5-Azacytidine suppresses EC9706 cell proliferation and metastasis by upregulating the expression of SOX17 and CDH1

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Abstract. 5-Azacytidine is a well-known anticancer drug that is clinically used in the treatment of breast cancer, melanoma and colon cancer. It has been reported that 5-azacytidine suppresses the biological behavior of esophageal cancer cells. However, corresponding mechanisms remain unclear. In this study, using Transwell invasion and cell proliferation assays, we demonstrated that 5-azacytidine significantly inhibited the metastasis and proliferation of EC9706 cells, and upregulated the expression of cadherin 1 (CDH1) and SRY-box containing gene 17 (SOX17). Moreover, the inhibition of the metastasis of the 5-azacytidine-treated EC9706 cells was impaired following transfection with siRNA targeting CDH1 (CDH1 siRNA), and the inhibition of cell proliferation was attenuated following the downregulation of SOX17 by siRNA targeting SOX17 (SOX17 siRNA). Furthermore, 5-azacytidine remarkably reduced the CDH1 and SOX17 promoter methylation levels, suggesting that 5-azacytidine upregulates the expression of SOX17 and CDH1 by inhibiting the methylation of the SOX17 and CDH1 promoter. The findings of our study confirm that 5-azacytidine suppresses the proliferation and metastasis of EC9706 esophageal cancer cells by upregulating the expression of CDH1 and SOX17. The expression levels of CDH1 and SOX17 negatively correlate with the promoter methylation levels. CDH1 and SOX17 are potential indicators of the clinical application of 5-azacytidine.
hypermethylation of the CDH1 and SOX17 promoters and the reduced expression of CDH1 and SOX17 have been reported to occur in esophageal cancer (33-35). These data suggest that these two genes are epigenetically involved in the development and progression of esophageal cancer.

Based on the facts that 5-azacytidine can affect DNA methylation and that the promoters of tumor suppressor genes are usually downregulated by high hypermethylation in esophageal cancer, in this study, we aimed to examine the effects of treatment with 5-azacytidine on esophageal cancer cells and to elucidate the mechanisms responsible for its antitumor activity in esophageal cancer. Our findings may help clinicians select an effective chemotherapeutic regimen for the treatment of esophageal cancer, and may also aid in the prognostic evaluation.

Materials and methods

Cell culture. The EC9706 esophageal cancer cell line was purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (both from Thermo Fisher Scientific, Waltham, MA, USA) and 100 U/ml penicillin/streptomycin (Invitrogen Life Technologies, Carlsbad, CA, USA), and were routinely incubated at 37°C under a 5% CO₂ atmosphere.

Treatment with 5-azacytidine. The EC9706 cells were seeded into 6-well culture plate (NEST Biotechnology, Jiangsu, China) at a density of 10⁴ cells/well and cultured overnight. The cells were treated with 5-azacytidine (50 µM) (5-aza; Sigma-Aldrich, St. Louis, MO, USA) for 72 h and dimethyl sulfoxide (DMSO; 1:100) as the negative control.

Reverse transcription-quantitative PCR (RT-qPCR). Following treatment with 5-azacytidine for 72 h, total RNA was isolated from the EC9706 cells using TRIzol reagent (Life Technologies) according to the manufacturer's instructions. RNA was reverse transcribed using the PrimeScript® First Strand cDNA Synthesis kit (Takara, Shiga, Japan) and then amplified with PCR primers on the iQ5 Real-time Quantitative PCR system (Bio-Rad, Hercules, CA, USA). The average Ct, from triplicate assays, was used for further calculations. Relative expression levels were normalized to the control and actin as the internal control. The interference efficiency was detected by RT-qPCR at 48 h post-transfection.

Cell viability assay. The EC9706 cells were seeded into 96-well culture plates (5,000 cells/well). 5-Azacytidine was added to the wells at a concentration of 50 µM. Cell viability was assayed using the Cell Counting kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) at 1-5 days following treatment with 5-azacytidine. The absorbance was measured at 450 nm using a microplate reader.

Cell colony formation assay. Soft agar assays were performed to detect the colony-forming ability of the EC9706 cells at 96 h following treatment with 5-azacytidine (50 µM). The cells were then resuspended and seeded into 6-well culture plates (1,000 cells/well). On day 7, the cells were washed with PBS twice and fixed with 75% ethanol for 30 min and stained with 0.2% crystal violet for visualization and photographing by microscope (Mi 8; Leica, Wetzlar, Germany).

Apoptosis assay. Cell apoptosis was determined by flow cytometry using the Annexin V-FITC Apoptosis Detection kit (Sungeen, Tianjin, China). The EC9706 cells were seeded in 6-well culture plates. 5-Azacytidine was added to the wells at the concentration of 50 µM. The cells were harvested 96 h following treatment with 5-azacytidine, washed in PBS and
5-Azacytidine suppresses the proliferation and invasion of EC9706 cells. To examine the effects of 5-azacytidine on the growth of esophageal cancer cells, the EC9706 cells were treated with 50 µM 5-azacytidine for 72 h, and CCK-8 cell viability assay and Transwell assay were then performed to determine the effects of 5-azacytidine on cell proliferation and invasion. The results of CCK-8 assay revealed that the viability of the EC9706 cells was markedly suppressed following treatment with 5-azacytidine (Fig. 1A). In addition, the results of Transwell invasion assay indicated that the invasive ability of the EC9706 cells was significantly decreased following treatment with 5-azacytidine (Fig. 1B and C).

Treatment with 5-azacytidine upregulates CDH1 and SOX17 expression in EC9706 cells. Studies have reported that CDH1 and SOX17 are involved in the inhibition of cancer metastasis and growth, respectively. CDH1 and SOX17 are downregulated in esophageal cancer (22,32). Therefore, in this study, RT-qPCR and western blot analysis were performed to analyze the expression of CDH1 and SOX17 in the EC9706 cells treated with 5-azacytidine (50 µM) for 72 h. The results revealed that the CDH1 and SOX17 expression levels were significantly increased in the 5-azacytidine treatment group, in comparison to the DMSO vehicle control (Fig. 2A). The results of western blot analysis also revealed that the CDH1 and SOX17 protein expression levels were significantly upregulated by 5-azacytidine treatment (Fig. 2B).

5-Azacytidine inhibits EC9706 cell metastasis via the upregulation of CDH1. To further confirm the involvement of CDH1 in the inhibition of EC9706 cell invasion induced by 5-azacytidine, the cells were transfected with CDH1 siRNA to knockdown CDH1 expression. The results of western blot analysis revealed that transfection with CDH1 siRNA suppressed the upregulation of CDH1 that was induced by 5-azacytidine (Fig. 3A). The results of Transwell invasion assay demonstrated that the inhibitory effects of 5-azacytidine on EC9706 cell invasion were impaired by transfection with CDH1 siRNA (Fig. 3B). Western blot analysis also revealed that 5-azacytidine significantly downregulated the expression of matrix metalloproteinase (MMP)2 and MMP9. The suppressive effects of 5-azacytidine on the expression of MMP2 and MMP9 were attenuated by transfection with CDH1 siRNA (Fig. 3C). These results indicate the involvement of CDH1 in the suppression of EC9706 cell metastasis by 5-azacytidine.

5-Azacytidine inhibits EC9706 cell proliferation through the upregulation of SOX17. To verify the involvement of SOX17 in the inhibition of EC9706 cell proliferation by 5-azacytidine, SOX17 siRNA was used to knockdown SOX17 expression. The results of western blot analysis revealed that transfection with SOX17 siRNA suppressed SOX17 expression which had been incubated with Annexin V and propidium iodide (PI) in binding buffer in the dark at room temperature for 10 min. The stained cells were analyzed using the BD FASARia Cell Sorter (BD Biosciences, Franklin Lakes, NJ, USA).

### Table I. Primer used for bisulfite-assisted genomic sequencing PCR (BSP).

<table>
<thead>
<tr>
<th>Name</th>
<th>Forward primer (5'-3')</th>
<th>Reverse primer (5'-3')</th>
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<tbody>
<tr>
<td>CDH1-1</td>
<td>TGATTTTAGGTTTTAGTGAG</td>
<td>CAAACTCACAAATACCTTTA</td>
</tr>
<tr>
<td>CDH1-2</td>
<td>GAATTGTTAATTTTGTAGTTTG</td>
<td>AATACCTACAACAAACAAAC</td>
</tr>
<tr>
<td>CDH1-3</td>
<td>GTGTGTTTGTGTTGTTAGTATT</td>
<td>CACTCCCCATCAAAACATCC</td>
</tr>
<tr>
<td>SOX17-1</td>
<td>AGAGTTAGGAAGAATTTTGA</td>
<td>CAAAACTAACCTACCCC</td>
</tr>
<tr>
<td>SOX17-2</td>
<td>GGGTTGAGTGTAGTTTTTG</td>
<td>TACCCCAAAACCCACC</td>
</tr>
<tr>
<td>SOX17-3</td>
<td>GGTGGGAGGTGGTTGGTGA</td>
<td>CACTTTCACCTCTACATCC</td>
</tr>
<tr>
<td>SOX17-4</td>
<td>GGTATAGGAAGGTTAAGGG</td>
<td>CTACACACCCCTAATT</td>
</tr>
<tr>
<td>SOX17-5</td>
<td>GTTTAAATTAGGGGTGTG</td>
<td>CTCCCCCTCAAACCTTA</td>
</tr>
</tbody>
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CDH, cadherin; SOX, SRY-box containing gene.
increased by 5-azacytidine (Fig. 4A). CCK8 growth curves proved that SOX17 siRNA suppressed the 5-azacytidine-induced growth inhibition (Fig. 4B). This finding was also supported by the results of colony formation assay (Fig. 4C). Flow cytometry displayed the effects of 5-azacytidine and SOX17 on cell apoptosis. The apoptotic rate in the 5-azacytidine-treated EC9706 cells increased by 13.32% compared to the negative control (DMSO-treated cells). Transfection with SOX17 siRNA abolished the 5-azacytidine-induced apoptosis of EC9706 cells (Fig. 4D). These results indicate the involvement of SOX17 in the inhibition of EC9706 cell proliferation by 5-azacytidine.

**Methylation of CDH1 and SOX17 promoters is decreased by 5-azacytidine in EC9706 cells.** Studies have confirmed that 5-azacytidine is able to regulate gene expression by decreasing the methylation of their promoters (14). We hypothesized that
5-azacytidine upregulates the expression of CDH1 and SOX17 via their promoter methylation. BSP methylation analysis was performed to evaluate the methylation of the CDH1 and SOX17 promoters. The results revealed that the promoters of CDH1 and SOX17 were hypermethylated in the EC9706 cells. Yet, the methylation levels in the EC9706 cell were significantly reduced following treatment with 5-azacytidine (Fig. 5).

**Discussion**

Recently, the incidence of esophageal cancer has gradually increased. Esophageal cancer has become the second cause of cancer-related mortality in China (36). To date, the most common treatment for esophageal cancer is surgery, chemotherapy and radiotherapy, in combination. However, the curative effects of 5-fluorouracil (5-FU) and cisplatin (DDP), two basic clinical chemotherapeutic drugs for esophageal cancer, is unsatisfactory in patients with highly metastatic esophageal cancer (37,38). It has been reported that epigenetic behaviors, such as DNA methylation, play important roles in the development and metastasis of esophageal cancer. Previous studies have found that tumorigenesis and the metastasis of esophageal cancer result from the downregulation of the expression of multiple tumor suppressor genes and the abnormal hypermethylation of their promoters (33-35). Studies have shown that 5-azacytidine, a clinical chemotherapeutic drug used in the treatment of various...
Figure 4. 5-Azacytidine inhibits EC9706 cell proliferation via the upregulation of SOX-box containing gene 17 (SOX17). The downregulation of SOX17 by siRNA attenuated the inhibitory effects of 5-azacytidine on cell proliferation. (A) Western blot analysis was used to detect the expression of SOX17 in the cells treated with 5-azacytidine and transfected with SOX17 siRNA. Compared with the DMSO vehicle control group, the protein expression of SOX17 was increased by 5-azacytidine treatment (**P<0.01). siRNA targeting SOX17 significantly attenuated the 5-azacytidine-induced upregulation of SOX17 (**P<0.01). (B) Cell counting kit-8 (CCK-8) assay of EC9706 cell viability. EC9706 cell viability was inhibited by 5-azacytidine treatment on days 3 and 4 (*P<0.05 and **P<0.01). SOX17 siRNA significantly impaired the effects of 5-azacytidine on days 3 and 4 (**P<0.01). (C) Cell colony-formation assay of EC9706 cells. 5-Azacytidine inhibited EC9706 cell colony formation, while SOX17 siRNA hampered the effects of 5-azacytidine. (D) Flow cytometry was carried out to examine the apoptosis of EC9706 cells. Compared with the negative control (DMSO-treated cells), 5-azacytidine promoted the apoptosis of EC9706 cells (**P<0.01). The 5-azacytidine-induced apoptosis was significantly inhibited by SOX17 siRNA (*P<0.05). 5-aza, 5-azacytidine.

Figure 5. Methylation of cadherin 1 (CDH1) and SRY-box containing gene 17 (SOX17) promoters is decreased by 5-azacytidine in EC9706. Methylation level analysis of CDH1 and SOX17 promoters. (A) Methylation analysis of CDH1 promoter in 5-azacytidine-treated EC9706 cells. Methylation level of the CDH1 promoter was decreased significantly by 5-azacytidine. (B) Methylation analysis of SOX17 promoter in 5-azacytidine-treated EC9706 cells. Methylation level of SOX17 promoter was decreased significantly by 5-azacytidine. 5-aza, 5-azacytidine.
types of cancer, inhibits the methylation of the promoter of multiple genes and affects the expression of these genes (39,40). In this study, we used EC9706 cells to assess the anticancer effects of 5-azacytidine in esophageal cancer.

This study confirmed that the proliferation and invasion of EC9706 cells were inhibited by 5-azacytidine, suggesting that 5-azacytidine is effective for the treatment of esophageal cancer. To investigate the mechanisms of action of 5-azacytidine, we screened numbers of genes that could be related to the proliferation and metastasis of cancer cells by western blot analysis. We found that the expression levels of SOX17 and CDH1 were significantly upregulated in the EC9706 cells by 5-azacytidine. Combined with the findings of previous studies, we hypothesized that SOX17 and CDH1 may participate in the 5-azacytidine-mediated inhibition of cell proliferation and metastasis. To further confirm our hypothesis, siRNAs were used to knockdown the expression of SOX17 and CDH1 in the EC9706 cells. We found that the siRNA-mediated downregulation of CDH1 was greatly impaired by 5-azacytidine, while the 5-azacytidine-induced inhibition of EC9706 cell growth and the induction of apoptosis were significantly attenuated by the siRNA-mediated downregulation of SOX17. These results support our hypothesis that SOX17 and CDH1 are involved in the 5-azacytidine-induced inhibition of the proliferation and metastasis of EC9706 cells, respectively.

To examine the mechanisms responsible for the regulation of CDH1 and SOX17 expression by 5-azacytidine, the methylation of the SOX17 and CDH1 promoters was analyzed. Our results revealed that the methylation of the SOX17 and CDH1 promoters was significantly decreased by 5-azacytidine treatment in the EC9706 cells. This may be an important regulatory pattern for 5-azacytidine to regulate SOX17 and CDH1 expression.

In conclusion, the findings of our study confirm that 5-azacytidine inhibits esophageal cancer cell proliferation and metastasis by exerting inhibitory effects on the methylation of SOX17 and CDH1 promoters. Our findings may prove to be beneficial to clinicians in selecting appropriate chemotherapeutic regimens and enhancing the therapeutic effects.

Acknowledgements

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References