ARTICLE

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Epigenetic modification regulates both expression of tumor-associated genes and cell cycle progressing in human colon cancer cell lines: Colo-320 and SW1116

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ABSTRACT

The aim of this study is to assess the effects of DNA methylation and histone acetylation, alone or in combination, on the expression of several tumor-associated genes and cell cycle progression in two established human colon cancer cell lines: Colo-320 and SW1116. Treatments with 5-aza-2'-deoxycytidine (5-aza-dC) and trichostatin A, alone or in combination, were applied respectively. The methylation status of the *CDKN2A* promoter was determined by methylation-specific PCR, and the acetylated status of the histones associated with the $p21^{WAF1}$ and *CDKN2A* genes was examined by chromatin immunoprecipitation. The expression of the *CDKN2A*, $p21^{WAF1}$, *p53, p73*, *APC, c-myc, c-Ki-ras* and *survivin* genes was detected by real-time RT-PCR and RT-PCR. The cell cycle profile was established by flow cytometry.

We found that along with the demethylation of the *CDKN2A* gene promoter in both cell lines induced by 5-aza-dC alone or in combination with TSA, the expression of both *CDKN2A* and *APC* genes increased. The treatment of TSA or sodium butyrate up-regulated the transcription of $p21^{WAF1}$ significantly by inducing the acetylation of histones H4 and H3, but failed to alter the acetylation level of *CDKN2A*-associated histones. No changes in transcription of *p53, p73, c-myc, c-Ki-ras* and *survivin* genes were observed. In addition, TSA or sodium butyrate was shown to arrest cells at the G_1 phase. However, 5-aza-dC was not able to affect the cell cycle progression. In conclusion, regulation by epigenetic modification of the transcription of tumor-associated genes and the cell cycle progression in both human colon cancer cell lines Colo-320 and SW1116 is gene-specific.

Keywords: human colon cancer cell lines, tumor-associated genes, DNA methylation, histone acetylation, cell cycle.

INTRODUCTION

Colon cancer is one of the most commonly occurring tumors and a major cause of cancer-related deaths worldwide. Colon cancer cell lines Colo-320[1-3] and SW1116 [4-6] are frequently used in molecular biological experiments. The transcription of *CDKN2A*[7], $p21^{WAF1}$ [8] and adenomatous polyposis coli (*APC*)[9] genes are down-regulated and *c-myc* proto-oncogene is over-expressed[10] in the Colo-320 cell line.

CDKN2A inhibits the catalytic activity of the cyclin-dependent kinases 4 (CDK4) /cyclin D complex and also blocks the G_1/S transition in cells[11, 12]. $p21^{WAF1}$ is one of the CIP/KIP family, which inhibits late G_1/S check point kinases. The increased expression of $p21^{WAF1}$ may induce growth arrest in transformed cells[13-15]. c-myc is a transcription factor whose normal function is to promote cell proliferation[8, 16].

Epigenetic modifications, mainly DNA methylation[17] and changes of histones, are now recognized as additional mechanisms contributing to malignant phenotypes[18]. Several studies have demonstrated that the inactivation of CDKN2A in human colon tissue may be due to *de novo* methylation of its 5' promoter-associated CpG island[19-23].

Hyperacetylation of histones has been shown to open chromatin and is required for transcriptional activation[24]. Because the inhibitory effects of complex endogenous genes may play a significant role in the G_1-S progression of the cell cycle, histone deacetylases (HDAC) inhibitors, trichostatin A (TSA, a hybrid polar compound specific inhibitor)[24-26] and sodium butyrate (butyrate, a short chain fatty acid, SCFA)[27-29] have been considered as candi-

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Abbreviations: 5-aza-dC, 5-aza-2'-deoxycytidine; HAT, histone acetyltransferase HDAC, histone deacetylase; APC, adenomatous polyposis coli; TSA, trichostatin A; SCFA, short chain fatty acid; CDK, cyclin-dependent kinase; CDKI, cyclin-dependent kinase inhibitor; Dnmt1, DNA methyltransferase 1.

dates for the treatment of cancer[20].

Previous studies focused on aberrant methylation or acetylation alone in a single gene. However, little is known about the effect of the regulation of methylation and histone acetylation on the expression of several tumor-associated genes including the tumor suppressor gene and proto-oncogene in human colon cancer. The DNA methylation status, gene-associated-histone acetylation pattern and the transcriptional level of tumor-associated genes in the presence of combined 5-aza-dC and HDAC inhibitors in the Colo-320 and SW1116 cell lines remain unclear. Furthermore, we want to know whether 5-aza-dC or TSA induces over-expression of proto-oncogene while regulating the transcription of tumor suppressor genes. The aim of this study is to gain a better understanding of the effect of the regulation of DNA methylation and histone acetylation on the regulation of tumor suppressor gene and proto-oncogene transcription.

MATERIALS AND METHODS

Cell culture

The colon cancer-derived cell lines Colo-320 and SW1116 were maintained by serial passages in RPMI1640 containing 10% heat-inactivated FCS, 100 U/ml penicillin and 100 μg/ml streptomycin, and incubated at 37°C, 5% CO_2-95% air using standard tissue culture incubators as described previously[30].

Treatment with 5-aza-dC , TSA and butyrate

5-aza-dC is an inhibitor of DNA methyltransferase (Dnmt 1) [31], but TSA and butyrate are HDAC inhibitors. To assess the restoration of *CDKN2A*, $p21^{WAF1}$, *APC, p53, p73, c-myc, c-Ki-ras* and *survivin* genes expression by 5-aza-dC, TSA or butyrate treatment, the colon cancer cell lines were exposed to different concentrations (2 μM, 5 μM or 10 μM) of 5-aza-dC (Sigma, St. Louis, MO) alone for 24 h and 72 h; 1 μM TSA or 5 μM butyrate (Sigma, St. Louis, MO) alone for 24 h. For the combined treatment of drugs, cells were incubated with 10 μM of 5-aza-dC for 2 d and then with 1 μM of TSA or 5 μM of sodium butyrate for 24 h. The control cultures were treated with phosphate-buffered saline (PBS) or ethanol (for TSA treatment, because TSA can only be dissolved in PBS containing ethanol). DNA and RNA were extracted at various time points.

Cell viability assays

The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenytetrazolium (MTT) assay measures both cytostatic and cytotoxic responses to drug treatment and thus provides an accurate overall measure of drug sensitivity. Cell viability assays were performed in quadruplicate wells of 24-well plates 3 d after the treatment of 5-aza-dC or TSA or sodium butyrate. MTT (Calbiochem, San Diego, CA) assay was used to assess drug-induced cell growth inhibition and cytotoxicity. No significant cytotoxic damage was observed during the drug treatment.

Bisulfite modification and methylation-specific PCR (MSP) for *CDKN2A* promoter

To address whether DNA methylation of *CDKN2A* changed during the drug treatment, we carried out bisulfite modification[32] and MSP to define a CpG-rich region in the *CDKN2A* gene promoter.

Bisulfite converts unmethylated cytosine residues to uracil, but methylated cytosines remain nonreactive. PCR amplifies uracil as thymine while methylated cytosines can only be amplified as cytosines. Genomic DNA treated by bisulfite was amplified with *CDKN2A* promoter gene fragment specific primers. PCR reaction buffer contained 0.1 mM dNTP, 2.0 mM $MgCl_2$, and 0.5 μM primers. PCR product was directly loaded on to 3% agarose gels and electrophoresed. The gel was stained with ethidium bromide and directly visualized under UV illumination. The sequences and PCR program are shown in Tab 1. Furthermore, wild-type *CDKN2A* primers were used to monitor the complete conversion of DNA obtained in the bisulfite reaction.

Chromatin immunoprecipitation (ChIP) assay

A ChIP assay kit from Upstate Biotechnology was used according to the manufacturer's protocol and Richon's report[33]. Colo-320 and SW1116 cells, mock-treated and treated with drugs, were plated at a density of 10×10^6 cells/T25 flask. $p21^{WAF1}$- and *CDKN2A* -specific primers were used to carry out PCR. The density of bands in ChIP-PCR was quantitated using a Molecular Dynamics PhosphorImager (Nucleo Tech Inc, San Mateo, CA). The sequences of one set of primers for *CDKN2A* and two sets of primers for $p21^{WAF1}$ PCR, as well as the PCR condition are shown in Tab 1. The 1st set of primers was used to amplify -576 to -293 and the 2nd set of primers was used to amplify -51 to +77 of $p21^{WAF1}$ promoter and exon 1, which contained the transcription factor E2A binding sites. The 395 bp band corresponds to the promoter of *CDKN2A* (21 CpG sites, positions -494 to -101)[34].

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted by using a commercial kit (Trizol) according to the manufacturer's instructions (Gibco BRL). RT reactions using 5 μg of total RNA were performed with Superscript II reverse transcriptase (Life Technologies, Inc.). The mRNA transcription levels of *p53*, *p73*, *c-Ki-ras* and *survivin* genes were determined by RT-PCR. The sequences and PCR reaction for each primer are shown in Tab 1. For the control of RT-PCR, a 612 bp fragment of *β-actin* cDNA was also amplified.

Real-time quantitative PCR

The mRNA levels of *CDKN2A*, $p21^{WAF1}$, *APC* and *c-myc* were measured using a real-time quantitative PCR system. Relative quantitation using the comparative Ct method with data from the ABI PRISM 7700 Sequence Detection System (version 1.6 software) was performed according to the manufacturer's protocol. The primers and Taqman fluorogenic probes for *CDKN2A*, $p21^{WAF1}$, *APC* and *c-myc* were provided by Shenyou Company, Shanghai. The sequences of forward and reverse primers and the probes are shown in Tab 2. Real-time PCR was also performed with primers and Taqman *β-actin* probes to normalize each of the extracts for amplifiable human DNA. The results were expressed as the ratio of copies of *CDKN2A*, $p21^{WAF1}$, *APC* and *c-myc* to *β-actin,* respectively. The Ct values were

Tab 1. Sequences and PCR programs of primers for RT-PCR, sequencing and ChIP-PCR

Gene	Reactions	Primer (Sense) (5'→ 3')	Primer (Antisense) (5'→ 3')	Size and PCR condition	GenBank accession number
β-actin	RT-PCR	GGC ATC GTG ATG GAC TCC G	GCT GGA AGG TGG ACA GCG A	612 bp 92°C 40 sec, 58°C 40 sec, 72°C 50 sec, 30 cycles	BC023204
c-Ki-ras	RT-PCR	ATG ACT GAA TAT AAA CTT GTG GTA	TGT CTT GTC TTT GCT GAT GTT TCA	449 bp 95°C 30 sec, 55°C 30 sec, 72°C 30 sec, 35 cycles	M54968
survivin	RT-PCR	ATT CGT CCG GTT GCG CTT TCC TTT	TTC CTA AGA CAT TGC TAA GGG GCC	297 bp 94 °C 1 min, 55°C 1 min, 72°C 1 min, 35 cycles	U 75285
p53	RT-PCR	CAG CCA AGT CTG TGA CTT GCA CGT AC	CTA TGT CGA AAA GTG TTT CTG TCA TC	292 bp 94°C 30 sec, 65°C 1 min, 72°C 1 min, 35 cycles	XM008679
p73	RT-PCR	AAC GCT GCC CCA ACC ACG AG	GCC GGT TCA TGC CCC CTA CA	226 bp 95°C 30 sec, 60°C 30 sec, 72°C 30 sec, 35 cycles	Y11416
CDKN2A	Methyl-MSP	TTA TTA GAG GGT GGG GCG GAT CGC	GAC CCC GAA CCG CGA CCG TAA	150 bp 95°C 1 min, 65°C 1 min, 72°C 1 min, 40 cycles	X94154
CDKN2A	Unmethyl-MSP	TTA TTA GAG GGT GGG GTG GAT TGT	CAA CCC CAA ACC ACA ACC ATA A	151 bp 95°C 1 min, 60°C 1 min, 72°C 1 min, 40 cycles	X94154
$p21^{WAF1}$ p1	ChIP-PCR	CGT GGT GGT GGT GAG CTA GA	CTG TCT GCA CCT TCG CTC CT	296 bp 95°C 1 min, 56°C 1 min, 72°C 1 min, 35 cycles	U 24170
$p21^{WAF1}$ p2	ChIP-PCR	GGT TGT ATA TCA GGG CCG	CTC TCA CCT CCT CTG AGT GC	128 bp 95°C 1 min, 52°C 1 min, 72°C 1 min, 35 cycles	U 24170
CDKN2A	ChIP-PCR	GGG CTC TCA CAA CTA GGA	CGG AGG AGG TGC TAT TAA CTC	395 bp 95°C 1 min, 58°C 1 min, 72°C 1 min, 40 cycles	AF527803

measured, and the average Ct of triplicate samples was calculated. Alteration of mRNA expression was defined as a 3-fold difference in the expression level after treatment, relative to that before treatment [35].

Flow cytometric (FCM) detection of cell cycle

Cell cycle analysis was carried out by FCM[36]. In brief, a total of 1×10^8 cells were removed from the treated and mock-treated cultures at specified time points. The cells were washed twice with PBS and fixed in ice-cold ethanol for 1 h. The samples were concentrated by removing ethanol and treated with 1% (v/v) Triton X-100 (Sigma, USA) and 0.01% RNase (mg/ml, sigma, St. Louis, Mo.) for 10 min at 37°C. The staining of cellular DNA was performed with 0.05% propidium iodide for 20 min at 4°C in darkness. The cell cycle distribution was detected using a flow cytometer (Model FACSCALIBAR, BD, USA) and 10,000 cells were analyzed with the MultiCycle software package (Phoenix, San Diego, CA, USA).

Statistical analysis

Differences in the cell cycle between those cells treated with drugs and those mock-treated were analyzed by the Fisher exact test.

Tab 2. Sequence of primers and probes for real-time PCR

Gene	Primer (Sence) (5'→3')	Primer (Antisense) (5'→3')	Probe	GenBank No
CDKN2A	CAT AGA TGC CGC GGA AGG T	CAG AGC CTC TCT GGT TCT TTC AA	CCT CAG ACA TCC CCG	NM_058197
p21^{WAF1}	CTG GAG ACT CTC AGG GTC GAA	GGA TTA GGG CTT CCT CTT GGA	ACG GCG GCA GAC CAG CAT GA	NM_078467
APC	TTG ACA AAC TTG ACT TTT GGA GAT G	CAT GCA GCC TTT CAT AGA GCA T	AGC CAA CAA GGC TAC	NM_000038
c-myc	ACA CCG CCC ACC ACC AG	CCA CAG AAA CAA CAT CGA TTT CTT	AGC GAC TCT GAG GAG G	V00568
β-actin	CTG GCA CCC AGC ACA ATG	GGA CAG CGA GGC CAG GAT	ATC ATT GCT CCT CCT GAG	BC016045

RESULTS

Demethylation of promoter and the restoration of expression of *CDKN2A* gene by 5-aza-dC

In the first part of this study, we examined the possibility of methylation in regulating the expression of *CDKN2A* in Colo-320 and SW1116 cells. As shown in Fig 1, mock-treated Colo-320 and SW1116 cells showed a positive 150 bp band and 151 bp band for methylated and unmethylated specific primer sets for *CDKN2A* respectively, indicating that the *CDKN2A* gene is partially methylated in the tested cell lines. The methylated bands of *CDKN2A* gene in the cells treated with 10 μM of 5-aza-dC for 24 h were consistently lower than those of the mock-treated cells (Fig 1). Thus, unmethylated product was significantly higher in the 5-aza-dC treated cells. The data from this study showed that untreated Colo-320 and SW1116 cells were methylated at some CpG sites in most alleles. After treatment with 10 μM of 5-aza-dC, the cytosine (C) in CpG changed to thymine (T) in some sites.

As shown in Tab 3, 5-aza-dC in a higher concentration (10 μM) induced transcription of *CDKN2A* at 24 h, but not in a lower concentration (2 μM or 5 μM). In contrast, at 72 h post-treatment, the level of *CDKN2A* mRNA of cells exposed to 5-aza-dC was less than that at 24 h.

However, the transcription of the *CDKN2A* gene can be induced by 5-aza-dC alone but not by TSA alone or the combination of 5-aza-dC and TSA. Also the treatment of TSA or sodium butyrate failed to induce the acetylation of histones H3 and H4 in colon cancer cells (Fig 2C). Thus, the above results indicate that DNA methylation but not histone acetylation is the major regulating mechanism for *CDKN2A* expression in human colon cancer cell lines.

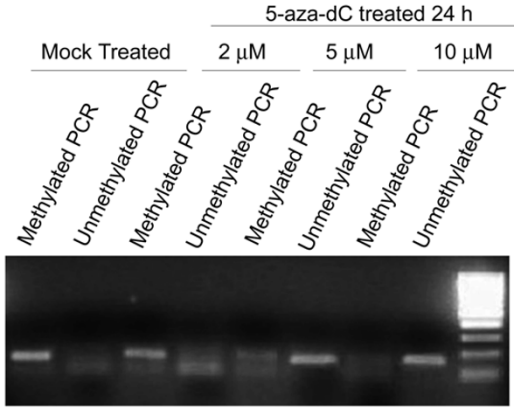
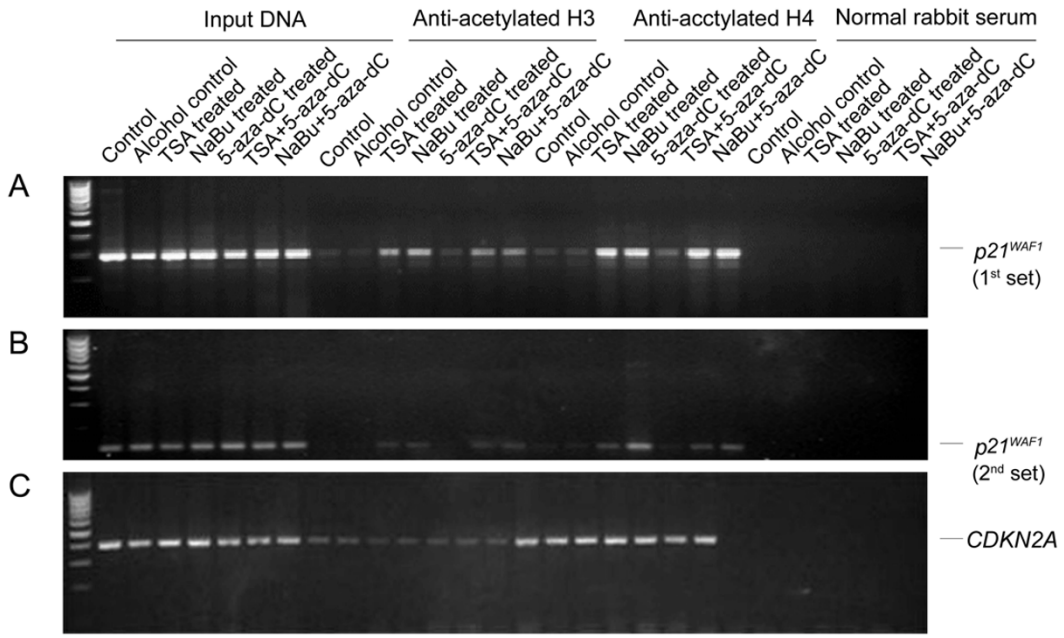


Fig 1. Methylation analysis of *CDKN2A* gene promoter by MSP. MSP was performed with the specific primers described in the Materials and Methods.

5-aza-dC but not TSA or sodium butyrate increased the transcription of the *APC* gene

To identify whether the transcription level of *APC* is regulated by DNA methylation or histone acetylation or both in human colon cancer cells, *APC* mRNA level was assayed. Colo-320 and SW1116 cells were cultured with



D *p21*^{WAF1}-assocaited histones acetylation*

Drugs		IP.DNA/Input DNA				
		TSA	NaBu	5-aza-dC	TSA+aza	NaBu+aza
Fold increased	Anti-H3	1.80	2.01	1.12	2.08	1.84
	Anti-H4	2.17	2.53	1.29	2.91	3.10

*Because the density of bands from the control and the ethanol control in PCR using 2nd set primers were not detected,the data indicated in table was the result from ChIP-PCR using 1st set primers only.

Fig 2. TSA and sodium butyrate (NaBu) but not 5-aza-dC induced accumulation of acetylated histones H3 and H4 in *p21*^{WAF1} gene but not *CDKN2A*. Soluble chromatin from Colo-320 cells treated with or without TSA or NaBu or 5-aza-dC was immunopreciptated with antiacetylated histones H3 and H4 antibodies. The 1st (**A**) and the 2nd (**B**) sets PCR primers for the regions of *p21*^{WAF1} , and (**C**) were PCR primers for the promoter of *CDKN2A* as indicated in Materials and Methods. (**D**) The Figs in A and B were scanned and quantified by Molecular Dynamics PhosphorImager. The ratio between input DNA and precipitated DNA was calculated for each treatment and primer set. The folds increased after treatment with TSA or NaBu or 5-aza-dC were calculated from the indicated ratios.

or without 5-aza-dC or TSA or sodium butyrate for 24 h and 72 h. An incubation of 24 h with 5-aza-dC resulted in the accumulation of *APC* mRNA, which remained at a higher level during a 72 h incubation period. The *APC* mRNA levels were normalized with *β-actin* mRNA (Tab 3). The effect of 5-aza-dC on the expression of *APC* was high even at a very low dosage (2 μM for Colo-320 and 5 μM for SW1116 cells), suggesting that methylation-induced silencing of this gene was the primary event. The restoration of *APC* expression by 5-aza-dC confirmed a causal relationship between DNA hypermethylation and *APC* silencing in colon cancer cell lines Colo-320 and SW1116.

No obvious change was seen during the treatment with TSA or sodium butyrate. The expression of the housekeeping gene *β-actin* was used as a control to ensure the similarity in quality and quantity of RNA samples amplified by real-time RT-PCR.

Either TSA or sodium butyrate but not 5-aza-dC induced the acetylation of *p21*^{WAF1} gene-associated histones and the re-expression of *p21*^{WAF1}

To determine whether histone acetylation affects *p21*^{WAF1} transcription, and whether a functional interaction occurs between *p21*^{WAF1} and TSA or sodium butyrate treatment, ChIP-PCR was performed. The input was 10% of the

Tab 3. Alteration of mRNA expression of *CDKN2A*, *p21^{WAF1}*, *APC*, *c-myc* and *β-actin* in Colo-320 and SW1116 cells, using real-time quantitative PCR

Groups	*CDKN2A*		*p21^{WAF1}*		*APC*		*c-myc*	
	Colo-320	SW1116	Colo-320	SW1116	Colo-320	SW1116	Colo-320	SW1116
Mock control	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Ethanol control	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
5-aza-dC treated								
2 μM, 24 h	1.72	1.05	0.37	0.73	4.66*	1.24	0.88	0.72
5 μM, 24 h	2.77	1.65	0.47	0.73	1.01	3.71*	0.81	1.07
10 μM, 24 h	3.41*	3.76*	0.45	1.09	1.30	0.81	0.93	0.67
2 μM, 72 h	1.00	1.73	0.38	1.46	1.87	1.38	0.73	1.19
5 μM, 72 h	1.22	1.85	0.98	1.03	3.05*	1.93	1.36	0.96
10 μM, 72 h	2.81	1.74	0.76	1.44	2.81	3.03*	1.09	1.10
TSA	0.77	0.85	27.1*	6.72*	1.59	0.69	0.52	0.90
Sodium butyrate	0.52	1.16	17.15*	3.63*	1.69	0.71	0.45	0.97
TSA +5-aza-dC	1.42	1.02	30.70*	6.82*	4.26*	0.95	1.17	0.99
Butyrate +5-aza-dC	1.29	1.25	16.11*	3.41*	3.14*	1.13	1.57	1.16

Reverse transcription of total RNA from Colo-320 and SW1116 cells and subsequent amplification of cDNA resulted in 56- ,98-, 66-, and 73 bp fragments for *CDKN2A* , *p21^{WAF1}, APC,* and *c-myc*, respectively. Using real-time quantitative PCR with *β-actin* (93 bp) as internal standard. *Alteration of mRNA expression was defined as a 3-fold difference in the transcription level in colon cancer cell lines after treatment relative to before treatment.

amount of chromatin which was used for immunoprecipitation.

As shown in Fig 2, the densities of bands of *p21^{WAF1}* gene-associated acetylated histone H4 and H3 were higher in chromatin extracted from either TSA or sodium butyrate treated Colo-320 cells than that from mock-treated cells. 285- and 128 bp fragments of *p21^{WAF1}* promoter region and exon 1 were amplified (Fig 2A and 2B). In the PCR product containing transcription factor E2A binding sites of promoter region, the positive bands that were not detected in controls were found after being cultured with TSA or sodium butyrate.

On the other hand, sodium butyrate or TSA or both combined with 5-aza-dC greatly increased the *p21^{WAF1}* transcription level in the cells. As shown in Tab 3, after treatment with 5-aza-dC alone, there was no significant difference between the expression of this gene in cells treated with and without drugs. In addition, in Colo-320 cells, *p21^{WAF1}* expression increased to a higher level than that in SW1116 cells.

Taken together, the above results indicated that TSA or sodium butyrate activated the transcription of *p21^{WAF1}* through acetylating histones H4 and H3 associated with the *p21^{WAF1}* promoter.

5-aza-dC or TSA or sodium butyrate treatment failed to induce expression of *p53*, *p73*, *c-myc*, *c-Ki-ras* and *survivin* in Colo-320 and SW1116 cells

To further define the modification status of proto-oncogene *c-myc* overexpression in colon cancer cells, we attempted to clarify whether *c-myc* expression is altered after treatment with Dnmt inhibitor or HDAC inhibitors. Although the significant overexpression of *c-myc* and *c-Ki-ras* has been found in mock-treated Colo-320 and SW1116 cells, our current study revealed that almost no change in transcription was seen when these two cell lines were treated by 5-aza-dC or TSA or sodium butyrate. In other words, no evidence was found that 5-aza-dC and

Tab 4. TSA and sodium butyrate induce the arrest of G1 to S phase in SW1116 cells

Treatment	G_0/G_1-phase (%)	S-phase (%)	G_2/M-phase (%)
Mock treatment	34.6±4.8	37.2±1.7	28.2±6.6
Ethanol control	30.3±4.6	38.8±2.8	31.0±6.3
TSA treatment	85.9±3.6**	4.8±0.1**	9.4±3.6**
Butyrate treatment	88.6±1.8**	5.0±0.5**	6.4±1.4**
5-aza-dC treatment	39.1±1.7	36.5±1.7	24.4±3.4
TSA + 5-aza-dC	89.6±1.8**	4.6±0.5**	5.8±1.9**
Sodium butyrate +5-aza-dC	89.8±1.3**	5.1±0.7**	5.1±1.9**

Cell cycle analysis was performed by FCM. TSA and sodium butyrate induce histone hyperacetylation and the re-expression of *p21^{WAF1}*, and induce a arrest of $G_1 \rightarrow S$ phase in SW1116 cells. mRNA from mock treatment (or ethanol control) *vs* that from drugs treatment, $^{**}P<0.01$, Fisher exact test.

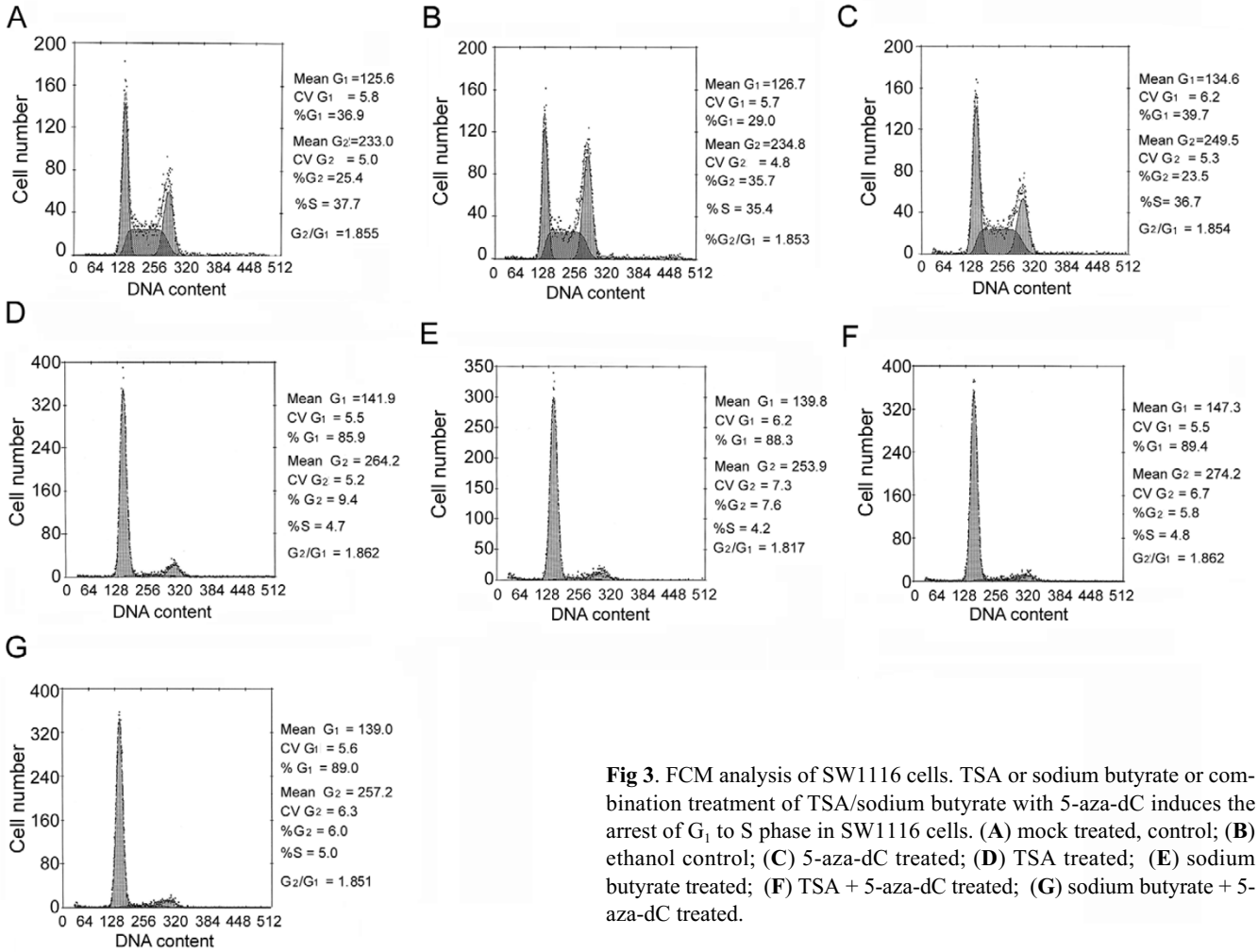


Fig 3. FCM analysis of SW1116 cells. TSA or sodium butyrate or combination treatment of TSA/sodium butyrate with 5-aza-dC induces the arrest of G_1 to S phase in SW1116 cells. (**A**) mock treated, control; (**B**) ethanol control; (**C**) 5-aza-dC treated; (**D**) TSA treated; (**E**) sodium butyrate treated; (**F**) TSA + 5-aza-dC treated; (**G**) sodium butyrate + 5-aza-dC treated.

TSA or sodium butyrate could induc the accumulation of *c-myc* gene mRNA (Tab 3). Taken together, the 5-aza-dC and TSA or sodium butyrate-induced changes in methylation and histone acetylation were localized at specific regions of the chromatin. Other mechanisms might explain a possibility in controlling the activity of the *c-myc* gene in colon cancer cell lines Colo-320 and SW1116. In addition, the effect of regulation of epigenetic modification on the expression of *p53*, *p73*, *c-Ki-ras* and *survivin* was not found (data not shown).

TSA or sodium butyrate induces cell cycle arrest

To address whether demethylation or acetylation of $p21^{WAF1}$ affects the cell cycle, FCM was performed as described above. As expected, the data demonstrated a significant accumulation of cells in the G_1 and G_2 phases, and a decrease in the S-phase, thus indicating that either TSA or sodium butyrate induced cell cycle arrest in these two cell lines. Tab 4 further elucidated the association between hypoacetylation and the cell cycle, showing that the ratio of the cell number in G_1/G_0 increased significantly, and that in the G_2/M and S-phases it decreased. Interestingly, 5-aza-dC-treated SW1116 cells did not present comparable cell cycle arrest although 5-aza-dC induced the restoration of *CDKN2A* expression; also there was no correlation between *CDKN2A* gene expression and changes in the cell cycle of SW1116 cells (see representative examples in Fig 3).

Taken together, these results suggested that due to the lack of $p21^{WAF1}$ expression in human colon cancer cells, and hence an absence of G_1-phase arrest in the cells, the genes were re-expressed after treatment with TSA or sodium butyrate. Similar results were obtained from another colon cancer cell line, Colo-320 (data not shown).

DISCUSSION

DNA methylation and chromatin modification are two global mechanisms for regulating gene expression. CpG

island chromatin is found to contain highly acetylated histones H3 and H4[37]. The process of DNA methylation mediated by Dnmt1 may depend on the generation of an altered chromatin status via histone deacetylase activity [38]. Many investigators showed that genes silenced by the promoter hypermethylation could be reactivated by treatment with the demethylating drug 5-aza-dC, either alone or in combination with an HDAC inhibitor[31, 39], suggesting that DNA methylation and chromatin architecture act together to silence genes. Furthermore, TSA induces $G_0 \rightarrow G_1$ cell cycle arrest[40].

Several studies indicate that *CDKN2A* is hypermethylated in colon cancer cells[41], and 5-aza-dC-induced growth inhibition may result from the release of methylation silencing of the cell cycle regulatory genes, *CDKN2A*[42]. However, it remains unclear whether histone acetylation regulates the transcription of *CDKN2A* and *APC* genes. Our finding that 5-aza-dC but not TSA or sodium butyrate induces the transcription of *CDKN2A* and *APC*, suggests that DNA methylation rather than histone acetylation is the major regulation mechanism for these two genes in Colo-320 and SW1116 cells. We noted that 5-aza-dC enhanced *CDKN2A* transcription, but the combination of 5-aza-dC and TSA did not induce more expression of *CDKN2A*. It is possible that apoptotic cell deaths occurred when cells were treated by a combination of demethylation and TSA [43]. However, for $p21^{WAF1}$, either TSA or the combination of TSA and 5-aza-dC exhibits a 17- to 30-fold (Colo-320 cells) or 3- to 6-fold (SW1116 cells) increase in transcription, suggesting that synergic gene activation occurs (Tab 3). Regarding the mechanism of sensitization mentioned above, these data indicated that the deme thylation of the gene promoters and the activity of 5-aza-dC could have complex effects on the localization and activity of HDACs that had not been previously contemplated[44].

Butyrate is known to alter the expression of a variety of genes by modulating histone acetylation[45-47]. Both sodium butyrate and TSA were shown to inhibit HDAC, leading to hyperacetylation of selective histone proteins such as histone H4[46]. However, most previous studies have demonstrated that the transcription of $p21^{WAF1}$ could be strongly enhanced by TSA in the colon cancer cell line, HT29[48], but have not elucidated the expression status of this gene during the treatment of 5-aza-dC or HDAC inhibitors in the human colon cancer cell lines Colo-320 and SW1116. As summarized in Tab 3 and 4, the hyperacetylation of histone but not demethylation induced the re-expression of $p21^{WAF1}$ and a G_1-phase arrest of Colo-320 and SW1116 cells. We also implied that either TSA or sodium butyrate or in combination with 5-aza-dC, but not 5-aza-dC alone, could induce $p21^{WAF1}$ expression. We used real-time quantitative PCR based on TaqMan methodology to analyze quantitatively the expression of these genes in routinely processed cells and demonstrated that the measurements of gene expression could be reliably and accurately conducted in such cells.

The inhibition of proliferation demonstrated a significant accumulation of cells in the G_1 and G_2 phases, and a decrease of cells in the S-phase of the two TSA-treated cell lines. TSA and sodium butyrate-induced growth inhibition could result from the release of hypoacetylation silencing of the cell cycle regulatory genes such as $p21^{WAF1}$. We also examined whether the re-expression of *CDKN2A* induced by promoter hypomethylation could induce the arrest of the cell cycle similar to that by $p21^{WAF1}$ in human colon cancer cells. The result was negative. Data from this report supports the idea that $p21^{WAF1}$ expression is more important than *CDKN2A* for the regulation of the cell cycle in the human colon cancer cell lines Colo-320 and SW1116.

Although reduced levels of methylation of genes including *c-myc* in human tumors have also been reported, it has not been shown convincingly that they are indeed responsible for increased levels of gene expression rather than merely a secondary characteristic observed in cancer cells. Overexpression and abnormal intracellular location of the product of the proto-oncogene *c-myc* in colon dysplasia and neoplasia may be related to alterations in epigenetic mechanisms which controll the function of this gene[49]. *p73* is a new member of the *p53* family. The transcriptional silencing of the *p73* gene by hypermethylation of a CpG island was observed in several leukemia and lymphomas [50]. Survivin is the first apoptosis inhibitor described todate to be expressed in G_2-M, in a cell cycle-dependent manner[45] and is detected in all cases of normal colon mucosa. Up to now, little is known about the relevance between the methylation and transcriptional level of *c-myc, p53*[47], *p73* and *survivin* genes during colon carcinogenesis or human colon cancer cell lines. Therefore, the coexistence of methylated and unmethylated status of several tumor suppressor genes and proto-oncogenes in the same colon cancer cell line, reflects the *in vitro* situation and has some functional significance.

The effect of the promoter methylation of several tumor- associated genes and histone acetylation of $p21^{WAF1}$ and *CDKN2A* transcription have been analyzed in human colon cancer cell lines Colo-320 and SW1116. In this study, the results from RT-PCR and real-time RT-PCR indicate first that *CDKN2A*, *APC, p53, p73, survivin* and *c-myc, c-Ki-ras* are expressed in Colo-320 and SW1116 cell lines. Furthermore, epigenetic modification did not seem to be involved in the transcription regulation of *p53, p73, survivin* and *c-myc, c-Ki-ras* genes in these two cell lines.

One of the most important findings from this study is that neither DNA methyltransferase inhibitor nor HDAC

inhibitor regulates the transcription of *c-myc* and *c-Ki-ras* proto-oncogenes in colon cancer cell lines Colo-320 and SW1116, although *c-myc*[51] and *c-Ki-ras*[52] were over-expressed in most human colon cancer. Previous clinical trials indicated that 5-aza-dC was devoid of anti-tumour activity in adult patients with colon cancer[53-55]. The data in present study suggests that the reason for its failing in colon cancer treatment is not *c-myc* over-expression from demethylation.

In conclusion, the major findings of this present study support the concept that histone acetylation but not methylation is the major mechanism in the regulation of $p21^{WAF1}$ gene expression, whereas methylation regulates the expression of *CDKN2A* and *APC*. The transcription level of *p53, p73*, *c-myc, c-Ki-ras* and *survivin* tumor associated genes are regulated by either methylation or histone acetylation in human colon cancer cell lines Colo-320 and SW1116. Our results not only enhance our understanding of the molecular epigenetic mechanisms underscoring colon carcinogenesis, but also facilitate the development of diagnostic tools based on the DNA methylation profile and histone acetylation status for the early diagnosis of human colon cancer.

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