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• COLORECTAL CANCER •

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

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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 $p16^{INK4A}$, $p21^{WAF1}$, *APC* and *c-myc* genes were observed by using RT-PCR. The methylation status of $p16^{INK4A}$ promoter in HT-29 cells was also determined by methylation-specific PCR (MSP).

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

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

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INTRODUCTION

DNA methylation is the main epigenetic modification after replication in humans[1]. 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[2,3].

Colon cancer is one of the most common tumors worldwide. The loss of $p21^{WAF1}$, $p16^{INK4A}$ 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[4]. As described in our previous review[5], mutation of $p16^{INK4A}$ 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 $p16^{INK4A}$ in human colon tissue might be due to *de novo* methylation of promoter-associated CpG island[6-8]. Colon cancer cell lines, Colo-320[9-11] and SW1116[12-14], 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[15-17]. 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 $p16^{INK4A}$, $p21^{WAF1}$, *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 ℃ using standard tissue culture incubators as described previously[18]. The cells were plated as 10^6 cells onto per 100-mm dish.

Treatment with 5-aza-dC

5-aza-dC was a DNMT inhibitor[19]. To assess the expression of $p16^{INK4A}$, $p21^{WAF1}$, *APC* and *c-myc* genes by 5-aza-dC, colon cancer cell lines were exposed to different concentrations (1 μmol/L and 10 μmol/L for HT-29 cells; 2 μmol/L, 5 μmol/L and 10 μmol/L for Colo-320 and SW1116 cells) of 5-aza-dC (Sigma, St. Louis, MO) 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 cytotoxics reactions from 5-aza-dC, even at 10 μmol/L 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^{INK4A}*, *p21^{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^{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[16]. RT-PCR was performed three times at least.

Methylation-Specific PCR (MSP) for p16^{INK4A}

We followed Clark's method of bisulfite treatment[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^{INK4A}* promoter, we used 0.1 μg aliquot of converted DNA. Methylation of the 5' CpG island in *p16^{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^{INK4A} promoter in HT-29 cells treated with 5-ada-dC

We examined the methylation status of *p16^{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^{INK4A}* respectively, indicating that *p16^{INK4A}* gene was partially methylated in this cell line. The methylated bands for *p16^{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^{INK4A}* gene was a target of the decreased methylation level in HT-29 cells treated with 5-aza-dC.

Restoration of p16^{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^{INK4A}*, *p21^{WAF1}* and *APC*, and proto-oncogene *c-myc* in human colon cancer cell lines HT-29 (*p16^{INK4A}* only), Colo-320 and SW1116. mRNA levels of the above genes were investigated by using semiquantitative RT-PCR. *p16^{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^{INK4A}* in three colon cancer cell lines. Increased levels of *p16^{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^{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 2 The expression of *p16^{INK4A}* gene in HT-29 cells (the band density)

5-aza-dC conc	Mock treated	1 μM, 24 h	10 μM, 24 h
Density	2257.7	2782.5	1975.3

The density of each band from RT-PCR in each lane of Figure 2 was normalized to the amount of total RNA as determined by the density of band in RT-PCR for β-*actin*.

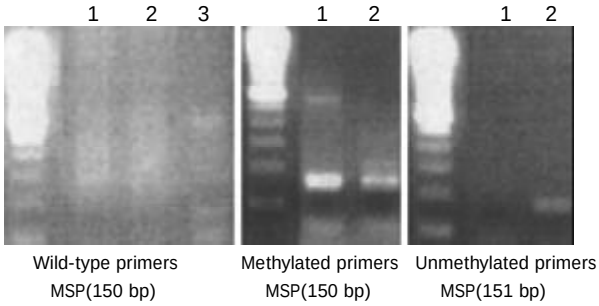

Figure 1 5-aza-dC induced hypomethylation of the promoter of *p16^{INK4A}* gene in HT-29 cells. Lane 1, untreated; lane 2, 5-aza-dC treated; lane 3, untreated with bisulfite. MSP was performed with the specific primers described in the Materials and Methods.

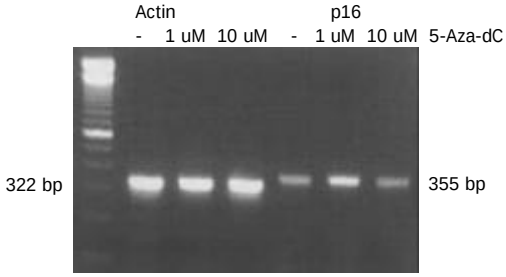

Figure 2 Up-regulated mRNA level of *p16^{INK4A}* by 5-aza-dC in HT-29 cells. RT-PCR was performed as described in Materials and Methods. β-*actin* was used as a loading / amplification control.

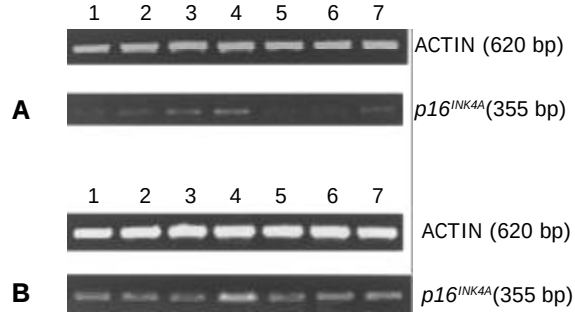

Figure 3 5-aza-dC increased the transcription of *p16^{INK4A}* gene in Colo-320 (A) and SW1116 cells. Lane 1: mock treatment. Lanes 2-7: after 5-aza-dC treatment; lane 2: 2 μmol/L, 24 h; lane 3: 5 μmol/L, 24 h; lane 4: 10 μmol/L, 24 h; lane 5: 2 μmol/L, 72 h; lane 6: 5 μmol/L, 72h; lane 7: 10 μmol/L, 72 h. The density of bands shown in 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 *p16*^{INK4A} RT-	TGG CAT CCA CG	TTT GCG GTG GA	94 ℃ 3 m; 94 ℃ 30 s, 60 ℃	
PCR in HT-29)			1 m, 72 ℃ 1 m, 27 X; 72 ℃ 5 m	
β-*actin* RT-PCR	GGC ATC GTG	GCT GGA AGG	612 bp	BC023204
(for RT-PCR	ATG GAC TCC G	TGG ACA GCG A	94 ℃ 5 min; 92 ℃ 40 s, 58 ℃ 40 s,	
in other cells)			72 ℃ 50 s, 30 X; 72 ℃ 5 min	
p16^{INK4A}	CCC GCT TTC	TTA TTT GAG	355 bp	L27211
RT-PCR	GTA GTT TTC AT	CTT TGG TTC TG	94 ℃ 5 min; 94 ℃ 1 min,58 ℃ 1 min,	
			72 ℃, 1 min, 35 X; 72 ℃ 5 min	
APC	GAG ACA GAA	GTA AGA TGA TTG	170 bp	AF209032
RT-PCR	TGG AGG TGC TGC	GAA TTA TCT TCT A	95 ℃ 5 min; 95 ℃ 1 min, 53 ℃ 1 min,	
			72 ℃, 1 min, 35 X; 72 ℃ 5 min	
p21^{WAF1}	CAG GGG ACA	GGG CGG CCA	335 bp	NM_000389
RT-PCR	GCA GAG GAA GA	GGG TAT GTA C	94 ℃ 5 min; 94 ℃ 1 min, 58 ℃ 1 min,	
			72 ℃ 1 min, 35 X; 72 ℃ 5 min	
c-myc	CCA ACA GGA	CTC GGT CAC CAT	290 bp	V00568
RT-PCR	GCT ATG ACC TC	CTC CAG CT	94 ℃ 5 min; 94 ℃ 1 min, 52 ℃ 1 min,	
			72 ℃, 1 min, 35 X; 72 ℃ 5 min	
p16^{INK4A} MSP	CAG AGG GTG	CGG GCC GCG	140 bp	X94154
(Wild-type)	GGG CGG ACC CGC	GCC GTG G	95 ℃ 5 min; 95 ℃ 1 min, 65 ℃ 2 min, 72 ℃ 3 min,	
			5 X; 95 ℃ 30 s, 65 ℃ 30 s, 72 ℃ 1 min, 35 X; 72 5 min	
p16^{INK4A} MS	TTA TTA GAG GGT	GAC CCC GAA CCG	150 bp	X94154
P-methyl-primers	GGG GCG GAT CGC	CGA CCG TAA	95 ℃ 5 min; 95 ℃ 1 min, 65 ℃ 2 min, 72 ℃ 3 min,	
			5 X; 95 ℃ 30 s, 65 ℃ 30 s, 72 ℃ 1 min, 35 X; 72 ℃ 5 min	
p16^{INK4A}	TTA TTA GAG GGT	CAA CCC CAA ACC	151 bp	X94154
MSP-unmethyl	GGG GTG GAT TGT	ACA ACC ATA A	95 ℃ 5 min; 95 ℃ 1 min, 60 ℃ 2 min, 72 ℃ 3 m ℃,	
primers			5 X; 95 ℃ 30 s, 60 ℃ 30 s, 72 ℃ 1 min, 35 X; 70 ℃ 5 min	

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, 24 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, 24 h
SW1116	786.2	1481.2	782.6	796.9	802.9	1173.5	1236.8
Colo320	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^{WAF1} *and c-myc in Colo-320 and SW1116 cells*

To further define the modification status of *p21*^{WAF1} and *c-myc* expression in colon carcinogenesis, we attempted to observe whether their transcription levels would change after treatment with DNMT inhibitor. Although no expression of *p21*^{WAF1} and

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.

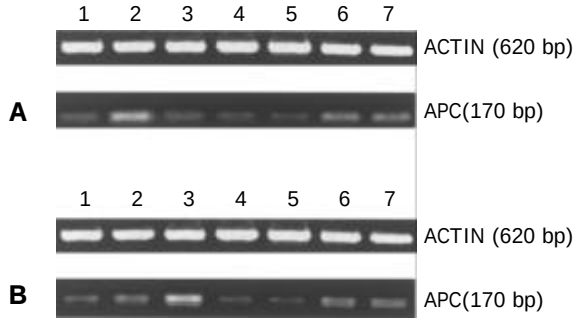

Figure 4 5-aza-dC increased the transcription of *APC* gene in Colo-320 (A) and SW1116 cells. Lane 1: mock treatment. Lanes 2-7: after 5-aza-dCtreatment; lane 2: 2 μmol/L, 24 h; lane 3: 5 μmol/L, 24 h; lane 4: 10 μmol/L, 24 h; lane 5: 2 μmol/L, 72 h; lane 6: 5 μmol/L, 72 h; lane 7: 10 μmol/L, 72 h. The 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[28]. Several studies indicat that 5-aza-dC induced growth inhibition might be resulted from the release of methylation silenced cell cycle regulatory gene $p16^{INK4A}$[29]. *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 it' s 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-oncoprotein 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.

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