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Hypermethylation-modulated down-regulation of *CDH1* expression contributes to the progression of esophageal cancer

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Abstract. *CDH1*, a cell adhesion molecule, which plays a key role in maintaining the epithelial phenotype, is regarded as an invasion-suppressor gene in light of accumulating evidence from *in vitro* experiments and clinical observations. In an attempt to clarify the mechanism responsible for inactivation of this gene in carcinomas, we investigated the methylation status of the *CDH1* gene 5'-CpG islands and its regulatory mechanism in the progression of esophageal squamous cell carcinoma. Real-time methylation-specific polymerase chain reaction (qMSP) and treatment with the demethylating agent 5-aza-2'-deoxycytidine (5-Aza-CdR) were conducted to analyze the methylation status at the *CDH1* promoter region in the human esophageal carcinoma cell lines, EC1 and EC9706. A total of 235 invasive esophageal squamous cell carcinomas (ESCC) at stages I-IV and their corresponding normal tissue samples, were included in an immunohistochemistry study and methylation analysis of *CDH1*. The results demonstrate that in EC1 and EC9706 cells, the *CDH1* promoter is methylated and treatment with 5-Aza-CdR restored *CDH1* expression. Enhanced *CDH1* expression decreased cell migration, invasion ability and increased adhesion ability. Decreased *CDH1* expression was detected in 59.6% of ESCC tissues, compared with their adjacent non-neoplastic epithelia, which had a close correlation with the primary tumor status, lymph node status, distant metatasis and clinicopathologic stage. Hypermethylation at the *CDH1* promoter was detected in 97.9% of 140 cases of ESCC with low *CDH1* expression. The methylation of *CDH1* promoters (P=0.929) was closely correlated with the lack of expression of their corresponding proteins. The Cox regression model for survival analysis showed that increases in *CDH1* methylation had a greater impact on the prognosis than tumor clinical stage. These findings suggest that *CDH1* gene silencing by promoter hypermethylation and the resultant reduction of *CDH1* expression may play an important role in the progression of ESCC. *CDH1* methylation was a significant predictor of survival in ESCC patients after surgery.

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Key words: CDH1, esophageal squamous cell carcinoma, methylation, tumor progression, metastasis, prognosis

Introduction

Esophageal carcinoma (EC) is a frequently occurring carcinoma in humans that is characterized by a high degree of malignancy, fast metastasis, poor post-operative quality of life, and significant regional differences in incidence (1-3). It ranks eighth in order of occurrence worldwide and sixth as the leading cause of death (4,5). China has the highest EC incidence and mortality rates in the world (1,2,5). Among new cases of EC in the world, China accounts for 52.8% (3,6). The mortality rate of EC in China ranks first in the world (3,6,7). In China, it ranks as the fourth and third cause of death in urban and rural areas, respectively (1,2,6,7). Areas of high incidence for EC in China include the regions of the Taihang Mountains in northern China, the Dabie Mountains, northern Sichuan, northern Jiangsu, the southeast coastal areas of the border of Fujian and Guangdong, the inhabited areas of Xinjiang Kazaks, including the Ci and She counties of Hebei Province, Lin county of Henan Province, and Yangcheng county in Shanxi Province, which exhibit 5 times the national average incidence and 10 times the world average incidence (1,2,6,7). EC development shows similarities to other tumors, which implies the involvement of multiple factors, multiple genetic changes, and a multistage development process (8,9).

Recent studies on the mechanisms of gene expression loss and progress in the understanding of the effect of promoter hypermethylation on tumor suppressor genes have promoted

the emergence of DNA methylation as an important mechanism of tumor suppressor gene inactivation (10-13). In-depth studies of the relationship between DNA methylation and gene expression and its mechanism will facilitate tumor pathogenesis research (10-13), and guide therapeutic approaches (14-16). *CDH1*, a tumor metastasis suppressor gene, is located on chromosome 16q22.1. Its product is a Ca^{2+}-dependent cell adhesion molecule, and mutations in this gene have been associated with the origin, development, invasion, metastasis, and prognosis of carcinomas derived from a variety of epithelial tissues (17,18). The product of *CDH1* expression, E-cadherin, mediates the adhesion reaction between the same types of cells and plays a role in the cytoskeleton, implying that the degree of its expression and function directly impact the detachment and re-attachment of tumor cells. When the activity of *CDH1* is normal, tumor cells are not easily detached from the primary tumor. On the contrary, *CDH1* inactivation results in decreased cell adhesion and abnormal polarity, which promotes tumor metastasis (18-21). *CDH1* methylation-modulated loss of gene expression has been shown to be important in the origin and development of many tumors (22-27). Various degrees of methylation in the *CDH1* promoter CpG islands and the consequent loss of E-cadherin expression were reported in many tumor issues, such as cervical carcinoma (22), prostate carcinoma (23), malignant melanoma (24), non-small cell lung carcinoma (25), liver carcinoma (26) and gastric carcinoma (27). *E-cadherin* expression was also shown to be associated with the level of methylation of promoter CpG islands, implying that this mechanism may be an early event in the malignant process, and that it is an important event in tumor occurrence and development (22-28).

In the present study, the demethylating agent 5-aza-deoxycytidine (5-Aza-CdR) was used in the treatment of EC exhibiting *CDH1* promoter methylation, and its effect on the biological behavior of carcinoma cells after *CDH1* promoter demethylation was observed. We also analyzed the *CDH1* methylation status in EC tissues and its relationship with clinicopathological parameters and prognosis.

Materials and methods

Patients and tissue samples. The Institutional Review Board on Medical Ethics, Zhejiang Province Cancer Hospital approved the method of tissue collection including informed consent. The present study analyzed the esophageal squamous carcinoma (ESCC) tissue and the corresponding para-carcinoma normal tissue of 235 cases. All the specimens were obtained surgically from ESCC patients between December 2002 and July 2009 in the Zhejiang Province Cancer Hospital. Para-carcinoma normal tissue was obtained from a region 5-cm from the tumor edge and was assessed microscopically for the presence of normal cells. A total of 211 patients were men and 24 were women, aged 39-85 years. Tumor-node-metastasis (TNM) staging was performed according to the criteria of the World Health Organization (WHO) and the International Union against Cancer (UICC) (29). A total of 48 patients had upper segment lesions, 70 had middle segment lesions, and 117 had lower segment lesions; 55 tumors of patients were well differentiated, 127 were moderately differentiated, and 53 were poorly differentiated; 16 patients were in stage I, 62 were in stage II; 113 were in stage III; and 44 were in stage IV. The follow-up ended on April 25, 2010.

Cell culture and drug treatment. The human ESCC cell lines, EC1 and EC9706, were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 U/ml streptomycin at 37˚C and 5% CO_2. 5-Aza-CdR (Sigma-Aldrich, USA) was dissolved in dimethyl sulfoxide (DMSO). Cultured cells were seeded at a density of $5x10^5$ cells/flask. The cells were in the logarithmic phase and the number of viable cells was 95-100% before the addition of 5-Aza-CdR to the culture medium at a final concentration of 1 μmol/l. The medium was changed daily and the drug concentration was maintained. The cells were collected after 72 h of drug treatment. Cells in the untreated group were cultured in normal complete culture medium for 72 h.

MTT assay. EC1 and EC9706 cells in the logarithmic growth phase were seeded in 96-well plates at a density of $5x10^3$ cells per well, with 5 replications in each group. After 24 h, the supernatant was discarded and 5-Aza-CdR was added to the culture medium at a final concentration of 1 μmol/l and different plates were cultured for additional periods of 24, 48, and 72 h. A total of 20 μl MTT solution (5 g/l) was added to each well and incubated for an additional 4 h. The reaction was stopped by aspiration of the media and the product was dissolved by the addition of 200 μl DMSO per well, followed by mixing on an oscillating plate for 10 min. Absorbance values were determined by a microplate reader at a wavelength of 570 nm and cell viability rates in the different groups were calculated.

Flow cytometry. EC1 and EC9706 cells were cultured in RPMI-1640 medium containing 5-Aza-CdR at a final concentration of 1 μmol/l. Cells were harvested after 72 h, washed twice in cold PBS, fixed in cold 70% ethanol at -20˚C and incubated with RNase A at a final concentration of 0.1 g/l for 30 min at 37˚C before the addition of 250 μl propidium iodide (0.05 g/l). After 30 min of staining in the dark at room temperature, FACS flow cytometry was used for determining cell cycle and apoptosis. Results were analyzed using ModFit software.

Tumor cell-matrix adhesion analysis. Serum-free RPMI-1640 culture medium was used for diluting Matrigel (Becton-Dickinson Labware) to 100 μg/ml and 50 μl Matrigel were added to each well of 96-well culture plates and placed in a laminar flow hood for drying overnight. Before use, 100 μl of serum-free RPMI-1640 medium was added to each well. After 90 min of standing at room temperature, the liquid was absorbed to remove unbound Matrigel; EC1 and EC9706 cells that were treated for 3 days with 1 μmol/l 5-Aza-CdR were prepared as a $4.0x10^5$ cell/ml cell suspension. A volume of 100 μl cell suspension was added to each well of the adhesion system and cultured for 2 h at 37˚C and 5% CO_2; phosphate-buffered saline (PBS) was used for washing non-attached cells and 0.5% crystal violet dye was added at a volume of 100 μl/well for 10 min for staining. Wells were washed with PBS and dried at 37˚C, before the addition of 100 μl of 1% acetic acid ethanol to each well, mixing and incubation for

10 min. The OD was measured at 570 nm in a microplate reader. The cell-matrix adhesion ability was calculated according to the OD.

Cell in vitro invasion assay. Serum-free RPMI-1640 culture medium pre-cooled to 0˚C was used to dilute Matrigel to 200 mg/l, and 50 μl Matrigel per well were added into the upper chamber of Transwell cell culture plates (Corning) and dried on a clean bench overnight. Before use, 100 μl of serum-free RPMI-1640 medium was added to each well. After 90 min of incubation at room temperature, the liquid was aspirated to remove unbound Matrigel. Esophageal carcinoma cells treated with 1 μmol/l 5-Aza-CdR for 3 days were prepared as a $5.0x10^8$ cell/l suspension and 100 μl of the cell suspension per well was added into the upper chamber of a Transwell cell culture plate. A volume of 600 μl of RPMI-1640 medium supplemented with 10% serum and 10 mg/l fiber binding protein was added into the lower chamber. The Transwell cell culture plate was placed in an incubator at 37˚C and 5% CO_2 for 24 h before fixing with methanol/acetone (1:1) for 10 min. Routine hematoxylin staining was performed. A moist cotton swab was used to wipe off the cells on the membrane facing the upper chamber. Neutral balsam was used for mounting the glass slides. Cells invading into the membrane of the lower chamber were counted under the microscope (magnification, x400). Cells in 5 different fields (up, down, left, right, and middle) of each membrane were counted and the average value was calculated. Three parallel membranes were set in each group.

In vitro cell migration experiment. In the cell migration experiment, all steps were the same as in the invasion assay described above, except that the Matrigel was not coated on the upper chamber of the Transwell cell culture plates. In the *in vitro* Transwell invasion and migration experiments, cell invasion and movement ability was shown as the number of cells crossing the recombinant basement membrane to reach the back of the polycarbonate membrane (PVPF membrane).

RT-PCR analysis. Cells treated for 3 days with 1.0 μmol/l 5-Aza-CdR were used for extraction of total cellular RNA using the Trizol (Gibco) one-step method. A total of 3 μg total RNA was subjected to reverse transcription using M-MLV reverse transcriptase (Promega). *CDH1* was amplified by PCR and glyceraldehyde phosphate dehydrogenase (GAPDH) was selected as the internal reference. The *CDH1* primer sequences were as follows: (F) 5'-GCTGGCTTCAGACCGTGAT-3', (R) 5'-GCAGCCCAAATACTCCTGG-3', and the amplicon size was 89 bp (GeneBank No. BC0512 87). The GAPDH primer sequences were as follows: (F) 5'-CATGAGAGATGA CAACAGCCT-3', (R) 5'-TAATTTTAGGTTAGAGGGTTAT TGT-3', and the amplicon size was 116 bp. The $2^{-\Delta\Delta Ct}$ method was used to calculate relative changes in gene expression determined from real-time RT-PCR experiments. The PCR product (5 μl) was used for 2.0% agarose gel electrophoresis.

DNA extraction, bisulfite treatment and real-time methylation-specific PCR (qMSP). Serial 5-μm sections that contained carcinoma and non-neoplastic tissues were mounted on non-coated glass slides and dried at 37˚C overnight. After deparaffinization and staining with hematoxylin and eosin (H&E), we collected 5000 nuclei from 5 to 10 serial sections using a 27G needle. The collected nuclei were treated with 40 μl of 200 μg/ml proteinase K (Sigma-Aldrich) at 42˚C, for 72 h. A Universal Genomic DNA Extraction kit Ver.3.0 (Takara Co., Japan) was used to extract the DNA from the cultured cells. DNAs were modified by sodium bisulfite and purified and recycled according to the EpiTect Bisulfite kit (Qiagen Inc.) instructions. The *CDH1* methylation (M) and non-methylation (U) specific primer sequences were as follows: *CDH1*(M): (F) 5'-TTAGGTTAGAGGGTATCGC GT-3', (R) 5'-TAATTTTAGGTTAGAGGGTTATTGT-3', and the amplification length was 116 bp; *CDH1*(U): (F) 5'-TAA CTAAAAATTCACCTACCGAC-3', (R) 5'-CACAACCAA TCAACAACACA-3', and the amplification length was 97 bp (GeneBank No. L34545). The primers were synthesized by Invitrogen. Modified DNAs with sodium bisulfite were analyzed by qMSP on the ABI 7500 PCR (Applied Biosystems) instrument. The procedure was performed following the instructions for the SYBR Premix Taq ExTaq kit (Takara). The quantitative methylation analysis of samples was carried out using methylation and non-methylation specific primers, respectively. The percentage of methylated DNAs in the samples were calculated according to the CT value and a standard curve. The methylation percentage was calculated according to a previous report (30). Human genomic DNA (NEB) treated by *Sss*I methyltransferase *in vitro* was used as a positive control. Peripheral blood DNA of healthy untreated subjects was used as a negative control.

Immunohistochemical analysis. The expression of E-cadherin protein in cultured cells was detected by immunohistochemistry. Cells were seeded in 24-well plates with a coverslip placed at the bottom of each well. After treatment with 5-Aza-CdR for 72 h, the cells were fixed with 40 g/l paraformaldehyde and the coverslip was removed. E-cadherin monoclonal antibody (dilution 1:30, Santa Cruz Biotechnology) were used for staining according to the manufacturer's instructions. The results were determined based on the percentage of positive cells and the staining intensity. The scoring according to the percentage of positive cells was as follows: <5%, 0 points; 5-25%, 1 point; 26-50%, 2 points; 51–75%, 3 points; and >76%, 4 points. The cell staining intensity was scored as follows: no staining, 0 points; yellow, 1 point; deep yellow, 2 points; and brown, 3 points. The sum of the score of the 2 items was considered for the total score, 0 to 1 point; negative (-); 2-3 points, weakly positive (+); and ≥4 points, positive (++).

Immunohistochemical staining for E-cadherin was carried out using representative paraffin-embedded specimens from 235 patients. Sections (4 μm) were cut from resected specimens fixed in 10% buffered formalin and embedded in paraffin. After deparaffinization, antigen retrieval in 0.01 M citrate buffer, and inactivation of endogenous peroxidase activity in 3% H_2O_2/methanol, we incubated the slides with an antibody against E-cadherin at 4˚C overnight, and immunohistochemical staining, following a standard avidin-biotin-peroxidase complex technique, was carried out using the Histofine SAB-PO (M) kit (Nichirei, Tokyo, Japan) and 3,3'-diaminobenzidine (DAB) as the chromogen. Nuclei were

counterstained with hematoxylin. The scores of staining results were given as follows: score 1, negative; score 2, reduction (weaker than the internal standard); score 3, positive (comparable to the internal standard).

Western blot analysis. After treatment of cells with 1.0 μmol/l 5-Aza-CdR for 3 days, the plates were treated with 0.25% trypsin for 5 min and the cells were collected, washed in 1% PBS (pH 7.0), and centrifuged. Cell lysis buffer was added at a volume equal to six times that of the cell pellet. Total protein was extracted and then quantified using the Lowry method (31). The protein supernatant was stored at -20˚C for further use. Heated and denatured samples were analyzed using 10% sodium dodecyl sulfate (SDS) polyacrylamide gels. Samples consisting of 80 μg protein each were separated by electrophoresis, transferred to a nitrocellulose membrane, which was subsequently blocked in non-fat dry milk at 4˚C overnight. Then the membrane was incubated with anti-E-cadherin (1:500) and anti-β-actin monoclonal antibodies (1:500, Santa Cruz, CA) at room temperature for 2 h. After TBST washing, a secondary goat anti-mouse IgG antibody (1:5000) was added and incubated at room temperature for 2 h. Alkaline phosphatase staining (Sigma) was performed for 5 min. Imaging was done using an automatic electrophoresis gel imaging analysis system (Chemi Imager 5500, AlPha InnCh). The data were collected with a Fluor Chen V.2.0 system. Bands were subjected to a density scan for quantitative analysis. The experiments were repeated three times and the mean value was calculated for statistical analysis.

Statistical analysis. The SPSS 14.0 statistical software was used for data analysis. Data comparisons between the groups were conducted with the χ^2-test and data not in accord with the χ^2-test conditions were analyzed using the Fisher's exact test. Data comparisons between the groups were performed using one-way ANOVA, and the results of the measured data were expressed as the mean ± SEM. A p-value <0.05 indicated a statistically significant difference. The survival analysis was computed by means of the Kaplan-Meier method and significant levels were assessed by means of the log-rank test. A univariate analysis with the Cox regression model was used to determine the identified prognostic factors, and multivariate analysis with the Cox regression model was used to explore the combined effects. p<0.05 was considered to indicate statistical significance.

Results

Effect of 5-Aza-CdR on the biological characteristics of EC1 and EC9706 cells. Treatment of cells with 5-Aza-CdR resulted in a reduction in cell size, rounding of cells, and a decrease in cell density. Prolonged exposure and increased concentration of the inhibitor caused the cells to become irregular, showing cell debris and small bubbles, followed by early apoptotic changes, including nuclear condensation, chromatin margination, membrane rupture, or even the formation of apoptotic bodies (Fig. 1). The changes in cell viability of EC1 and EC9706 cells treated with 1.0 μmol/L 5-Aza-CdR are shown in Fig. 2. Cell viability of EC1 and EC9706 cells decreased gradually with prolonged exposure to the inhibitor.

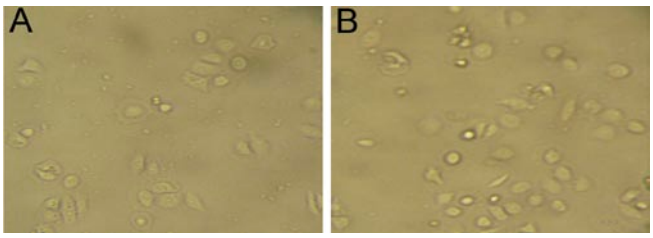

Figure 1. Changes in morphology of (A) EC1 and (B) EC9706 cells treated with 1.0 μmol/l 5-Aza-CdR for 24 h Original magnification x200.

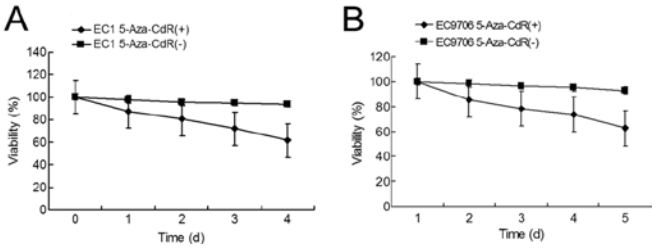

Figure 2. Changes in cell viability of (A) EC1 and (B) EC9706 cells treated with 1.0 μmol/l 5-Aza-CdR for 4 days.

Table I. Flow cytometry analysis.

Group	G0/G1	S	G2/M
EC-1			
5-Aza-CdR (+)	70.45±3.2[a]	20.21±2.8[a]	9.34±1.8[a]
5-Aza-CdR (-)	57.44±2.1	30.31±2.7	13.25±2.9
EC9706			
5-Aza-CdR (+)	70.23±3.4[a]	29.62±2.8[a]	0.90±0.6[a]
5-Aza-CdR (-)	59.22±2.9	37.48±1.3	3.30±0.9

[a]P<0.05 when compared with the 5-Aza-CdR (-) group. Flow cytometry results results revealed that the number of cells in the S phase increased and the number of cells in G0/G1 and G2/M phase decreased, in the 5-Aza-CdR experimental group, in comparison with the control group (mean ± SD, %).

After EC1 and EC9706 cells were treated by 5-Aza-CdR for 72 h, flow cytometry results revealed that in comparison with the control group, the number of cells in G0/G1 and G2/M phase decreased, the number of cells in S phase increased and the apoptosis rate increased in the 5-Aza-CdR experimental group (Table I and Fig. 3).

Effect of 5-Aza-CdR on the promoter methylation, mRNA and protein expression of CDH1 in EC1 and EC9706 cells. The qMSP results revealed that the methylation rate of *CDH1* was 100% in EC1 cells and 60% in EC9706 cells; after treatment with 1.0 μmol/l 5-Aza-CdR, the percentage of non-methylated DNA increased significantly, reaching 95% and 98%, respectively. Analysis by 2.0% agarose gel electrophoresis showed that EC1 cells contained only methylated bands (116 bp), and EC9706 cells had methylated bands (116 bp) and unmethylated bands (97 bp) before treatment with 5-Aza-CdR; after

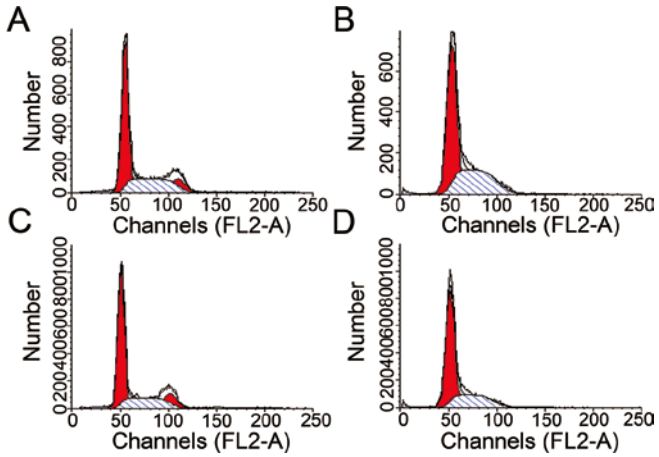

Figure 3. Changes in the cell cycle of EC1 and EC9706 cells treated with 1.0 μmol/l 5-Aza-CdR for 72 h (A) EC1 cells without 5-Aza-CdR treatment, (B) EC1 cells treated with 5-Aza-CdR, (C) EC9706 cells without 5-Aza-CdR treatment, (D) EC9706 cells with 5-Aza-CdR treatment.

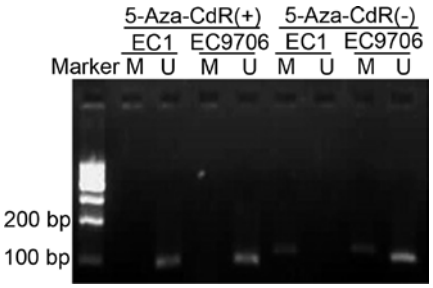

Figure 4. The *CDH1* promotor region methylation status in EC1 and EC9706 cells demonstrated by 2% agarose gel electrophoresis. DNAs extracted from each EC cell line were treated with sodium bisulfate. U, unmethylated; M, methylated.

5-Aza-CdR treatment, only unmethylated bands (97 bp) were detected in EC1 and EC9706 cells (Fig. 4), suggesting that the *CDH1* promoter in EC1 and EC9706 cells had abnormal methylation and after demethylation treatment with 5-Aza-CdR, *CDH1* methylation was reversed to a certain degree.

Real-time RT-PCR revealed the absence of CDH1 expression in EC1 cells (T/R: 0.07) and showed low expression levels in EC9706 cells (T/R: 0.63). After demethylation treatment with 5-Aza-CdR, EC1 and EC9706 cells showed high *CDH1* mRNA expression (T/R values were 3.2 and 2.9, respectively). At the protein level, immunocytochemistry showed a strong positive expression of the E-cadherin protein in EC1 and EC9706 cells after treatment with 5-Aza-CdR (Fig. 5). Western blot analysis further confirmed the immunohistochemistry results. Before 5-Aza-CdR treatment, E-cadherin protein expression was not detected in EC1 cells, while EC9706 cells showed weak expression. After treatment with 1.0 μmol/l 5-Aza-CdR, a band representing 24 kDa relative molecular weight was found in both groups of cells. EC9706 cells showed bands with different density before and after drug treatment (Fig. 6). Computer scanning and analysis of OD in the different groups showed that the expression of E-cadherin protein increased after 5-Aza-CdR treatment and the protein expression in the 1.0 μmol/l treatment group was

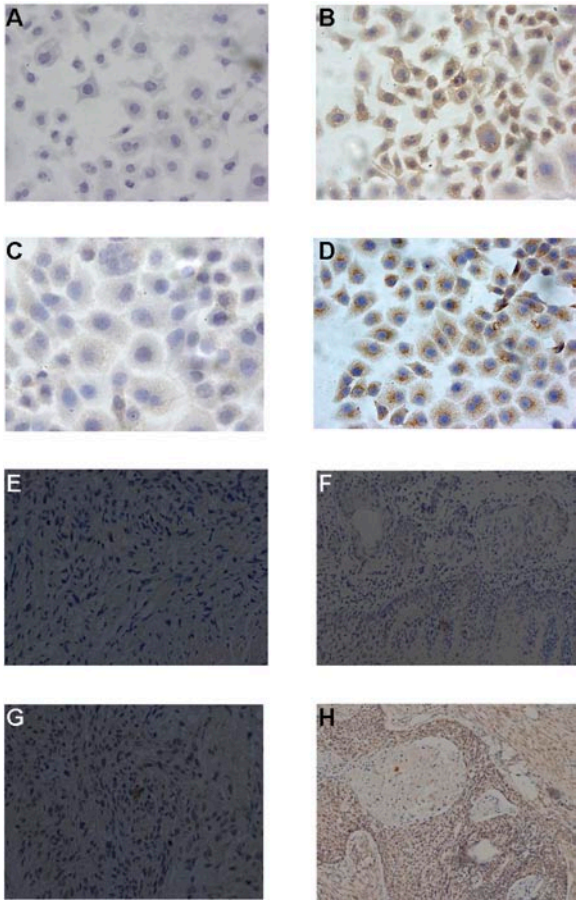

Figure 5. Immunohistochemical analysis of E-cadherin expression in EC1 and EC9706 cells treated with 1.0 μmol/l 5-Aza-CdR for 72 h, as well as in ESCC tissues (A) EC1 cells without 5-Aza-CdR treatment, (B) EC1 cells treated with 5-Aza-CdR, (C) EC9706 cells without 5-Aza-CdR treatment, (D) EC9706 cells with 5-Aza-CdR treatment, (E) absence of E-cadherin expression in ESCC tissues, (F and G) partial and (H) positive expression of E-cadherin in ESCC tissues. (A,B,C and D) Original magnification x200, (E,F,G and H) Original magnification x100.

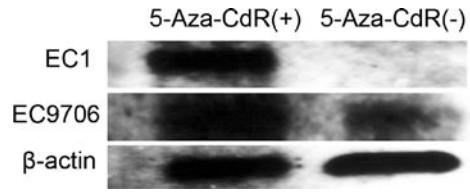

Figure 6. Western blot analysis of E-cadherin expression in EC1 and EC9706 cells treated with 1.0 μmol/l 5-Aza-CdR for 72 h.

3.5-fold higher than in the untreated group; statistical analysis indicated that these differences were statistically significant ($P<0.05$).

Effect of 5-Aza-CdR on the adhesion between tumor cell and matrix. In the experiment of attachment, the average OD of EC1 and EC9706 cells treated with 1.0 μmol/l 5-Aza-CdR for 72 h were 0.55±0.03 and 0.61±0.04 respectively, which were significantly lower than that of the control group (those without 5-Aza-CdR treatment) (0.87±0.07, 0.90±0.08, $P<0.05$). However, there was no significant difference between the EC1 and EC9706 cells whether treated or not with 5-Aza-CdR.

Table II. Effect of 5-Aza-CdR on the invasion and migration of EC1 and EC9706 cells (cells/HPF).

Group	Invasion	Migration
EC-1		
5-Aza-CdR (+)	19.23±2.12[a]	39.68±4.72[a]
5-Aza-CdR (-)	77.39±4.72	127.73±5.84
EC9706		
5-Aza-CdR (+)	20.37±3.71[a]	41.28±4.85[a]
5-Aza-CdR (-)	79.38±4.19	137.32±5.36

[a]P<0.05 when compared with 5-Aza-CdR (-) group.

Effect of 5-Aza-CdR on tumor cell invasion and migration. The Transwell *in vitro* invasion and migration experiments showed cell invasion and movement ability as the number of cells crossing the recombinant basement membrane to reach the back of the polycarbonate membrane (PVPF membrane). After treatment with 5-Aza-CdR, the cell invasion and migration of EC1 and EC9706 cells were significantly lower than in the control group (P<0.01) (Table II).

Expression of E-cadherin protein in ESCC tissues. Of the 235 cases in this series, E-cadherin protein expression was preserved in cancer specimens from 95 patients (40.4%). In the other 140 cases, E-cadherin expression was completely absent (50.2%) or partially absent (9.4%) (Fig. 5

Table III. Clinicopathological correlations of E-cadherin expression in primary ESCC tissues.

	E-cadherin expression				
	Positive	Absent			
Variables	+ to +++ (n=95)	± (n=22)	- (n=118)	p-value[a]	Correlative analysis
Age					
≥60	46	8	53	0.896	γ=0.054
<60	49	14	65		
Gender					
Male	83	19	109	0.204	γ=-0.281
Female	12	3	9		
Tumor location[b]					
Upper	21	0	27	U vs. M: 0.851	γ=-0.129
Mid	26	7	37	M vs. L: 0.454	
Lower	45	17	55	U vs. L: 0.307	
Grade					
G1	22	6	27	G1 vs. G2: 1.000	γ=0.000
G2	46	17	64	G2 vs. G3: 1.000	
G3	22	5	26	G1 vs. G3: 1.000	
Tumor status					
T1	22	1	3	T1 vs. T2: 0.003	γ=0.471
T2	25	2	23	T2 vs. T3: 0.247	
T3	43	17	79	T3 vs. T4: 0.149	
T4	4	1	15	T1/T2 vs. T3/T4: 0.000	
Lymph node status					
N0	46	2	26	0.001	γ=0.443
N1	44	23	94		
Metastasis					
M0	93	12	86	0.000	γ=0.652
M1	0	9	35		
Staging					
I	14	0	2	I vs. II: 0.501	γ=0.785
II	46	2	14	II vs. III: 0.000	
III	33	8	72	III vs. IV: 0.060	
IV	1	8	35	I /II vs. III/IV: 0.000	

[a]Fisher's exact test was used when cases <40. [b]U, upper segment lesion; M, mid segment lesion; L, lower segment lesion.

Table IV. Clinicopathological correlations of *CDH1* hypermethylation in primary ESCC tissues.

Variables	Methylation status			p-value[b]	Correlative analysis
	Absent	Present			
	U (n=95)[a]	U/M (n=25)[a]	M (n=115)[a]		
Age					
≥60	47	9	51	0.274	γ=0.145
<60	48	16	64		
Gender					
Male	83	22	106	0.381	γ=-0.213
Female	12	3	9		
Tumor location[d]					
Upper	22	1	25	U vs. M: 0.15	γ=-0.092
Mid	26	8	36	M vs. L: 0.768	
Lower	47	16	54	U vs. L: 0.084	
Grade					
G1	25	4	26	G1 vs. G2: 0.302	γ=0.005
G2	46	18	63	G2 vs. G3: 0.211	
G3	24	3	26	G1 vs. G3: 0.939	
Tumor status					
T1	22	2	2	0.000	γ=0.634
T2	27	2	21		
T3	42	19	78		
T4	4	2	14		
Lymph node status					
N0	47	3	24	0.000	γ=0.608
N1	48	22	91		
Metastasis					
M0	94	14	83	0.000	γ=0.953
M1	1	11	32		
Staging					
I	13	1	2	0.000[c]	γ=0.842
II	47	3	12		
III	34	10	69		
IV	1	11	32		

[a]U, unmethylated; M, methylated. [b]Fisher's exact test. [c]There was a signifficant difference between any group and the other group by analysis of variance (P<0.000). [d]U, upper segment lesion; M, mid segment lesion; L, lower segment lesion.

and Table III). E-cadherin protein expression was preserved in the corresponding adjacent normal esophageal tissues from all 235 patients (100.0%). There was a significant difference between the cancer tissues and the corresponding adjacent normal esophageal tissues (P<0.001). Esophageal cancers exhibiting deleted E-cadherin expression tended to invade more aggressively. The depth of tumor invasion (T1/T2 vs. T3/T4), lymph node metastasis (N0 vs. N1), metastasis status (M0 vs.. M1) and staging (I/II vs. III/IV) were significantly correlated with the deletion of E-cadherin expression (P<0.001). Correlation analysis showed that the absence of E-cadherin protein expression was positively correlated to lymph node metastasis, depth of invasion, metastasis, and staging (γ-values were 0.443, 0.471, 0.652 and 0.785, respectively). In contrast, E-cadherin expression status was not associated with the other clinicopathologic parameters, including age, gender, tumor location as well as grade (P>0.05).

Methylation of the CDH1 promoter region in ESCC tissues. Among 235 ESCC specimens, 140 (59.6%) cases had 5'-CpG island abnormal methylation in the *CDH1* promoter region, including 115 (48.9%) cases of M and 25 (10.6 %) cases of U/M; in the corresponding adjacent normal esophageal tissues, only 22 (9.4%, 22/235) cases of U/M were found. The difference between ESCC specimens and normal esophageal tissues was statistically significant (P<0.05) (Table IV). *CDH1* methylation correlated significantly to lymph node metastasis,

Table V. Correlative analysis between E-cadherin expression and *CDH1* hypermethylation in primary ESCC tissues.

	Methylation status				
	Absent	Present			
Variables	U (n=95)	U/M (n=25)	M (n=115)	p-value[a]	Correlative analysis
E-cadherin expression					
+ to +++ (n=95)	92	3	0	0.000	0.929
± (n=22)	0	22	0		
- (n=118)	3	0	115		

[a]Fisher's exact test. U, unmethylated; M, methylated

depth of invasion, metastasis and staging (P=0.001), and not related to gender, age, tumor location and degree of differentiation (P>0.05). Correlation analysis showed that *CDH1* methylation was positively correlated to lymph node metastasis, depth of invasion, metastasis, and staging (γ-values were 0.608, 0.634, 0.953, and 0.842, respectively), suggesting that an increase in *CDH1* methylation is an important factor in the development of ESCC.

Correlation of CDH1 promoter methylation and the loss of corresponding protein expression in tumor tissues. The result showed that the loss rate of E-cadherin protein expression in tumor tissues was 59.6% (140/235). Hypermethylation at the *CDH1* promoter was detected in 97.9% of 140 cases of ESCC with low E-cadherin expression. The methylation of *CDH1* promoters (P=0.929) was closely correlated with the lack of expression of their corresponding proteins (Table V).

Prognostic analysis

Relationship between esophageal carcinoma staging and survival. Three deaths were recorded among 16 stage I cases with an average survival time of 38.6 months; 13 deaths occurred among 62 stage II cases with an average survival time of 34.6 months; 71 deaths occurred among 113 stage III cases with an average survival time of 19.4 months; 35 deaths were recorded among 44 stage IV cases with an average survival time of 17.6 months. Patients in stages III/IV and I/II showed significant differences in average survival time (P<0.05) (Fig. 7).

Relationship between CDH1 methylation and survival. Two patients died among 95 cases of ESCC with unmethylated *CDH1*, showing a median survival time of 45.4 months; 6 patients died among 25 cases of ESCC with partly-methylated *CDH1*, revealing a median survival time of 41.1 months; 114 patients died among 115 cases of ESCC with completely methylated *CDH1*, with a median survival time of 14.1 months. The comparison between the three groups showed significant differences (P<0.05) (Fig. 8).

Cox regression model for survival analysis. The Cox regression model was used for survival analysis and a comparison of the effect of double factors (*CDH1* methylation and tumor clinical stage) on patient survival was performed. The results showed that compared to tumor clinical stage increases in *CDH1* methylation had a greater impact on the prognosis (Fig. 9).

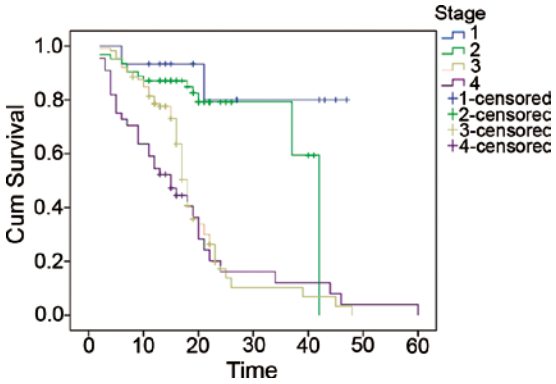

Figure 7. The relationship between esophageal carcinoma staging and survival.

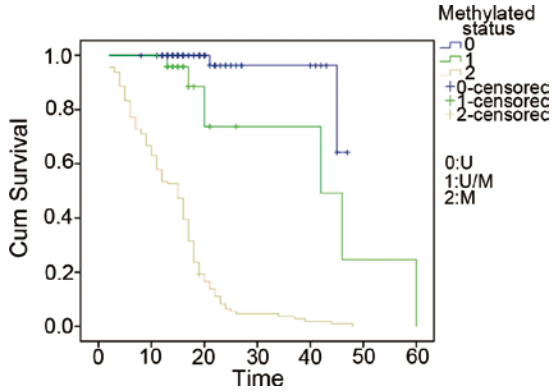

Figure 8. The relationship between the methylated status of *CDH1* and survival.

Discussion

Gene expression and DNA function can be regulated at the transcriptional level by DNA chemical modifications (32). This effect of DNA modifications on tumor formation has recently received increased attention. One of these

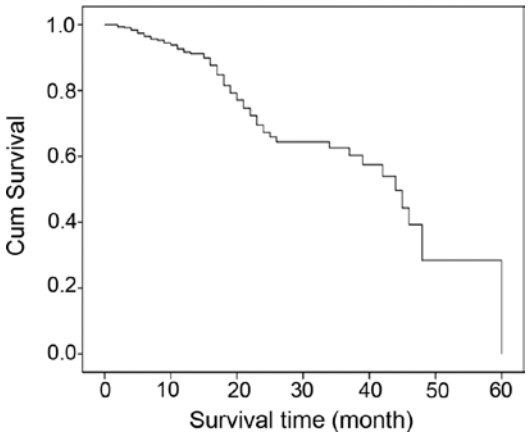

Figure 9. Cox regression model of survival analysis based on double factors (*CDH1* methylation and tumor clinical stage).

significant DNA modification mechanisms is CpG island methylation (33). CpG islands are C and G rich sequences and hypermethylation of CpG islands can inhibit or silence gene transcription, leading to a reduction or loss of functional protein and promote tumor development (33-36). Methylation is the most common DNA modification and in some cases it is the only mechanism of inactivation of tumor suppressor genes (37). Genes with transcriptional inactivation due to methylation are very sensitive to DNA methylation inhibitors and can easily be re-activated (36). The methylation inhibitor 5-Aza-CdR has been extensively studied. As a deoxycytidine analog, 5-Aza-CdR can be incorporated during DNA synthesis, which reduces the capacity for DNA methylation by methyltransferase enzymes. At the same time, it can covalently bind to DNA methyltransferases (DNMTs) and inhibit their biological activity, reversing the methylation status of tumor suppressor gene promoters. Gene silencing caused by methylation, particularly in tumor suppressor genes, can therefore be reactivated, resulting in gene expression and effectively suppressing tumor development (36-39).

CDH1 encodes a transmembrane protein subtype in the cadherin family, composed of 723-748 amino acid residues with extracellular, transmembrane, and intracellular regions. The extracellular region contains a ligand binding site or adhesion region. CDH1 binds to itself to mediate the adhesion of the same cell types. *CDH1* is found mainly in human and animal epithelial cells and it plays an important role in the maintenance of normal epithelial cell morphology, structural integrity and polarity. *CDH1* gene expression can inhibit tumor metastasis (17-21,40). The protein product of the *CDH1* gene is reduced or absent in many tumor tissues through several possible mechanisms such as gene mutation (40), promoter hypermethylation (22-27) and transcriptional repression (18). When *CDH1* expression is decreased or lost, cell adhesion ability decreases, resulting in cell dispersion and outward infiltrating growth (17-21). Once the metastasis conditions are met, the cells can detach from the primary lesion and cause invasion and metastasis (19-21). Many epithelial malignant tumors show a loss of *CDH1* expression; pathological grading, invasive growth, lymph node metastasis, and distant metastasis are related to this decreased *CDH1* expression (41,42).

Matrigel is a matrix component extracted from mouse EHS (Engelbreth-Holm-Swarm) sarcoma. Matrigel coated on a PVPF membrane can form a structure very similar to the natural basement membrane after recombination in culture medium. Cells with an invasive ability can cross the membrane by induction of chemotactic agents, which makes Matrigel a useful tool to study cell invasion and movement ability (43). In the present study, EC1 and EC9706 esophageal carcinoma cells with *CDH1* promoter methylation were treated with 5-Aza-CdR. After the demethylation treatment, adhesion, migration, and the invasion ability of esophageal carcinoma cells significantly changed. To verify whether the inhibitory effect of 5-Aza-CdR on the malignant behavior of EC1 and EC9706 esophageal carcinoma cells was associated with *CDH1* up-regulation, qMSP, RT-PCR and Western blot analysis were used to assess the methylation status of the *CDH1* promoter in EC1 and EC9706 esophageal carcinoma cells. The results showed that 5-Aza-CdR can successfully reverse *CDH1* promoter methylation. *CDH1* mRNA was not expressed in EC1 cells and showed reduced expression in EC9706 cells. Treatment of EC1 and EC9706 cells with 5-Aza-CdR caused the up-regulation of *CDH1* expression, suggesting that the loss of *CDH1* mRNA expression was related to methylation. Analysis of E-cadherin protein expression in EC1 and EC9706 cells before and after 5-Aza-CdR treatment revealed a correlation between E-cadherin protein and mRNA expression, indicating that *CDH1* methylation may lead to loss of protein expression. The results showed that 5-Aza-CdR can restore the methylation-inhibited transcriptional activity of the *CDH1* gene in EC1 and EC9706 esophageal carcinoma cells, resulting in the restoration of the expression of *CDH1* mRNA and protein and of its tumor inhibiting activity. Assessment of cell viability and cell cycle progression showed that treatment of cells with 5-Aza-CdR for 72 h caused an increase in the cell growth inhibition rate and changes in the cell cycle, with G0/G1 phase arrest. In addition, treatment with the inhibitor decreased the cell migration and invasion ability and increased the adhesion ability in association with an increase in E-cadherin protein levels, suggesting that 5-Aza-CdR inhibited the malignant behavior of EC1 and EC9706 esophageal carcinoma cells by increasing the expression of the E-cadherin protein. Increased E-cadherin protein levels enhanced carcinoma cell adhesion and promoted the survival of these cells, protecting them from apoptosis and preventing a loose carcinoma structure (17-21,44).

CDH1 is an important cell adhesion molecule and it can inhibit the detachment of cells from primary tumors, which is considered an important factor in tumor metastasis suppression (17-21,44,45). Alterations in *CDH1* structure and function often cause a reduction in the adhesion between tumor cells, which may cause the detachment of cells from primary tumors and the acquisition of invasion and metastatic properties (18-21,44,45). However, few reports have addressed the role and mechanism of *CDH1* methylation in the progression of ESCC. Our immunohistochemical analysis demonstrated reduced E-cadherin expression in more than half of the ESCC cases in the present series (59.6%). Furthermore, our study found that reduced E-cadherin expression correlated with primary tumor status, pT, lymph node status, pN, and distant

metastasis, pM. E-cadherin is a cell adhesion molecule that is reported to have an important role in cancer metastasis, these results suggest that reduced E-cadherin induces cancer metastasis via tumor invasion by suppressing cell-to-cell attachment, and activates cancer growth and invasion by loss-of-function as a tumor suppressor gene (46,47). It is very important to conduct further investigations to clarify the mechanisms regulating E-cadherin expression in ESCC, because the control of epigenetic silencing of *CDH1* might be critical for suppressing cancer invasion or metastasis and might be a potential target of gene therapy. In the present study, the positive rate of *CDH1* methylation in ESCC was 59.6% (140/235), in which M was 48.9% (115/235) and U/M was 10.6% (25/235), which was significantly higher than the rates in the adjacent normal esophageal tissue (8.1%, 19/235, both U/M). Moreover, hypermethylation was associated significantly with the loss or reduction in E-cadherin expression (P=0.929). This result suggests that hypermethylation might be an important mechanism in *CDH1* silencing in ESCC. When invasive growth of esophageal carcinoma and lymph node involvement occurred, *CDH1* expression was often lost through hypermethylation. The results in this study showed that *CDH1* methylation was significantly correlated to lymph node metastasis, depth of invasion, metastasis and staging (P=0.001, respectively), but not related to gender, age, tumor location and degree of differentiation (P>0.05). Correlation analysis showed that *CDH1* methylation was positively correlated with lymph node metastasis, depth of invasion, metastasis, and staging (γ=0.608, 0.634, 0.953, and 0.842, respectively), suggesting that an increase in *CDH1* methylation is an important factor in ESCC development. The present results demonstrated that *CDH1* loss was associated with the development of tumors, causing a decrease in adhesion between epithelial cells, which easily detached from the tumor leading to the development of metastases (17-21,44,45). Normal *CDH1* expression levels can inhibit the invasion and metastasis of esophageal carcinoma. Decreased expression of *CDH1* caused the development of invasive and metastatic tumors and was associated with a poor prognosis, which is consistent with other reports (17-21,40-45). *CDH1* hypermethylation in esophageal carcinoma patients was associated with more metastatic lymph nodes, reflecting the presence of malignant invasiveness and poor prognosis. *CDH1* methylation may serve as a significant poor prognostic indicator in patients with ESCC in this study. The Cox regression model was used for survival analysis and for a comparison of the effect of double factors (*CDH1* methylation and tumor clinical stage) on patient survival. The results showed that compared to tumor clinical stage increases in *CDH1* methylation had a greater impact on the prognosis. In conclusion, *CDH1* methylation is a useful marker to predict invasion, metastasis, and the prognosis of esophageal carcinoma due to its association with tumor development and tumor cell migration, and the restoration of *CDH1* expression could provide a new approach for the development of cancer therapeutics.

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