

• Basic Research •

Effects of 5-Aza-CdR on expression and methylation of E-cadherin gene in human colon carcinoma cells

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[Abstract] Background and Objective: Colon cancer is one of the most common malignant tumors, and its pathogenesis is not fully understood. Transcriptional silencing by DNA methylation is believed to be an important mechanism of carcinogenesis. E-cadherin can suppress tumor cell invasion and metastasis, and is considered as an invasion/metastasis suppressor gene. Inactivation of E-cadherin gene often occurs in colon carcinoma. This study was to investigate the correlation between E-cadherin gene expression and the methylation status of E-cadherin 5' CpG islands in human colon carcinoma cell line HT-29, and to explore the mechanism of carcinogenesis of colon cancer. **Methods:** Immunocytochemical di-cho-step method and reverse transcription-polymerase chain reaction (RT-PCR) were used to detect the expression of E-cadherin protein and mRNA in HT-29 cells after 5-Aza-CdR treatment; methylation specific PCR was used to analyze the methylation status at promoter of E-cadherin gene. **Results:** The expression of E-cadherin gene could be restored by 5-Aza-CdR treatment, immunocytochemical staining showed the positive expression ratio of E-cadherin increased from (21±7)% (1 μmol/L) to (39±13)% (5 μmol/L); E-cadherin genes were methylated and not expressed in HT-29 cells in the colon carcinoma. **Conclusions:** E-cadherin methylation plays an important role in the carcinogenesis of colon carcinoma cells and can re-express after the treatment with 5-Aza-CdR.

Key words: Colon neoplasm, E-cadherin gene, methylation

Colorectal cancer is one of the most common human malignancies in North America and Europe, and the main cause of cancer-related death.¹ In China, its incidence is increasing year by year. Like other tumors, the occurrence and development of colon cancer is a complex multi-stage process involving multi-gene changes. The malignant behavior of tumor cells depends on three characteristics: uncontrolled growth, local infiltration and metastasis, all of which are dependent on decline and even loss of functions of the adhesive system, in which cell adhesive molecules are involved and play an important role.²

E-cadherin protein is a type of calcium-dependent transmembrane glycoproteins mediating mutual adhesion between cells, which plays an important role in embryonic development, morphogenesis, epithelial polarity and maintenance of the integrity. and it is an important tumor suppressor gene as well.^{3,4} In this study, we used immunocytochemistry, RT-PCR and methylation-specific PCR to explore the function and clinical

significance of E-cadherin expression and its promoter methylation levels in human colon cancer cell lines at the molecular and protein levels.

Materials and Methods

Materials

Human colon cancer cell line (HT-29) was supplied by Central Laboratory of 2nd Xiangya Hospital, Central South University, which is taken from ATCC. 5-Aza-2'-deoxycytidine (5-Aza-CdR), hydroquinone, sodium bisulfite were purchased from Sigma; monoclonal concentrated antibody against E-cadherin (ZM-0092), and two-step kit (PV-9000) were purchased from Beijing Zhongshan Inc.; SV total RNA isolation system, reverse transcription system, Wizard Genomic DNA purification kit, Wizard DNA clean-up system were purchased from Promega.

Methods

Cell culture and drug treatment HT-29 cells were cultured with 10% fetal bovine serum, 100 u/mL penicillin and 100 u/mL streptomycin, DMEM high-glucose culture medium, placed in 37°C culture boxes with 5% CO₂, 100% saturation humidity. Logarithmic growth cells were produced into 1 × 10⁵/mL cell suspension after digestion by trypsin. The suspension was added to 15 six-well culture plates, which were categorized into three groups, with 5 culture plates in each group. Two groups were

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cultured with 1 $\mu\text{mol/L}$ and 5 $\mu\text{mol/L}$ 5-Aza-CdR in medium in the first 24 h, then cultured in drug-free medium; the other group served as a control group, cultured in medium without 5-Aza-CdR.

Immunocytochemistry Logarithmic growth cells were inoculated into the six-well culture plates with a pre-placed 1.5 cm \times 1.5 cm cover slip on each well. Grouping and drug treatment were the same as stated above. After 24 h of culture, 2 cover slips were randomly selected from each group. They were picked out and washed with PBS and then fixed with 4% paraformaldehyde for 30 min for slides making. The resulting slips were stained following the instructions of immunocytochemical test kit, and yellow or brown colored fine particles appearing on the cell membrane as a positive signal. Five high power fields with 1000 cells were randomly selected, and the number of positive cells was counted to determine the effect. Monoclonal anti-E-cadherin antibody worked as primary antibody, and PBS instead of primary antibody served as negative control, and normal tissue biopsies as positive control.

Reverse transcription-polymerase chain reaction (RT-PCR)

Cells without drug treatment and treated by 1 $\mu\text{mol/L}$ or 5 $\mu\text{mol/L}$ 5-Aza-CdR for 24 h were collected. SV Total RNA Isolation System kit was used to extract the total RNA of these cells, and the A_{260}/A_{280} ratio was 1.8–2.0 according to the absorbance value measured by UV spectrophotometer, which verified the RNA purity. Reverse transcription was done under the guidelines of Reverse Transcription System kit. The resulting cDNA was used as templates to amplify E-cadherin gene, with GAPDH as an internal control. E-cadherin upstream primer was 5'-GGTTCAAGCTGCTGACCTTC-3', the downstream primer was 5'-CTCAAAATCCTCCCTGTCCA-3', and the resulting PCR product length was 630 bp; GAPDH upstream primer was 5'-ACAGTCCATGCCATCACTGCC-3', and downstream primer 5'-GCCTGCTTACCACCTTCTTG-3', with the PCR product length of 266 bp (primers synthesized by Shanghai Bio-Engineering Company). PCR reaction was done with PE-2400 PCR Amplifier, and the obtained PCR products run agarose gel electrophoresis after fully mixed with loading buffer in a ratio of 10:2.

Methylation-specific PCR (MSP) Cells without drug treatment but treated by 1 $\mu\text{mol/L}$ or 5 $\mu\text{mol/L}$ 5-Aza-CdR for 24 h were collected. Wizard Genomic DNA Purification Kit was used to extract the DNA of these cells, and the A_{260}/A_{280} ratio was > 1.8 according to the absorbance value measured by UV spectrophotometer, which suggested the required DNA purity. These DNA went through bisulfite modification according to Herman *et al.*⁵ and then purified by Wizard DNA Clean-up System. The purified DNA solution served as templates for PCR amplification.

According to the sequence of E-cadherin gene (Genbank accession No. L34545), methylation-specific PCR amplification primers were designed following the approach described by Tamura *et al.*⁶ The methylated primer sequences were 5'-GGTGAATTTTAGTTAATTAGCGGTAC-3' and 5'-CATACTAACCGAAACGCCG-3'; the non-methylated primer sequences were 5'-GGTAGGTGAATTTTAGTTAATTAGTGGTA-3' and 5'-ACCCATA

ACTAACCAAAAACACCA-3' (all primers were synthesized by Shanghai Biological Engineering Company). The above-mentioned primers were used to amplify the E-cadherin promoter region 5'CpG island methylation and non-methylation alleles, respectively. The resulting PCR products were 204 bp for the methylation-specific primers, and 211 bp for the non-methylation specific primers.

PCR reaction was done with PE-2400 Amplifier, with the replacement of template DNA with sterile ddH₂O as negative control. The obtained PCR products run agarose gel electrophoresis after fully mixed with loading buffer in a ratio of 6:1.

Statistical analysis

SPSS13.0 statistical software was used for data analysis, data are expressed with the mean \pm standard deviation (SD), and comparison between groups was analyzed by analysis of variance. A value of $P < 0.05$ was considered significant.

Results

Changes in cell morphology

HT-29 cell morphology changed apparently after 5-Aza-CdR treatment while they became spindle or polygonal, irregular and sometimes grew into a cluster without 5-Aza-CdR treatment, and after treatment for 24 h, some cells had reduced cell volume, nuclear pyknosis, chromatin margination, reduced nuclear-cytoplasmic ratio, and the number of split-phase cells declined; 72 h after treatment, nucleolus disappeared in some cells surrounded by the cell membrane, and cell shape changed from the prism, polygon into a round or oval shape. Besides, a small number of cell debris analogue and some dead cells appeared.

HT-29 cell growth under interventional factors

The initial number of cells was defined as 1, and the growth curve drawing method was applied to cell counting. The growth of HT-29 cells under intervention of 5-Aza-CdR had a significant difference as compared with the control group ($P < 0.01$); with the increase in drug concentration, the cell growth rate showed a slowing trend (Figure 1).

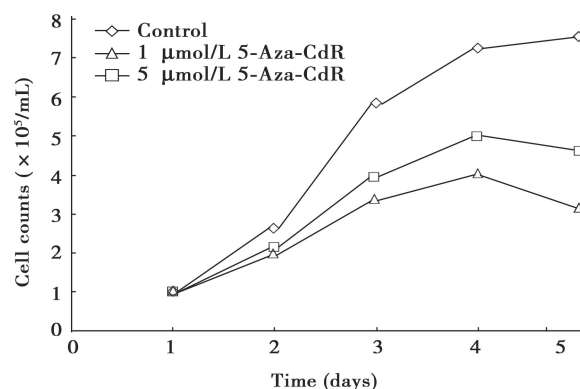


Figure 1 Growth curve of HT-29 cells after treatment with 5-Aza-CdR

Test results of immunocytochemistry

Compared with before intervention, E-cadherin expression increased in cells under intervention of 5-Aza-CdR for 24 h, difference being significant ($P < 0.01$). E-cadherin positive

expression rate increased from $(21 \pm 7)\%$ in $1 \mu\text{mol/L}$ 5-Aza-CdR treated cells to $(39 \pm 13)\%$ in $5 \mu\text{mol/L}$ 5-Aza-CdR treated cells (Figure 2).

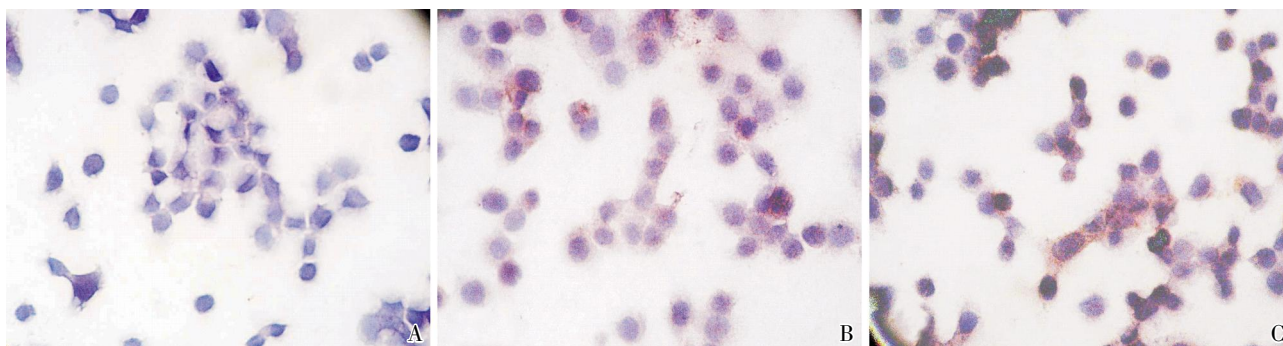


Figure 2 E-cadherin protein expression before and after treatment of 5-Aza-CdR in HT-29 cells (SP $\times 400$)

A, no treatment; B, $1 \mu\text{mol/L}$ 5-Aza-CdR; C, $5 \mu\text{mol/L}$ 5-Aza-CdR.

RT-PCR test results

In HT-29 cells without 5-Aza-CdR treatment, 630 bp specific band of E-cadherin gene mRNA could not be amplified; and this band could be detected in HT-29 cells treated by $1 \mu\text{mol/L}$ and $5 \mu\text{mol/L}$ 5-Aza-CdR for 24 h (Figure 3).

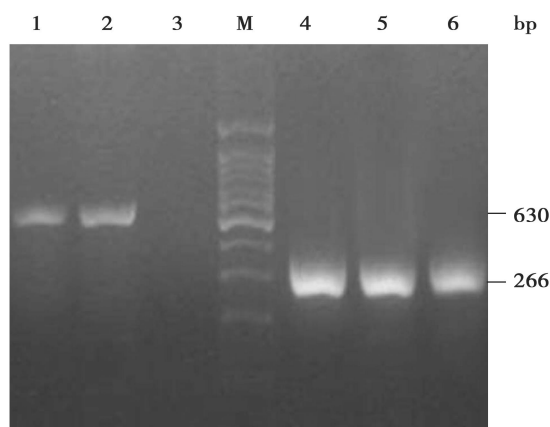


Figure 3 E-cadherin mRNA expression before and after treatment of 5-Aza-CdR in HT-29 cells

Lane 1, $1 \mu\text{mol/L}$ 5-Aza-CdR; lane 2, $5 \mu\text{mol/L}$ 5-Aza-CdR; lane 3, no treatment; lanes 4–6, GAPDH.

MSP test results

E-cadherin gene promoter region was methylation positive in HT-29 cells not treated with 5-Aza-CdR; but it was negative when treated by $1 \mu\text{mol/L}$ and $5 \mu\text{mol/L}$ 5-Aza-CdR for 24 h (Figure 4).

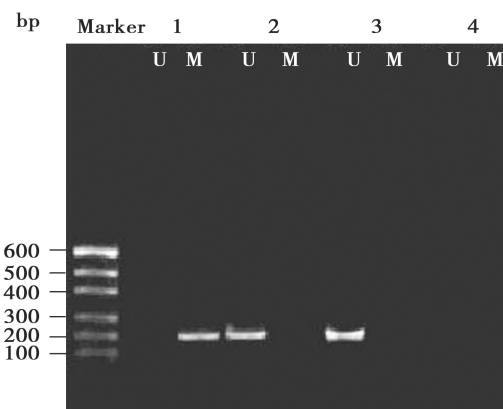


Figure 4 MSP production of E-cadherin expression in colon carcinoma cells

Lane 1, no treatment; lane 2, $1 \mu\text{mol/L}$ 5-Aza-CdR; lane 3, $5 \mu\text{mol/L}$ 5-Aza-CdR; lane 4, control. U, unmethylated; M, methylated; Marker, DNA marker.

Discussion

DNA methylation is an important epigenetic modification

regulating mode in mammals, which is the only natural chemical modification of DNA in spinal animals. Studies have shown that abnormal DNA methylation patterns occur in the process of tumor development in a variety of human cancers. Those patterns include increased methylase expression level, decreased genome-wide methylation level, and the most common pattern, the partially enhanced methylation level.⁷ Hypermethylation in CpG island of tumor suppressor gene promoter region, is an important factor for gene transcription silencing.⁸ It has been found that in tumor cells, gene silencing of multiple tumor (metastasis) suppressor was caused by CpG island methylation in their promoter regions. E-cadherin gene is located on chromosome 16q22.1.⁹ By combination of two identical E-cadherin molecules through a calcium ion to form dimers, two adjacent cells were stabilized. This stability plays a role in mediation of homogeneous adhesion and maintenance of integrity

of tissue structure. The most important physiological function of E-cadherin in epithelial malignant tumors is the inhibition of tumor cell transfer after detachment. Much data have indicated that inactivation of E-cadherin gene expression caused by methylation plays an important part in the occurrence and development of many tumors. In vitro experiments showed that E-cadherin could inhibit tumor cell invasion and metastasis, so it was known as a metastasis-suppressor factor and its inactivation of tumors was closely related to the infiltration growth and metastasis of tumor cells.¹⁰

Colon mucosa is a kind of glandular epithelium and the maintenance of epithelial morphology depends on the expression of adhesive molecules. Kinsella *et al.*¹¹ and Hu *et al.*¹² found in the histological studies of colon carcinoma and colon adenoma that decreased E-cadherin expression in colorectal carcinoma was related to tumor differentiation, invasion and metastasis, as well as Duke's staging. This study explored the expression of E-cadherin in colon cancer cells. The results showed that with the normal colon tissue expression of E-cadherin as a complete expression control, expression of E-cadherin decreased or missing in human colon cancer HT-29 cells. This is in accordance with the decreased or missing expression of E-cadherin in colon carcinoma, suggesting that down-regulated E-cadherin expression might be an important mechanism in the development of colon cancer.

Is the decreased or missing of E-cadherin expression in colon cancer related with methylation? In this study, demethylation agents (methyl-transferase inhibitor, 5-Aza-CdR) were used for intervention of methylation in HT-29 cells, and E-cadherin methylation was tested. The results showed that under the intervention of 5-Aza-CdR, there were apparent morphologic changes in HT-29 cells in terms of reduced cell volume, nuclear pyknosis, chromatin margination, reduced split-phase cell number, and nucleolus disappearance in some nucleated cells. With the increase in drug concentration, cell growth rate showed a slowing trend. According to immunocytochemical staining after 24 h of drug intervention, E-cadherin positive rate increased from $(21 \pm 7)\%$ when treated with $1 \mu\text{mol/L}$ 5-Aza-CdR to $(39 \pm 13)\%$ treated with $5 \mu\text{mol/L}$ 5-Aza-CdR. RT-PCR tests showed that without 5-Aza-CdR treatment, HT-29 cells had no E-cadherin mRNA expression while E-cadherin gene mRNA re-expression in HT-29 cells could be detected by $1 \mu\text{mol/L}$ and $5 \mu\text{mol/L}$ 5-Aza-CdR treatment after 24 h. MSP tests showed that E-cadherin gene methylation was positive in untreated HT-29 cells, but it was negative in HT-29 cells exposed to $1 \mu\text{mol/L}$ and $5 \mu\text{mol/L}$ 5-Aza-CdR for 24 h, which was in accordance with the results from Nojima *et al.*,¹³ Darwanto *et al.*¹⁴ and Garinis *et al.*¹⁵ This phenomenon suggested that changes in promoter methylation might be a significant mechanism of E-cadherin gene inactivation in colon cancer. In addition, no partial methylation was found in this study, which might be related to primer design.

Yoshiura *et al.*¹⁶ and Si *et al.*¹⁷ reported that over methylation

of E-cadherin gene promoter caused loss of E-cadherin protein expression in cell lines of gastric cancer, liver cancer, bladder cancer and esophageal cancer, which could be recovered with 5-Aza-CdR treatment. Our study also confirmed that E-cadherin gene promoter methylation was positive in E-cadherin expression down-regulated HT-29 cells detected by MSP. It was also found that after being treated with 5-Aza-CdR, E-cadherin expression regained in these cells according to immunocytochemical staining and RT-PCR. These phenomena are in agreement with the epigenetic mechanisms that high methylation inhibits gene expression.¹⁸

E-cadherin is a hot topic in cancer research, and its dynamic expression, DNA methylation regulation, and their relationship with cancer are worthy of further in-depth studies. In combination with E-cadherin expression and CpG island methylation of its gene promoter region, it is of great significance to investigate the relationship between the tumor and E-cadherin at protein and molecular levels so as to reveal the mechanism of tumor occurrence and development, and to predict the malignant degree and prognosis of the tumors. Besides, these studies will provide some molecular theory basis and experimental evidence for clinical early diagnosis, detection and treatment of the tumors.

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