Effects of DNA methylation on expression of tumor suppressor genes and proto-oncogene in human colon cancer cell lines

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Supported by the National Natural Science Foundation of China, No.30170413, and Ph.D Funds from the Ministry of Education of China, No.1999946, and the Key Subject Funds of Shanghai Education Committee to Jing-Yuan Fang  
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Received: 2002-12-22 Accepted: 2003-01-14

Abstract
AIM: To investigate the effects of DNA methylation on the expression of tumor suppressor genes and proto-oncogene in human colon cancer cell lines.

METHODS: Three colon cancer cell lines (HT-29, SW1116 and Colo-320) treated with different concentrations of DNA methyltransferase inhibitor, 5-aza-2’-deoxycytidine (5-aza-dC) were used to induce DNA demethylation. The expressions of p16\textsuperscript{INK4A}, p21\textsuperscript{WAF1}, APC and c-myc genes were observed by using RT-PCR. The methylation status of p16\textsuperscript{INK4A} promoter in HT-29 cells was also determined by methylation-specific PCR (MSP).

RESULTS: Weak expressions of p16\textsuperscript{INK4A} and APC in the three colon cancer cells were detected, and p21\textsuperscript{WAF1} expression was not found in SW1116 and Colo-320 cells before treatment. After treatment of 1 \mu M or not 10 \mu M of 5-aza-dC, the methylation level of p16\textsuperscript{INK4A} gene promoter decreased significantly, and the hypomethylation led to the up-regulation of p16\textsuperscript{INK4A} gene transcription in HT-29 cells. In the cell lines of SW1116 and Colo-320, p16\textsuperscript{INK4A} and APC mRNA expressions were obviously enhanced after treatment of either 10 \mu M or 5 \mu M of 5-aza-dC for 24 h. However, no evidence was found that methylation regulated the expression of p21\textsuperscript{WAF1} and c-myc genes in human colon cancer cell lines.

CONCLUSION: Expression of p16\textsuperscript{INK4A} and APC genes is regulated by DNA methylation in three human colon cancer cell lines.

Fang JY, Lu J, Chen YX, Yang L. Effects of DNA methylation on expression of tumor suppressor genes and proto-oncogene in human colon cancer cell lines. World J Gastroenterol 2003; 9 (9): 1976-1980  
http://www.wjgnet.com/1007-9327/9/1976.asp

INTRODUCTION
DNA methylation is the main epigenetic modification after replication in humans\cite{4}. DNA (cytosine-5)-methyltransferase (DNMT) catalyzes the transfer of a methyl group from S-adenosyl-L-methionine (SAM) to C5 of cytosine within CpG dinucleotide sequences in genomic DNA of higher eukaryotes. The expression of some genes can be frequently inactivated by reversible epigenetic events rather than genetic events\cite{2,3}.

Colon cancer is one of the most common tumors worldwide. The loss of p21\textsuperscript{WAF1}, p16\textsuperscript{INK4A} and adenomatous polyposis coli (APC) gene expression, or/and the over-expression of c-myc gene are believed to play a crucial role in colon carcinogenesis\cite{4-6}. As demonstrated in our previous review\cite{7}, mutation of p16\textsuperscript{INK4A} was not found but the frequency of hypermethylation was 10-53 % in colon cancer. Previous studies by two independent groups of investigators have demonstrated that inactivation of p16\textsuperscript{INK4A} in human colon tissue might be due to de novo methylation of promoter-associated CpG island\cite{8,9}. Colon cancer cell lines, Colo-320\cite{10,11} and SW1116\cite{12,13}, were frequently used in molecular biological experiments.

To date, most of these studies were focused on aberrant methylation in a single gene. However, little is known about the regulation of methylation on the expression of several tumor suppressor genes and proto-oncogenes in the same human colon cancer cell line. Furthermore, several clinical trials indicated that methylation inhibitor, 5-aza-2’-deoxycytidine (5-aza-dC) was devoid of antitumour activity in adult patients with colon cancer\cite{14,15}. We want to know whether 5-aza-dC induces over-expression of proto-oncogene while regulates the transcription of tumor suppressor gene.

In this study, we investigated the transcriptional level of p16\textsuperscript{INK4A}, p21\textsuperscript{WAF1}, APC tumor suppressor genes, and c-myc proto-oncogenes. We examined whether the expression of these genes was influenced by methylation in colon cancer cell lines. The focus of this work was to gain a better understanding of the factors involved in regulating DNA methylation.

MATERIALS AND METHODS

Cell culture
Colon cancer-derived cell lines HT-29, Colo-320 and SW1116 were maintained by serial passages in MEM containing 10 % heat-inactivated FCS, 20 mmol/L of L-glutamine, 62.5 mg/L of penicillin, and incubated at 37 °C using standard tissue culture incubators as described previously\cite{16}. The cells were plated at 10\textsuperscript{4} cells onto per 100-mm dish.

Treatment with 5-aza-dC
5-aza-dC was a DNMT inhibitor\cite{17}. To assess the expression of p16\textsuperscript{INK4A}, p21\textsuperscript{WAF1}, APC and c-myc genes by 5-aza-dC, colon cancer cell lines were exposed to different concentrations (1 \mu M and 10 \mu M for HT-29 cells; 2 \mu M for HT-29 cells; 2 \mu M and 5 \mu M for Colo-320 at 10 \mu M) for 24 hours and 72 hours. The control cultures were treated simultaneously with PBS. The media were changed, DNA and RNA were harvested at various time points, respectively. We did not find cytotoxicities reactions from 5-aza-dC, even at 10 \mu M concentration.

Reverse transcription polymerase chain reaction (RT-PCR)
Total RNA was isolated by using a commercial kit (Trizol)
according to the manufacturer's instructions (Gibco BRL). Reverse transcription reactions using 5 µg of total RNA in a total reaction volume of 20 µl were performed with Superscript II reverse transcriptase (Life Technologies, Inc.). The mRNA transcription levels of p16\textsuperscript{INK4A}, p21\textsuperscript{WAF1}, APC and c-myc genes were evaluated by using RT-PCR. Primer sequence and PCR reaction for each primer are shown in Table 1. For control of RT-PCR, a 612 bp (322 bp for p16\textsuperscript{INK4A}) RT-PCR in HT-29 fragment of β-actin CDNA was also amplified. The density of bands in RT-PCR were quantitated by using a molecular dynamics phosphorImager (Nucleo Tech Inc., San Mateo, CA), which were normalized to the amount of total RNA as determined by the density of β-actin band from RT-PCR\textsuperscript{[16]}. RT-PCR was performed three times at least.

**Methylation-Specific PCR (MSP) for p16\textsuperscript{INK4A}**

We followed Clark's method of bisulfite treatment\textsuperscript{[20]} with some modifications as follows. Two µg of total genomic DNA (from at least two independent treatments corresponding to RT-PCR experiments) was isolated by using QIAamp DNA blood mini kit (QIAGEN Inc.), then denatured by NaOH and modified by sodium bisulfite solution (2.35 mol/L containing hydroquinone (0.04 mol/L)) freshly prepared. The bisulfite-treated DNA was desalted using Wizard DNA clean up kit (Promega). To amplify the p16\textsuperscript{INK4A} promoter, we used 0.1 µg aliquot of converted DNA. Methylation of the 5’ CpG island in p16\textsuperscript{INK4A} gene was also determined in samples from HT-29 cells treated by 5-aza-dC. The bisulfite treated DNA was amplified by PCR using primers specific for the methylated or unmethylated primer. The GenBank accession number, sequences of primers and program of PCR are also shown in Table 1. PCR product was directly loaded onto 3 % agarose gels and electrophoresed. The gel was stained with ethidium bromide and directly visualized under UV illumination.

**RESULTS**

**Methylation in p16\textsuperscript{INK4A} promoter in HT-29 cells treated with 5-aza-dC**

We examined the methylation status of p16\textsuperscript{INK4A} following 5-aza-dC treatment using MSP. Bisulfite treatment converted the cytosine residues in the genomic DNA to uracil, which were amplified as thymine during subsequent PCR. As shown in Figure 1, HT-29 cells showed a positive 150-151 bp band for methylated and unmethylated specific primer sets for p16\textsuperscript{INK4A} respectively, indicating that p16\textsuperscript{INK4A} gene was partially methylated in this cell line. The methylated bands for p16\textsuperscript{INK4A} gene in the mock treated HT-29 cells were consistently stronger than the products of 5-aza-dC treated HT-29 cells. Thus, the product level from PCR using unmethylated primer was significantly higher, and methylated product level was correspondingly lower in HT-29 cells treated with 5-aza-dC.

Three days after treatment with 1 µM of 5-aza-dC, MSP revealed a significant increase in the amount of unmethylated product (Figure 1). These results suggested that p16\textsuperscript{INK4A} gene was a target of the decreased methylation level in HT-29 cells treated with 5-aza-dC.

**Restoration of p16\textsuperscript{INK4A} gene expression by 5-aza-dC**

We initially tried to find out whether there were expressions of several tumor suppressor genes such as p16\textsuperscript{INK4A}, p21\textsuperscript{WAF1} and APC, and proto-oncogene c-myc in human colon cancer cell lines HT-29 (p16\textsuperscript{INK4A} only), Colo-320 and SW1116. mRNA levels of the above genes were investigated by using semiquantitative RT-PCR. p16\textsuperscript{INK4A} gene was expressed in these three cell lines slightly prior to the treatment with 5-aza-dC.

In the first part of the present study, we examined the possibility of methylation on expression regulation of p16\textsuperscript{INK4A} in three colon cancer cell lines. Increased levels of p16\textsuperscript{INK4A} expression were seen in HT-29 cells treated with lower (1 µmol/L, 24 hours) but not higher (10 µmol/L, 24 hours) concentrations of 5-aza-dC (Figure 2, Table 2). In contrast, 5-aza-dC induced transcription of p16\textsuperscript{INK4A} at higher concentration (10 µmol/L) for 24 hours or 72 hours, but not at the lower concentration (2 µmol/L or 5 µmol/L) for the same duration (Figures 3A and 3B, lanes 3 and 4, Table 3).
Table 1: Sequences of primers and program of PCR

| Primers          | Sense(5' → 3') | Antisense(5' → 3') | Size of product and PCR condition | GenBank accession number |
|------------------|----------------|--------------------|-----------------------------------|-------------------------|
| β-actin RT-PCR   | GGA GTC CTG    | CTA GAA GCA        | 322 bp                            | XM004814                |
| (for \(5′\)-aza-dC RT-PCR in HT-29) | TGG CAT CCA CG    | TTT GCG GTG GA        | 94 °C 3 min; 94 °C 30 s, 60 °C 1 m, 72 °C 1 m, 27 X; 72 °C 5 m | BC023204                |
| β-actin RT-PCR   | GGC ATC GTG    | GCT GGA AGG        | 612 bp                            |                         |
| (for RT-PCR in other cells) | ATG GAC TCC G    | TGG ACA GCG A        | 94 °C 5 min; 92 °C 40 s, 58 °C 40 s, 72 °C 50 s, 30 X; 72 °C 5 min | L27211                  |
| p16^INK4A         | CCC GTC TTC    | TTA TTT GGA        | 355 bp                            |                         |
| RT-PCR           | GTA GTG TTC AT | CTT TGG TTC TG      | 94 °C 5 min; 94 °C 1 min, 58 °C 1 min, 72 °C, 1 min, 35 X; 72 °C 5 min |                         |
| APC              | GAG ACA GAA    | GAA GGA TTA TTA TTT GAA TTT GGC TGT CTC A 95 °C 5 min; 95 °C 1 min, 53 °C 1 min, 72 °C, 1 min, 35 X; 72 °C 5 min | AF209032                |
| RT-PCR           | TGG AGG TGC TGC | GAA TTA TCT TCT A  | 95 °C 5 min; 95 °C 1 min, 53 °C 1 min, 72 °C, 1 min, 35 X; 72 °C 5 min | NM_000389                |
| p21^INK4A        | CAG GGG ACA    | GGG CGG CCA        | 335 bp                            |                         |
| RT-PCR           | GCA GAG GAA GA GA | GGG TAT GTC A    | 94 °C 5 min; 94 °C 1 min, 58 °C 1 min, 72 °C, 1 min, 35 X; 72 °C 5 min |                         |
| c-myc            | CCA ACA GGA    | CTC GTG CAG CAT CAG CT 94 °C 5 min; 94 °C 1 min, 52 °C 1 min, 72 °C, 1 min, 35 X; 72 °C 5 min | V00568                  |
| RT-PCR           | GCT ATG ACC TC | CTC CGT CT         | 94 °C 5 min; 94 °C 1 min, 52 °C 1 min, 72 °C, 1 min, 35 X; 72 °C 5 min |                         |
| p16^INK4A MSP CAG AGG GTG | CGG GCC GGC   | GCC GTG G         | 140 bp                            | X94154                  |
| (Wild-type)      | GGG GCC ACC CGC | GCT GGG G         | 95 °C 5 min; 95 °C 1 min, 65 °C 2 min, 72 °C 3 min, 5 X; 95 °C 30 s, 65 °C 30 s, 72 °C 1 min, 35 X; 72 °C 5 min |                         |
| p16^INK4A MSP GAC CCC GAA CGG | CCA CGA TGA CAG TAA | 150 bp          | 95 °C 5 min; 95 °C 1 min, 65 °C 2 min, 72 °C 3 min, 5 X; 95 °C 30 s, 65 °C 30 s, 72 °C 1 min, 35 X; 72 °C 5 min | X94154                 |
| P-methyl-primers | TTA TTA GAG GGT | TGA GCA GGG CCG | 95 °C 5 min; 95 °C 1 min, 65 °C 2 min, 72 °C 3 min, 5 X; 95 °C 30 s, 65 °C 30 s, 72 °C 1 min, 35 X; 72 °C 5 min |                 |
| p16^INK4A MSP GAC CCC GAA CGG | CCA CGA TGA CAG TAA | 150 bp          | 95 °C 5 min; 95 °C 1 min, 65 °C 2 min, 72 °C 3 min, 5 X; 95 °C 30 s, 65 °C 30 s, 72 °C 1 min, 35 X; 72 °C 5 min |                 |
| MSP unmethyl    | CAA CCC CAA ACC ACC A | 151 bp         | 95 °C 5 min; 95 °C 1 min, 60 °C 2 min, 72 °C 3 m °C, 5 X; 95 °C 30 s, 60 °C 30 s, 72 °C 1 min, 35 X; 70 °C 5 min | X94154               |
| primers         | GGG GTG GAT GGT | CAA ACC ACC A  |                         |                         |

Table 3: Expression of p16^INK4A gene in SW1116 and Colo-320 cells (the band density)

|       | 5-aza-dC treatment | Mock treated | 2 µmol/L, 24 h | 5 µmol/L, 24 h | 10 µmol/L, 24 h | 2 µmol/L, 72 h | 5 µmol/L, 72 h | 10 µmol/L, 72 h |
|-------|-------------------|--------------|----------------|----------------|----------------|----------------|----------------|----------------|
| SW1116| 1494.7            | 2055.5       | 2436.9         | 3487.3         | 1592.0         | 2074.8         | 2774.0         |
| Colo-320| 809.1            | 860.6       | 829.2          | 1298.8         | 875.7          | 923.5          | 1189.6         |

The density of each band from RT-PCR in each lane of Figure 3 was normalized to the amount of total RNA as determined by the density of band in RT-PCR for β-actin.

Table 4: Expression of APC gene in SW1116 and Colo-320 cells (the band density)

|       | 5-aza-dC treatment | Mock treated | 2 µmol/L, 24 h | 5 µmol/L, 24 h | 10 µmol/L, 24 h | 2 µmol/L, 72 h | 5 µmol/L, 72 h | 10 µmol/L, 72 h |
|-------|-------------------|--------------|----------------|----------------|----------------|----------------|----------------|----------------|
| SW1116| 786.2             | 1481.2       | 782.6          | 796.9          | 802.9          | 1173.5         | 1236.8         |
| Colo-320| 1804.6           | 2388.2       | 4055.2         | 1923.9         | 1803.0         | 3197.8         | 3271.7         |

The density of each band from RT-PCR in each lane of Figure 4 was normalized to the amount of total RNA as determined by the density of band in RT-PCR for β-actin.

5-aza-dC increased transcription level of APC gene

To identify whether the transcription level of APC was regulated by DNA methylation in human colon cancer cell lines, we cultured Colo-320 and SW1116 cells with or without 5-aza-dC treatment for 24 hours and 72 hours. The data from RT-PCR implied that before incubation with 5-aza-dC, the levels of APC transcription in these cells were lower (Figure 4, line 1, Table 4). Incubation for 24 hours with 5-aza-dC resulted in the accumulation of APC mRNA, whose levels remained unchanged during the 72 hour incubation period. APC mRNA levels were normalized with respect to the level of β-actin mRNA, which did not change during culture with 5-aza-dC (Figure 4, Table 4). RT-PCR was repeated twice and the results were consistent.

The effectiveness of 5-aza-dC on the expression of APC was high even at lower concentration (2 µmol/L), suggesting that methylation-induced silencing of this gene was the primary event. Restoration of APC expression by 5-aza-dC treatment confirmed a causal relationship between DNA hypermethylation and APC silencing in colon cancer cell lines Colo-320 and SW1116.

5-aza-dC treatment failed to induce expression of p21^INK4A and c-myc in Colo-320 and SW1116 cells

To further define the modification status of APC unmethylated CpG islands in Colo-320 and SW1116, we attempted to observe whether their transcription levels would change after treatment with DNMT inhibitor. Although no expression of p21^INK4A and c-myc was observed, further experiments were required to determine whether the silenced state of APC in Colo-320 and SW1116 cells was the primary event.
significant over-expression of c-myc were seen in mock treatment. Our current study revealed that almost no change in activity was seen when these two cell lines Colo-320 and SW1116 cells were treated by 5-aza-dC. In other words, regulation of methylation on the expression of p21^{WAF1} and c-myc genes was not found (data not shown).

Taken these together, it was suggested that the methylation silencing transcription be localized at specific regions of the chromatin. Other mechanisms might play a role in controlling the activity of p21^{WAF1} and c-myc genes in colon cancer cell lines Colo-320 and SW1116.

Figure 4 5-aza-dC increased the transcription of A PC gene in Colo-320 (A) and SW1116 cells. Lane 1: mock treatment. Lanes 2-7: after 5-aza-dC treatment; lane 2: 2 µmol/ L, 24 h; lane 3: 5 µmol/ L, 24 h; lane 4: 10 µmol/ L, 24 h; lane 5: 2 µmol/ L, 72 h; lane 6: 5 µmol/ L, 72 h; lane 7: 10 µmol/ L, 72 h. The density of bands shown in Table 4.

DISCUSSION
Compelling evidences for the role of epigenetic modification on the regulation of gene transcription have been published[21-26]. p16^{INK4A} was a tumor suppressor gene originally identified by Serrano et al[27], and the methylation profile of p16^{INK4A} promoter differed in each cancer type[26]. Several studies indicate that 5-aza-dC induced growth inhibition might be resulted from the release of methylation silenced cell cycle regulatory gene p16^{INK4A}[26]. APC gene hypermethylation is frequent but not universal in colon cancer cell line. Previous studies showed that p21^{WAF1} transcription was regulated by histone acetylation, another modification of epigenetics in human colon cancer[30], but little is known about the effect of DNA methylation on this gene expression.

In the current study, our findings indicated firstly that p16^{INK4A} was expressed in these three human colon cancer cell lines, and APC was expressed with p21^{WAF1} inactivated in Colo-320 and SW1116 cells. 5-aza-dC induced hypomethylation of p16^{INK4A} promoter and the restoration of p16^{INK4A} transcription, suggesting that DNA methylation is the major regulation mechanism for p16^{INK4A} in HT-29, Colo-320 and SW1116 cells. Previously it was suggested that lack of p21^{WAF1} expression appeared to be the result of hypermethylation of its promoter region, as p21^{WAF1} protein expression could be induced by growth of Rat-1 cells in the presence of 5-aza-dC[31]. However, the influence of methylation on p21^{WAF1} gene expression was dependent on differentiation of cells and tissues[30]. An important finding from this study indicated that reduction of DNA methylation might not play a crucial role in the regulation of p21^{WAF1} transcription in human colon cancer cell lines, Colo-320 and SW1116.

c-Myc proto-oncogene has been found to be deregulated in colon cancer. Over-expression of c-Myc in tissue culture caused an increase in cell proliferation with a shortened G1 phase, whereas loss of c-Myc resulted in slow growth and longer G1 phase[32]. Over-expression and abnormal intracellular location of the product of proto-oncogene c-myc in colon dysplasia and neoplasia might be related to the alteration in epigenetic mechanisms controlling the function of this gene[33]. Although hypomethylation of c-myc in human tumors has also been reported, it is not clear whether demethylation induces the over-expression of c-myc in human tumor cell lines. This paper reports that 5-aza-dC did not up-regulate c-myc transcription, while the expression of p16^{INK4A} and APC tumor suppressor genes responded to 5-aza-dC treatment in colon cancer cell lines. The reason why 5-aza-dC failed to colon cancer treatment was not due to c-myc over-expression from demethylation.

In conclusion, our study results support the concept that there are significant differences in the regulatory response to DNA methylation in different genes including tumor suppressor gene and proto-oncogene, even in the same colon cancer cell lines Colo-320 or SW1116.

ACKNOWLEDGEMENTS
We are grateful to Ms.Hong-Yin Zhu and Ju-Fang Tong for performing the RT-PCR and cell culture, and Dr.Xie-Ning Wu for his assistance in preparing this manuscript.

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Edited by Zhu LH and Wang XL