptional activation and a deacetylated state often leads to transcriptional silencing. The levels of histone H3-acetylation at the SOX11 locus is also associated with transcriptional activity (44). Vegliante *et al* (45) treated mantle cell lymphomas (MCL) cells with the histone acetylation inhibitor (SAHA) and/or methyltransferase inhibitor (Aza). The treatment with SAHA can reversed the expression of the *SOX11* gene despite the methylation state in SOX11 low-expressing cell lines, while Aza did not increase the expression of *SOX11*, suggesting that the repression of *SOX11* through promoter methylation is not the only mechanism involved, as the expression of SOX11 is associated with histone acetylation. Wasik *et al* (46) demonstrated high SOX11 mRNA expression in the majority of the MCL examined. The mRNA and protein expression of SOX11 in the MCL cell lines, Granta 519 and Rec-1, was decreased following the administration of the demethylating 5-Aza-CdR agent, which further indicated that the regulation of *SOX11* gene expression in MCL was not caused by high methylation of the promoter region. Additionally, there may be other regulatory mechanisms, such as the common regulatory mechanisms of the SOXC group (SOX11, SOX4 and SOX12) (46). In the present study, it was demonstrated *SOX11* expression was regulated by its promoter methylation status in CC. More clinical samples and experimental data are required to be studied to confirm the involvement of such a mechanism.

Furthermore, a significant association between SOX11 expression and the presence of HPV was revealed in patients with CC, LSIL and HSIL. Persistent infection with HPV is a key factor during the carcinogenesis of CC. The E6 gene of HPV can induce the degradation of tumor protein 53 (TP53) via ubiquitin-proteasome pathway, which sequentially inhibits the function of TP53 as a tumor suppressor gene. Chang *et al* (47) used a luciferase assay and glutathione S-transferase pull-down experiments to demonstrate that SOX11 could interact with TP53 *in vitro* and promote the transcriptional activity of TP53. These findings suggested that the expression of SOX11 during the initiation and development of CC may be associated with the E6 gene of HPV and the tumor suppressor gene *TP53*; however the specific mechanism requires further investigation.

There are also certain limitations of the present study. Immunohistochemistry was performed to detect SOX11 and the expression was quantified. The sections were obtained from the adjacent sections of which the pathological types had been confirmed by H&E staining. However, LSIL and HSIL sample lysates could not be obtained to determine the protein level of SOX11 by western blotting and to detect the methylation state of SOX11. The aberrant expression of SOX11 was reversed by the wide-spectrum demethylating drug, 5-Aza-dC, which suggested the effects 5-Aza-dC are associated with SOX11, at least in part. A knock-in SOX11 on CC cells using the inducible Crispr-Cas9 system to investigate the specific effects of SOX11 demethylation on cell proliferation and also investigating the role of SOX11 in the invasive potential CC cells are planned for future studies.

In conclusion, SOX11 expression was significantly downregulated in CC compared with normal tissue, suggesting that SOX11 may have a role as a tumor suppressor gene. In addition, hypermethylation of the *SOX11* promoter in CC samples and cell lines was observed compared with NC and Tara-1 cells. Additionally, the downregulation of SOX11 were reversed by a demethylating drug at the mRNA and protein levels, and cell viability was also inhibited. The *SOX11* promoter methylation is anticipated to be a new molecular marker for the diagnosis of CC and a treatment target. More experiments are required to confirm the function of SOX11 in CC. Studies with larger sample sizes and a long-term follow-up period are required to further investigate the clinical significance of SOX11 in CC.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

YL, YC, XL, NS, MD and YY collected the samples. YL and YC performed the experiments; XL and RT analyzed and interpreted the data; YG, XL, RT and NS drafted the article; XL, YG and XW designed the work; NS polished the language; XL, MD, RT, NS and YY revised it critically. YG ORCID no. 0000-0002-7894-7312. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The design and implementation of the study were approved by the Ethics Committee of Medical School of Xi'an Jiaotong University (Shannxi, China; no. 2017-266).

Patient consent for publication

During the study, a written informed consent for the publication was obtained from all participants.

Competing interests

The authors declare that they have no competing interests.

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