

Original Article

Promoter methylation and expression of RASSF1A genes as predictors of disease progression in colorectal cancer

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Abstract: Aberrant DNA methylation of RAS-association domain family (RASSF) is believed to be involved in gene inactivation process in various types of cancers. Therefore, this study tried to investigate the possible involved mechanisms along with clinical effects of RASSF gene methylation on patients with colorectal cancer. Promoter methylation status in four reported CRC related RASSF family genes, such as RASSF1A, RASSF2, RASSF4, RASSF5 were identified with methylation-specific polymerase chain reaction and validated with sequencing in 97 CRC patients and adjacent normal tissues. Then mRNA and protein expression was further examined. Afterwards, survival analysis was performed over a median follow-up of 60 months. Lastly, human CRC cell lines were treated with demethylating agent 5-azacytidine *in vitro*. Aberrant methylation of RASSF1A, RASSF2 was found to be associated with TNM stage and tumor differentiation ($P < 0.01$). Methylated RASSF1A and RASSF2 were associated with significant suppression of expression in tumors ($P < 0.05$). Hypermethylation status of RASSF1A was also associated with significantly worse overall survival ($P < 0.05$). Reduced DNA methylation and increased expression of RASSF1A genes *in vitro* was observed. Lastly, increased cell apoptosis as well as G₁-S arrest was further confirmed following 5-azacytidine treatment. Promoter hypermethylation significantly suppressed RASSF1A gene expression in patients' tissue samples and therefore may represent a prospective therapeutic candidate for CRC therapy.

Keywords: Colorectal cancer, methylation, RAS-association domain family, apoptosis, cell cycle

Introduction

Colorectal cancer is a common and high prevalence cancer in the world [1]. Many oncogenes and tumor suppressor genes were reported to regulate genetic and epigenetic status in CRC, thus affecting the biological function and clinical outcome of the carcinoma [2]. Despite of the rise in recent identified cancer related genes, RAS family still draw significant attention in cancer studies as a well-known tumor suppressor gene. RAS-association domain family (RASSF) members were a newly identified family of putative tumor suppressor RAS effectors. Loss or altered expression of this gene was also found to be correlated with the hypermethylation of its CpG-island promoter region

along with pathogenesis of many different cancers [3-5].

RASSF family of proteins includes members from RASSF1 to RASSF10. Among them, RASSF1A, RASSF2, RASSF4 and RASSF5 were most widely reported to be associated with colorectal carcinoma. Moreover, down regulation of RASSF1A expression was found in many types of human cancers [6-9]. Due to the highly conservative characteristic of the promoter region, methylation becomes the major reason of RASSF1A inactivation [10-13]. Evidence from previous studies confirmed a direct correlation between promoter methylation and loss of RASSF1A expression [14-17]. Additionally, RASSF2 is another RASSF family member which has a

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Table 1. All MSP Primers

Gene name	MSP primer (5'-3')	Un-MSP primer (5'-3')
RASSF1A	CGAGAGCGCGTTTAGTTTCGTT	GGTTTTGTGAGAGTGTGTTA
	CGATTAACCCGTA CTTCGCTAA	CACTAACAAACACAAACCAAAC
RASSF2	GGTTTA AGT TTTTCG GTT TAT TC	GGTTTA AGT TTT TTG GTT TAT TTG GA
	CACGTCTAA CCGACC CGC CAA ATC G	TCA CAT CTAACCAACCCA CCA AAT CA
RASSF4	CGATTAACCCGTA CTTCGCTAA	GTAGCGGTTTTTGTGGAAAGTTTAGGAGTT
	GGTTTAAGT TTTTGGTT TAT TTGGA	AGTTGAATAATGGTTTGGGGATATTTGGT
RASSF5	CGTCGTTTGGTACGGATTTATTTTTCGGTTC	ATTTATATTTGTGTAGATGTTGTTTGGTAT
	GACAACTTAAACAACGACGACTTTAACGACTACG	ACTTTAACAAACAACAACCTTTAACAACTACA

relatively high prevalence of methylation percentage in primary colorectal tumors [18]. Another RASSF member, RASSF4, has also been found to have a lower expression in CRC. However, the gene's inactivation status could be reversed by administering a general demethylation agent, 5-aza-2'-deoxycytidine [19,20]. RASSF5, also known as a novel Ras effector 1 (NORE1), is the closest homolog of RASSF1. Additionally, NORE1 gene is located on chromosome 1q32.1, and its two major transcripts (*NORE1A* and *NORE1B*) are derived from different promoter usage [21]. *NORE1A* is epigenetically inactivated by promoter hypermethylation in various cancer cell lines and primary tumors [22-24]. In this study, we investigated the expression and hypermethylation status of RASSF1A, RASSF2, RASSF4 and RASSF5 in a group of primary colorectal carcinoma tissues and cancer cell lines to explore its candidacy as a tumor suppressor in colorectal tumor genesis.

Materials and methods

Study patients

Ethics Committee from the first affiliated hospital of Xinxiang Medical University approved the ethical requirements for this study. Also, all patients consented to the procedures of molecular analysis. Moreover, tissues samples from 97 CRC patients and adjacent normal tissue, that underwent curative surgery without the use of chemotherapy, were analyzed and compared. American Joint Committee was used on Cancer TNM system in order to determine the tumor burden [19]. Moreover, the CRC patient group contained 32 females and 65 males with a mean age of 58.64 ± 10.75 years. Lastly, tumor tissue was collected from the central part of the lesion at the time of surgery, and all

the respective tissue samples were collected and frozen in liquid nitrogen at the First Affiliated Hospital of Xinxiang Medical University.

Methylation analysis

Genomic DNA was extracted and treated with bisulfite by using the EpiTect Fast Bisulfite Conversion Kit (Qiagen, US). Methylation specific PCR (MSP) primers of four selected candidate genes were prepared as described previously [26]. MSP was performed using 50 nanograms of modified DNA and Taq DNA polymerase (Roche, US) in 7500 Real-Time PCR system (Applied Biosystem, US). Furthermore, the methylation was calculated from threshold cycles (CT) values. Lastly, MSPs were analytically validated using methylated DNA as positive control and primary keratinocyte DNA as unmethylated controls.

Genetranscript expression analysis by real-time quantitative PCR

Total cellular RNA was extracted from CRC tissue and cell pellets with TRIZOL (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's instructions. Additionally, the total RNA was frozen in liquid nitrogen and stored at -80°C . Complementary DNA (cDNA) was synthesized using M-MLV RTase cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, United States) with RNA as the template. Transcript expression was evaluated using gene-specific primers (**Table 1**) in a 20 μL reaction (2 μL of template cDNA, 1 μL of 10 $\mu\text{mol/L}$ primers, 10 μL of 2 \times SYBR Green Master Mix, 4 μL of 25 mmol/L Mg^{2+} and 2 μL of ddH_2O) on an ABI Prism 7500HT sequence detection system (Applied Biosystems) with the following cycling parameters: 3 min at 95°C followed by 40 cycles at 95°C for 10 s and 60°C for 30 s.

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Table 2. The association of promoter methylation frequencies with some pathological and clinical features of patients

Clinical and pathological features	N	RASSF1A					RASSF2					RASSF4					RASSF5			
		M	%	U	%	P	M	%	U	%	P	M	%	U	%	P	M	%	U	%
All cases	97	42	43.3	55	56.7		37	38.1	60	61.9		34	35.1	63	64.9		39	40.2	58	59.8
Age																				
<50	34	10	23.8	24	43.6		12	32.4	22	36.7		12	35.3	22	34.9		9	23.1	25	43.1
≥50	63	32	76.2	31	56.4		25	67.6	38	63.3		22	64.7	41	65.1		30	76.9	33	56.9
Sex																				
Female	55	23	54.8	32	58.2		19	51.4	36	60.0		7	20.6	48	76.2		21	53.8	34	58.6
Male	42	19	45.2	23	41.8		18	48.6	24	40.0		27	79.4	15	23.8		18	46.2	24	41.4
Tumor location																				
Proximal	58	27	64.3	31	56.4		23	62.2	35	58.3		27	79.4	31	49.2		22	56.4	36	62.1
Distal	39	15	35.7	24	43.6		14	37.8	25	41.7		7	20.6	32	50.8		17	43.6	22	37.9
TNM stage																				
I	26*	2	4.8	24	43.6		2*	5.4	24	40.0		4	11.8	22	34.9		5	12.8	21	36.2
II	32	12	28.6	20	36.4		14	37.8	18	30.0		13	38.2	19	30.2		11	28.2	21	36.2
III	26	18	42.9	8	14.5		11	29.7	15	25.0		8	23.5	18	28.6		12	30.8	14	24.1
IV	13	10	23.8	3	5.5		10	27.0	3	5.0		9	26.5	4	6.3		11	28.2	2	3.4
Tumor differentiation																				
Well	55*	11	26.2	44	80.0		15*	40.5	40	66.7		11	32.4	44	69.8		15	38.5	40	69.0
Moderate	42	31	73.8	11	20.0		22	59.5	20	33.3		23	67.6	19	30.2		24	61.5	18	31.0
Histological type																				
Non-mucinous	59	27	64.3	32	58.2		21	56.8	38	63.3		22	64.7	37	58.7		21	53.8	38	65.5
Mucinous	38	15	35.7	23	41.8		16	43.2	22	36.7		12	35.3	26	41.3		18	46.2	20	34.5

*Compared to Unmethylation status. P<0.01.

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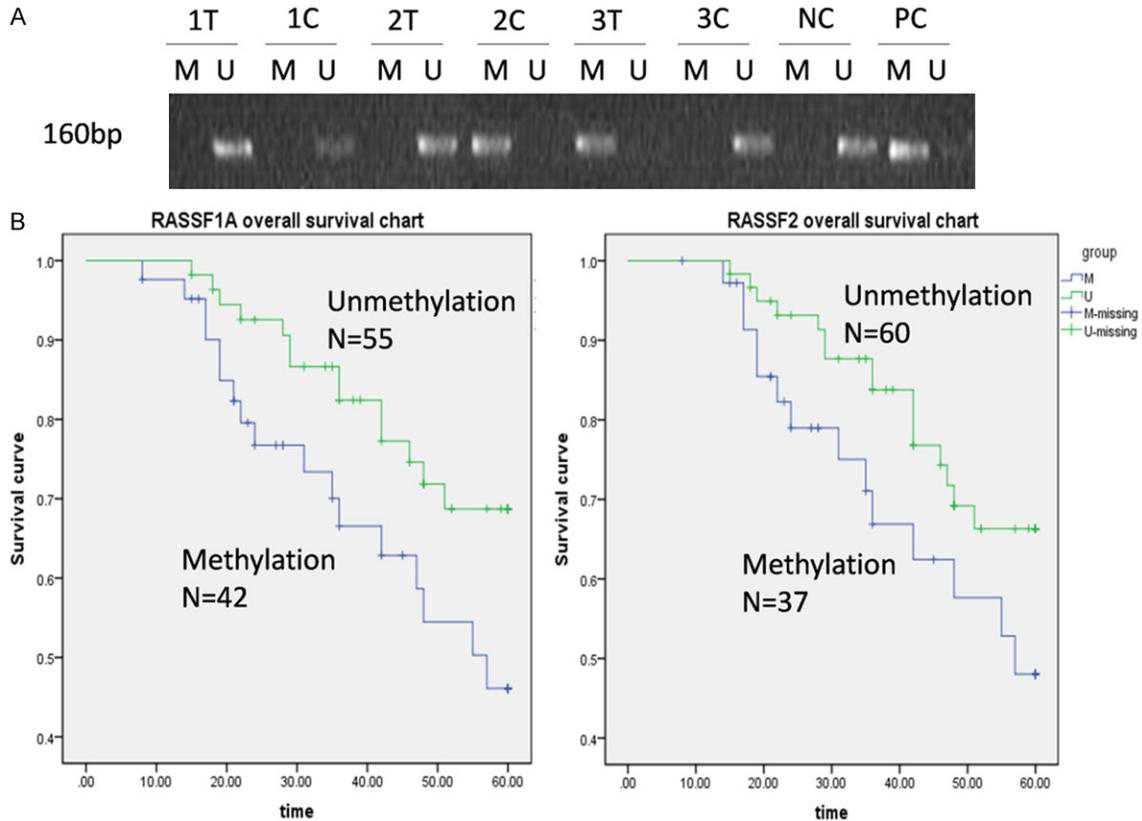


Figure 1. Overall survival analysis in colorectal patients. A. Methylation status in colorectal tissue samples and adjacent normal tissue. T: tumor tissue; C: normal control; NC: none methylation control; PC: positive methylation control. B. Kaplan-Meier curve was preformed to identify significant clinical relevance promoter methylation in all four test genes. Among the genes, only patients with methylated RASSF1A and RASSF2 gene promoter had shorter overall survival. M, Methylated; U, Unmethylated.

Transcript levels were calculated according to the comparative cycle threshold (CT) method using β -actin as an endogenous control. The final results were estimated by using $2^{-(\Delta\Delta Ct)}$, where ΔCt for each sample was determined by subtracting the Ct value for each gene from the Ct value of β -actin. Only triplicates with Ct values with a standard deviation <0.20 were acceptable. The product of MSP was kept at 4°C , and analyzed by gel electrophoresis.

Western blot

Tissues and cells were collected and lysed. After quantification via Bradford method, proteins were resolved by 10% sodium dodecyl sulfate (SDS) in polyacrylamide gel electrophoresis (PAGE) and transferred onto nylon membranes. The membranes were blocked in 5% non-fat milk and 0.1% Tween 20 in Tris-buffered saline and probed with mouse anti-RASSF1A

antibody (Abcam, Cambridge, MA, US) and an anti- β -actin antibody (Abcam, Cambridge, MA, US). Afterwards, the signals were visualized using a chemiluminescence kit (Life science, US).

Cell lines and drug treatments

The HCT116, SW1116, and SW480 CRC cell lines were obtained from cell bank at the Chinese Academy of Sciences. The cells were cultured in T75 flask with RPMI 1640 (Gibco, Life Technologies, Grand Island, NY, United States). Then the cells were supplemented with 10% fetal bovine serum (FBS; Gibco) and allowed to grow to 70% confluence in 5% CO_2 at 37°C . 5-Aza-CdR (Sigma-Aldrich, St. Louis, MO, United States) was diluted in DMSO and added to the culture media at 0.1, 1, 5 and 10 $\mu\text{mol/L}$. Similarly, the control cells were incubated with an equivalent volume of DMSO. Cells were collected 3 d after treatment.

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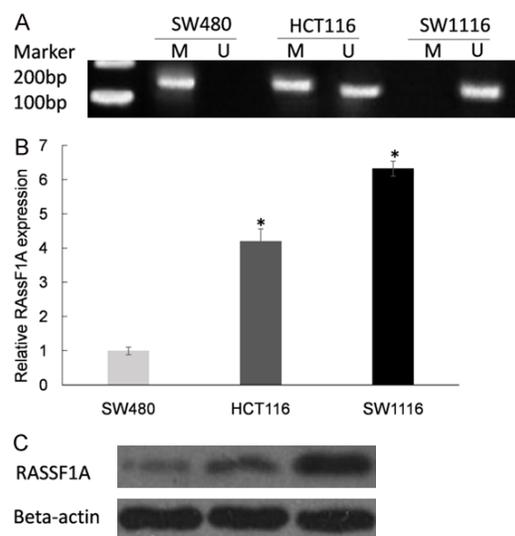


Figure 2. Association of RASSF1A methylation and RASSF1A expression in three human colorectal cancer cell lines. A. MS-PCR results for RASSF1A in all three human colorectal cancer cell lines. M indicates a methylated RASSF1A gene and U indicates an unmethylated RASSF1A gene; B. Real-time PCR result for RASSF1A gene expression in three CRC cell lines; C. RASSF1A protein level was determined by Western blot. β -actin antibody as a loading control; * $P < 0.05$ vs. control.

Cell cycle and apoptosis analysis in a FCM

Apoptosis kits were purchased from Life science company (Life science, USA). Harvested cells were washed in cold PBS and then treated with annexin-binding buffer. Then the suspension cells were fixed in 70% cold ethanol, and treated with 10 g/l RNase. Later, 5 μ l Alexa Fluor 488 annexin V and 1 μ l 100 μ g/mL PI was added to the working solution to each 100 μ l of cell suspension. After incubation for 15 min at room temperature, 400 μ l annexin-binding buffer was added and, mixed gently. Lastly, the samples were left on ice for further testing. In this experiment, cells with early apoptotic signals (stained with Annexin V) and cells with late death signals (stained with PI) were quantified and analyzed using the CellQuest software. The percentages of cells in G₀/G₁, S and G₂/M phases were determined by FACS Calibur. Each assay was performed in triplicate.

Statistical analysis

All the analyses were carried out with SPSS version 13.0 (SPSS, Chicago, IL, United States). Gene methylation in groups was demonstrated with percentages. The association of gene methylation distribution and clinical variables

were analyzed using the χ^2 and Fisher's exact tests. The relationship between methylation status and expression was compared using the independent samples t-test. The changes in expression, apoptosis and cell cycle with 5-Aza-CdR were evaluated via ANOVA analysis. All the reported P values are two sided, and P values < 0.05 were considered significant.

Results

Association of the promoter methylation status and clinical features

The aberrant promoter methylation distribution of four RASSF encoding genes were detected in 78.4% of patients (78 of 97) and none of the adjacent non-tumor samples showed methylation. The promoter methylation status of the four genes including RASSF1A, RASSF2, RASSF4, RASSF5 in 42 samples was discovered to be (43.3%), 37 (38.1%), 34 (35.1%), 39 (40.2%) respectively (Table 2). As shown in Table 2, the methylation status of RASSF1A and RASSF2 gene was significantly associated with tumor stage ($P < 0.01$). Furthermore, the methylation frequency of RASSF1A and RASSF2 gene was also associated with tumor differentiation ($P < 0.01$). No significant association was observed between gene methylation status and other clinical features. Furthermore, Kaplan-Meier survival analysis indicated that RASSF1A gene promoter methylation was correlated with poor survival in CRC patients as compared to unmethylated carriers for a follow-up period of 60 months ($P < 0.001$) (Figure 1A and 1B).

Correlation of RASSF1A methylation and expression in human CRC cell lines

Methylation status in three human CRC cell lines (SW480, HCT116 and SW1116) was evaluated with MS-PCR (Figure 2A). The most methylated cell line was SW480 compared to two other cell lines. Accordant with methylation level, RASSF1A expression levels obtained by real-time quantitative PCR and Western blotting was significantly suppressed in SW480 (Figure 2B and 2C).

Changed RASSF1A methylation and expression after 5-Aza-CdR treatment

To further explore the link between hypermethylation and transcript levels, the SW480 cell

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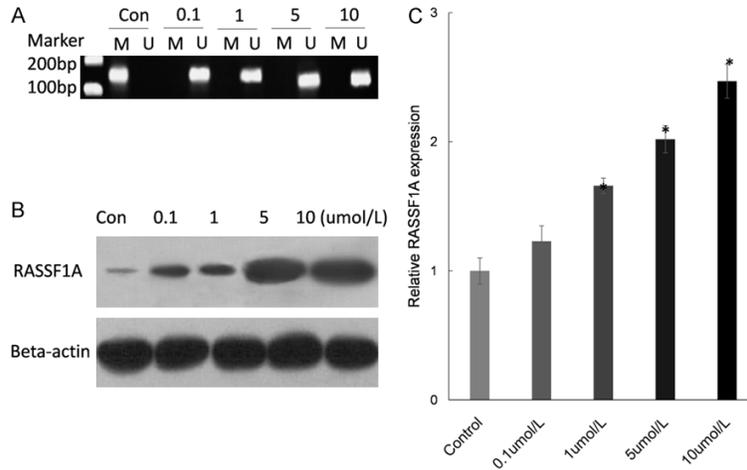


Figure 3. Changes in RASSF1A methylation and restored expression after treatment with 5-Aza-CdR in SW 480 cells. A. Cells were treated with 0 μmol/L (control), 0.1, 1, 5 or 10 μmol/L 5-Aza-CdR. A PCR band in lane M indicates a methylated RASSF1A gene; a band in lane U indicates an unmethylated RASSF1A gene. B. RASSF1A protein levels were further detected by Western blotting following a series of concentrations of 5-Aza-CdR. C. mRNA level of RASSF1A gradually increased with increasing concentrations of 5-Aza-CdR. * $P < 0.05$ vs. control.

line was treated with the demethylating agent 5-Aza-CdR. RASSF1A methylation was further investigated in SW480 cells by MS-PCR (**Figure 3A**) following different doses of 5-Aza-CdR. The results showed decreased levels of hypermethylation along with restoration of RASSF1A gene expression following 5-Aza-CdR treatment in dose-dependent manner (**Figure 3B**).

Increased apoptosis and induced G₁/S arrest after 5-Aza-CdR treatment

Apoptosis and cell cycle stages were evaluated by flow cytometry to further explore the link between cell biological function and RASSF1A gene expression after 5-Aza-CdR treatment. Apoptosis percentage (**Figure 4A** and **4B**) was significantly increased in a dose-dependent manner after 3 d of treatment with 5, or 10 μmol/L 5-Aza-CdR. Furthermore, the cells were significantly arrested at G₁/S phase after 5-Aza-CdR treatment, as compared to untreated controls (**Figure 4C** and **4D**).

Discussion

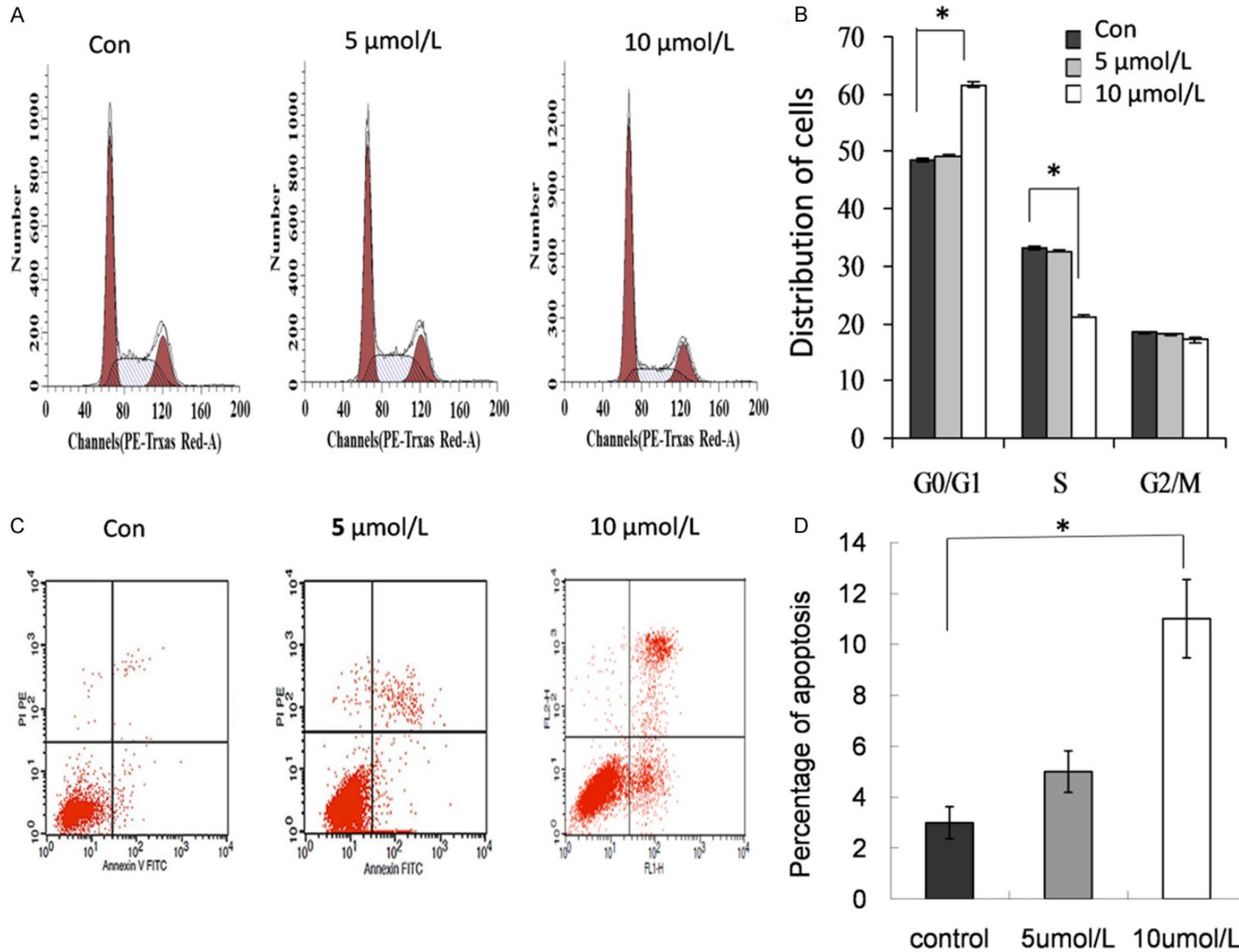
RASSF family members were found to be frequently inactivated in many cancers and such inactivation was correlated with promoter region methylation [27-29]. However, the effects of reduced expression on clinical out-

comes in CRC patients still requires further clarification. Moreover, our current study reported the methylation status in 97 CRC patients and their adjacent normal tissue in four RASSF family genes. Even though the promoter region methylation was detected in all four test genes in patients' samples, the distribution and their association with clinical outcomes were different. Furthermore, analyzed clinical and lab data illustrated a significantly negative correlation between RASSF1A and RASSF2 promoter methylation and survival time. Both RASSF1A and RASSF2 methylation status was also tightly related to tumor stage and tumor differential in current study patient.

These results indicated that both RASSF1A and RASSF2 participate in the tumor development process and could potentially be clinical outcome predictor.

RASSF1A is the major form of seven transcripts (RASSF1A-G) generated by RASSF1 gene [30, 31]. The CpG island methylation of RASSF1A was reported in previous studies [32, 33]. The lowest recorded percentage of methylation at the CpG island of the RASSF1A gene was 12% (3 out of 26) in Yoon and colleagues' study [32]. However, other studies found a higher prevalence of RASSF1A methylation in CRC samples that ranged from 36% (4/11) to 45% (13/29) [33]. But all these derived the distribution of methylation from small study groups. In contrast, our relatively large patient sample provides more confidence to report the percentage of methylation at 43.3% in 97 CRC patients.

Despite of previous efforts, the role of RASSF1A in CRC clinical development still remains elusive. Wagner et al explored the association between clinical features of CRC and RASSF1A methylation status, but they did not discover any significant correlation [33]. However, Engeland et al only detected a correlation between age and methylation status [34]. On the other hand, our study found a correlation between tumor stage and differential with



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Figure 4. Demethylation of RASSF1A induces cell cycle arrest in G₁/S-phase and increased apoptosis. A. Fluorescence-activated cell sorting analysis of cell cycle phase in cells treated with 5-Aza-CdR. B. Cells treated with 5-Aza-CdR exhibited a greater distribution of cells in G₀/G₁-phase. Values are presented as the mean ± standard deviation of three independent experiments. C. Flow cytometry analysis of cell apoptosis percentage in cells treated with 5-Aza-CdR. D. Cells treated with 5-Aza-CdR exhibited a greater distribution of apoptosis cells. *P<0.05; Scale bars, 10 μm.

RASSF1A methylation status along with a better understanding of the role of RASSF1A in CRC development.

It is well accepted that DNA promoter region methylation could result in loss and alteration of gene expression, and it subsequently affects cell biological function. Since RASSF1A is the most characterized RASSF family member, we focused our attention on the relationship between RASSF1A methylation and inactivation of gene expression in three human CRC cell lines. Our results revealed contrastingly different methylation statuses in the three cell lines, which indicated a variation of methylation in cells even with the same cancer. Furthermore, the negative association of gene expression and methylation status was then found in all three cell lines. Following treatment with demethylation agent 5-Aza-CdR, hypermethylation was reversed in cells and gene expression was subsequently increased in dose dependent manner. Therefore, all the combined data from our study confirmed the epigenetic effect on gene expression.

To further clarify the role of RASSF1A hypermethylation on cell function, cell cycle and apoptosis were investigated. Results showed colorectal cancer cells had increased G₁ phase arrest and elevated apoptosis level after demethylation agent 5-Aza-CdR treatment compared to the methylated parental cell line. These results strongly suggest that RASSF1A is an important human tumor suppressor protein that acts at the level of G₁/S-phase cell cycle progression. Previous studies also observed that RASSF1A could promote apoptosis and cell cycle arrest through interacting with multiple pro-apoptotic proteins [35, 36]. Additionally, RASSF1A has the ability to form a cell death induction complex with MST1 [37]. Also, it can interact with a pro-apoptotic scaffold protein CNK1 and can also bind to modulator of apoptosis 1 (MOAP1) respectively [38]. Similarly, RASSF1A was shown to induce cell cycle arrest at G₁/or G₂/M stage according to two studies [39, 40]. The regulation of the cell cycle by

RASSF1A was achieved through inhibition of cyclin D1 accumulation and activation of Cdc20. Other distinct set of proteins were believed to be involved in cell cycle regulation process but they were not fully confirmed [41]. Even though we discovered meaningful results, some flaws need to be improved in future studies. Firstly, all the methylation was detected by MSP method and bisulfite genomic sequencing should be performed latter to further confirm the methylation status. Secondly, the function of RASSF2 and the interaction of RASSF2 and RASSF1A were unknown and should be further investigated.

In summary, our current study provided a thorough understanding of the role of RASSF1A methylation in CRC clinical outcome and it also investigated the involved mechanisms in vitro. Consequently, our results suggested a potential function of RASSF1A as a predictable biomarker in colorectal cancer.

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Disclosure of conflict of interest

None.

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