

Adenovirus-mediated expression of spermidine/spermine N¹-acetyltransferase gene induces S-phase arrest in human colorectal cancer cells

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Abstract. Spermidine/spermine N¹-acetyltransferase (SSAT) is a key enzyme of polyamine catabolism. In a previous study, we constructed a recombinant adenovirus, Ad-SSAT, which can express human SSAT. In the present study, we investigated the effect of Ad-SSAT on the growth and cell cycle of colorectal cancer cells. We found that Ad-SSAT increased the expression of SSAT and inhibited the growth of HT-29 and LoVo cells. The growth inhibition was caused by cell cycle arrest in the S phase. Furthermore, Ad-SSAT was shown to suppress the expression of cyclin A and nuclear factor E2F-1 in HT-29 and LoVo cells. The inhibitory effect of Ad-SSAT on cyclin A promoter activity was also observed in a reporter gene assay. Our results suggest that the expression of SSAT mediated by Ad-SSAT infection inhibits the growth of colorectal cancer cells and induces cell cycle arrest at the S phase, through a mechanism involving the suppression of cyclin A and E2F-1 expression.

Introduction

Polyamines are naturally occurring aliphatic polycations found in almost all living organisms. Polyamines include spermidine, spermine and their diamine precursor, putrescine. At a physiological pH, polyamines carry a positive charge on each nitrogen atom, which facilitates their interactions with polyanionic molecules such as DNA and RNA (1). Polyamines have critical physiological functions in cell growth and differentiation. The levels of polyamines are

typically determined by the integrated contributions of biosynthesis, catabolism, uptake and export, each of which is sensitively regulated by effector molecules that, in turn, are controlled by intracellular polyamine pools (2). Ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (AdoMetDC) control polyamine biosynthesis, while spermidine/spermine N¹-acetyltransferase (SSAT) regulates polyamine catabolism and efflux out of the cell.

Polyamines are known to be critically involved in cell growth and have been implicated in the process of cell transformation (3,4). Polyamine levels are high in cancer cells and tissues, and rapid tumor growth has been associated with significant polyamine accumulation (5,6). High polyamine levels have been detected in most types of cancers (7-9). In colorectal cancer, the polyamine contents are increased 3- to 4-fold over those found in the equivalent normal tissue (10,11). Depletion of the intracellular polyamine pools invariably inhibits cell growth. Although this is usually accomplished by inhibiting polyamine biosynthesis, this may be more effectively achieved by activating polyamine catabolism at the level of SSAT, a strategy that has been validated in MCF-7 breast carcinoma cells (12).

Previously, we constructed a replication-deficient recombinant adenovirus (Ad-SSAT) to transfer the SSAT gene into cells. In this study, we examined the inhibitory effect of Ad-SSAT on the growth of colorectal cancer cells, evaluated the effects of Ad-SSAT on cell cycle distribution and investigated the underlying regulatory responses. Our findings indicate that Ad-SSAT significantly suppresses the growth of colorectal cancer cells, and that there is a close association between S-phase arrest induced by Ad-SSAT and the suppression of cyclin A. We presume that Ad-SSAT inhibits cyclin A through the down-regulation of nuclear factor E2F-1.

Materials and methods

Cells and reagents. The human colorectal cancer cell lines HT-29 and LoVo, routinely maintained in our laboratory, were cultured in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin. Luciferase reporter plasmids pGL3-basic and pRL-TK were kindly supplied by Dr Yaoqin Gong

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(Institute of Medical Genetics, School of Medicine, Shandong University). MTS [3-C(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt]/PMS (phenazine methosulfate) kit and the dual-luciferase reporter assay kit were purchased from Promega (Madison, WI, USA). A mouse anti- β -actin monoclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-human polyclonal antibodies against cyclin A, Cdk2 and the E2F-1 protein were purchased from Neomarkers.

Cell growth analysis. Cells were seeded at a density of 4000 cells/well in 96-well plates and cultured overnight. Owing to different infection efficiencies, HT-29 and LoVo cells were then infected with Ad-GFP and Ad-SSAT at 50 and 25 PFU/cell (MOI, multiplicity of infection). All experiments were performed six times. After 24, 48, 72, 96 and 120 h, each well received 20 μ l of MTS solution (with PMS) and was maintained for 2 h at 37°C. The optical density was measured by an ELISA plate reader at 490 nm. Cell viability was assessed by absorbance.

Cell cycle analysis. HT-29 and LoVo cells were seeded at a density of 3×10^5 cells/well in 6-well plates and infected with Ad-GFP and Ad-SSAT at MOI 50 and 25. After 72 h, the cells were harvested via trypsinization, washed once with cold PBS and fixed with 70% ethanol. The cells were washed with ice-cold PBS and treated with RNase. DNA was subsequently stained with propidium iodide. The cell cycle phases were analyzed using FACScan (Becton-Dickinson, Franklin Lakes, NJ, USA).

RT-PCR analysis. The HT-29 and LoVo cells were infected with Ad-GFP and Ad-SSAT at MOI 50 and 25 in RPMI-1640 medium containing 5% FBS. After 72 h, the total RNA from these cells was isolated by a TRIzol reagent (Invitrogen Co., Carlsbad, CA, USA) and 3.5 μ g of RNA of each sample was used for subsequent reverse transcription according to the manufacturer's protocol (Invitrogen). Polymerase chain reaction (PCR) analysis was performed to analyze the mRNA levels of cyclin A, Cdk2, E2F-1 and the internal housekeeping gene β -actin. The primers were: cyclin A forward, 5'-CCTCG TGGACTGGTTAGTTGAAG-3' and reverse, 5'-CTGGTGA AGGTCCATGAGACAAG-3'; Cdk2 forward, 5'-GGAGAG GTGGTGGCGCTTAAGAA-3' and reverse, 5'-TGGCTTGG TCACATCTGGAAGA-3'; E2F-1 forward, 5'-ACCATCA GTACCTGGCCGAGAGC-3' and reverse, 5'-CTACGAAGG TCCTGACACGTAC-3'; β -actin forward, 5'-CACGATG GAGGGGCCGACTCATC-3' and reverse, 5'-TAAAGACC TCTATGCCAACACAGT-3'. PCR cycling (28 cycles) was as follows: 94°C for 45 sec, 56°C for 45 sec, and 72°C for 1 min with initial inactivation of the enzyme at 94°C for 5 min and another 7 min for elongation at 72°C after the cycles were finished. PCR products were analyzed by electrophoresis on a 1% agarose gel containing ethidium bromide.

Western blot analysis. After the HT-29 and LoVo cells were infected with Ad-GFP and Ad-SSAT at MOI 50 and 25 in RPMI-1640 medium containing 5% FBS for 72 h, they were collected with a cell scraper and washed three times with ice-

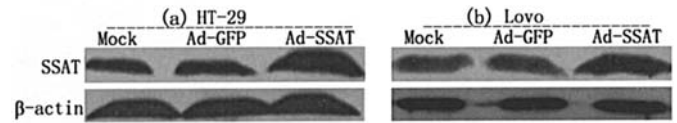


Figure 1. Western blot analysis for SSAT expression in HT-29 (a) and LoVo cells (b). Total protein from the HT-29 and LoVo cells infected with adenoviruses at MOI 50 and 25 was subjected to Western blot analysis using an anti-SSAT antibody. Equal loading was verified with anti- β -actin antibody. The results are representative of three separate experiments.

cold PBS. Total cell lysates were prepared in an extraction buffer containing 0.05 M Tris (pH 8.0), 0.15 M NaCl, 0.02% sodium azide, 0.1% SDS, 100 mg/ml phenylmethylsulfonyl fluoride (PMSF), 1 mg/ml aprotinin, and 1% NP-40. The nuclear protein was prepared as previously described (13). Sample protein concentrations were determined by bicinchoninic acid (BCA) protein assay. Proteins were electrophoresed on 5% stacking and 12% resolving SDS-PAGE gels and transferred onto nitrocellulose membranes (Millipore, Bedford, MA, USA). The membranes were blocked for 2 h at room temperature in TBS containing 5% non-fat dry milk and 0.02% Tween-20. After reaction with the appropriate primary antibody, the membranes were incubated with a secondary antibody conjugated to horseradish peroxidase. The membranes were then reacted for 5 min with luminol substrate and exposed to X-ray film (AGFA). The X-ray film was analyzed using light-density analysis software (Alpha Imager).

Construction of luciferase reporter plasmid pGL3-cyclin A. Total DNA was extracted from human blood. The DNA was used as the template to perform PCR for the amplification of the cyclin A promoter region from -516 to +245, harboring the E2F response elements (14). The primers were: forward, 5'-CGGCTGTACTACTATGTGAGCTCCGTGTTA-3' and reverse, 5'-ATTCTTGGAGCGCCGTCTGCTGCAATG CTA-3'. PCR cycling was as follows: initial activation of the enzyme at 94°C for 5 min, followed by 35 cycles of 94°C for 45 sec, 62°C for 45 sec, 72°C for 1.5 min, followed by elongation for 7 min at 72°C. The amplified products were purified by a Qiagen gel extraction kit (Qiagen, Valencia, CA, USA). The two purified PCR products and pGL3-basic null vector were digested with *Sac*I and *Sma*I. The desired fragments, 762 bp and 4.8 kb, respectively, were purified. The two purified DNA fragments were ligated with T4 ligase at 16°C overnight and then transformed into competent DH5 α cells. The positive clones (pGL3-cyclin A) and their insert directions were confirmed by digestion and sequencing.

Transient transfection and luciferase activity assay. The HT-29 and LoVo cells were seeded in 24-well plates at a density of 1×10^5 cells/well prior to transfection. Transient transfection was performed using LipofectamineTM 2000 (Invitrogen) according to the manufacturer's instructions. Briefly, the cells were transfected with pGL3-basic or pGL3-cyclin A reporter constructs or pRL-TK control vector. At 72 h after transfection, the cells were harvested for the promoter activity assay. Using the same procedure as described above, pGL3-cyclin A and pRL-TK were co-transfected into HT-29

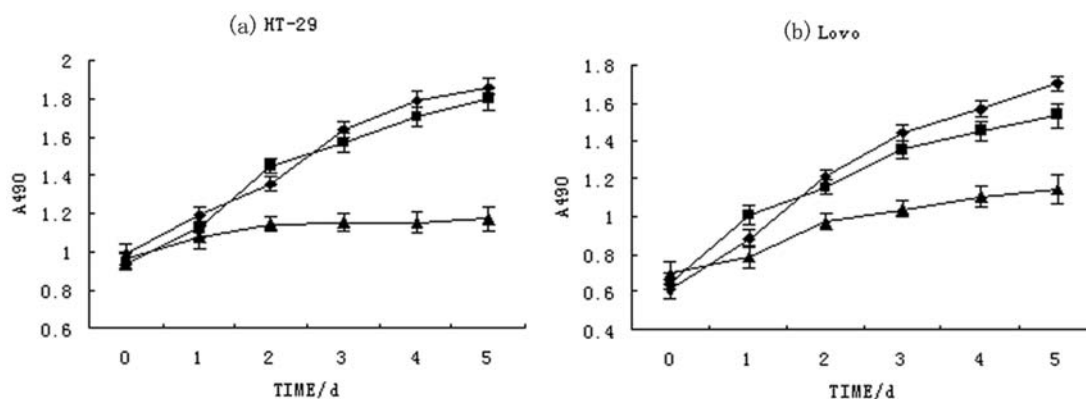


Figure 2. Effect of Ad-SSAT on the growth of HT-29 and LoVo cells. HT-29 and LoVo cells were infected with either Ad-SSAT (▲) or Ad-GFP (■) at MOI 50 and 25. After 24 h of incubation, absorbance was measured every day for 5 days. Growth curves of Ad-SSAT-infected (▲), Ad-GFP-infected (■) and PBS-treated cells (●). Each bar represents the mean ± SD of three experiments.

and LoVo cells in 24-well plates (1×10^5 cells/well). After 24 h, the cells were then infected with Ad-GFP and Ad-SSAT, respectively, at MOI 50 and 25. After 72 h, the cells were harvested for the promoter activity assay. The promoter activity was assayed using the dual-luciferase reporter system (Promega) according to the manufacturer's protocol.

Statistical analysis. Data were presented as mean ± SD from three separate experiments. Student's t-test was used to compare the data, and $P < 0.05$ was considered statistically significant. All results were analyzed by SPSS 10.0 statistical software.

Results

Effect of Ad-SSAT on the SSAT gene expression in colorectal cancer cells. Western blot analysis was performed to detect the effect of replication-deficient Ad-SSAT infection on intracellular SSAT protein levels. The SSAT levels in Ad-SSAT-infected cells were significantly higher than in cells infected with Ad-GFP or treated with PBS. The results analyzed using light-density analysis software (Alpha Imager) showed that SSAT expression in Ad-SSAT-infected HT-29 cells was 220% of the level in cells infected with Ad-GFP. The corresponding figure in LoVo cells was 175% (Fig. 1a and b). These data indicate that Ad-SSAT increased SSAT expression.

Effect of Ad-SSAT on the growth of colorectal cancer cells. We examined the growth inhibition effects of Ad-SSAT on HT-29 and LoVo cells *in vitro* using an MTS assay and observed the cell growth curves shown in Fig. 2a and b. The expression of SSAT affected the growth of colorectal cancer cells. Ad-SSAT inhibited the proliferation of the two cell types by ~50% compared to the growth of cells infected with Ad-GFP or treated with PBS.

Ad-SSAT arrests colorectal cancer cells in the S phase. To examine the mechanism by which Ad-SSAT retards the growth of colorectal cancer cells *in vitro*, the distribution of HT-29 and LoVo cells at different points of the cell cycle was analyzed using flow cytometry 48 h after infection. Results

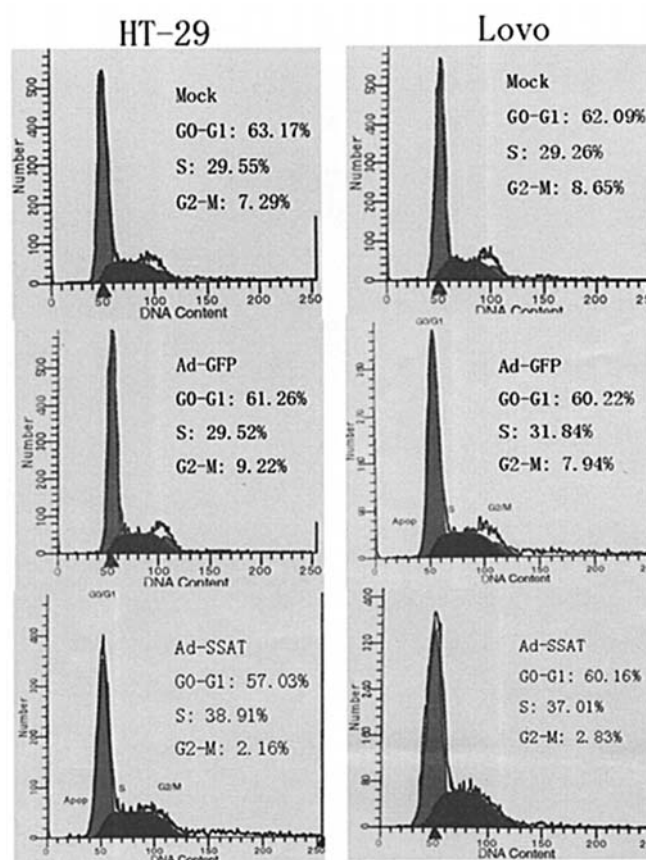


Figure 3. Effects of Ad-SSAT on the cell cycle of HT-29 and LoVo cells. Cells were infected for 3 days at MOI 50 or 25 with Ad-GFP, Ad-SSAT or PBS (mock), then collected and dyed with propidium iodide for cell cycle analysis. The data shown are of three separate experiments.

showed that Ad-SSAT caused more HT-29 and LoVo cells to halt their progression through the cell cycle compared to the controls ($P < 0.05$) (Fig. 3 and Table I). HT-29 cells were arrested in the S phase: $38 \pm 3\%$ in Ad-SSAT-infected cells compared with $30 \pm 2.1\%$ in PBS-treated cells and $29 \pm 2.5\%$ in cells infected with Ad-GFP. In the LoVo cells, Ad-SSAT elicits cell cycle arrest compared with the control vectors

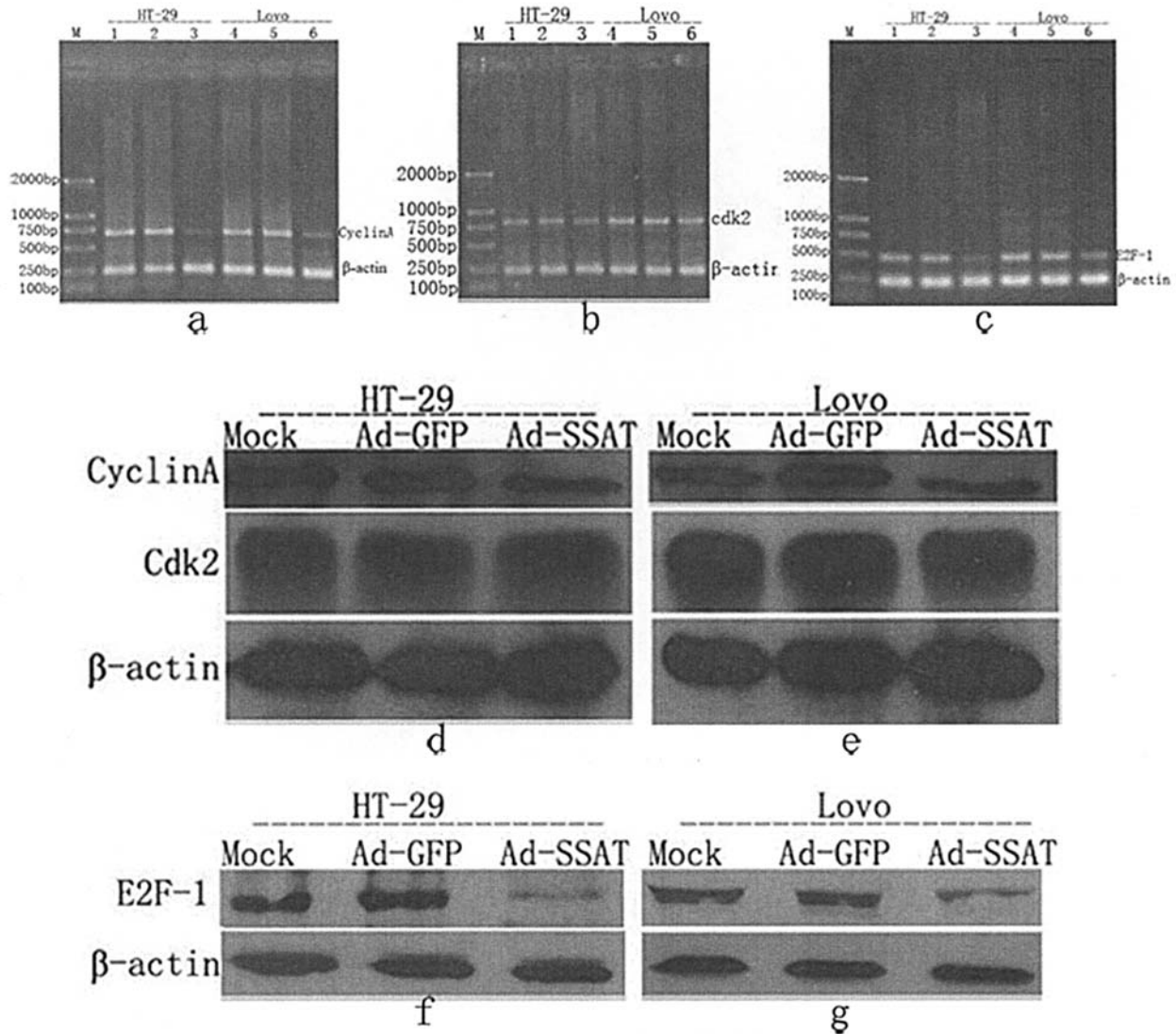


Figure 4. Expression of cyclin A, Cdk2 and E2F-1 expression in HT-29 and LoVo cells infected with Ad-SSAT. i) mRNA expression of cyclin A (a), Cdk2 (b) and E2F-1 (c) was assessed by RT-PCR. The HT-29 and LoVo cells were transfected with Ad-SSAT or the control vector. Total RNA was isolated from HT-29 and LoVo cells infected with Ad-SSAT or the control vector and used as the template for RT-PCR analysis. β -actin was used as an internal control. ii) Protein levels of cyclin A and Cdk2 (d and e) and the E2F-1 (f and g) protein were determined by Western blot analysis. HT-29 and LoVo cells were transfected with Ad-SSAT or the control vector. Whole cell extracts were subjected to Western blot analysis with anti-cyclin A, anti-Cdk2 and anti-E2F-1 antibodies. Equal loading was verified using an anti- β -actin antibody.

Table I. Distribution of HT-29 and LoVo cells in the S phase of the cell cycle.

Cell lines and treatment	Percent of total cells	
	S ($\bar{X} \pm SD$)	
HT-29 cell (control)	30 \pm 2.1	
+Ad-GFP	29 \pm 2.5	
+Ad-SSAT	38 \pm 3 ^a	
LoVo cell (control)	30 \pm 2.7	
+Ad-GFP	31 \pm 3.0	
+Ad-SSAT	37 \pm 2.7 ^a	

^aP<0.05, vs. Ad-GFP- and PBS-treated cells.

(P<0.05). Ad-SSAT treatments caused 37 \pm 2.7% of the cells to arrest in the S phase. There was no significant difference in cell cycle distribution between uninfected cells and cells infected with Ad-GFP (P>0.05).

Ad-SSAT inhibited expression of cyclin A. We further analyzed whether the expression levels of the major cell cycle regulatory proteins of the S phase were altered by an adenoviral gene transfer and whether they correlated with cell cycle arrest. The levels of cyclin A and Cdk2 mRNA in HT-29 and LoVo cells were detected by RT-PCR (Fig. 4). The results (Fig. 4a) showed that cyclin A mRNA levels decreased >80% in Ad-SSAT-infected HT-29 cells and 60% in Ad-SSAT-infected LoVo cells relative to the cells infected with Ad-GFP or treated with PBS. In contrast, there were no obvious changes in Cdk2 mRNA levels in the three groups (Fig. 4b). We also analyzed the expression of cyclin A and Cdk2 at the

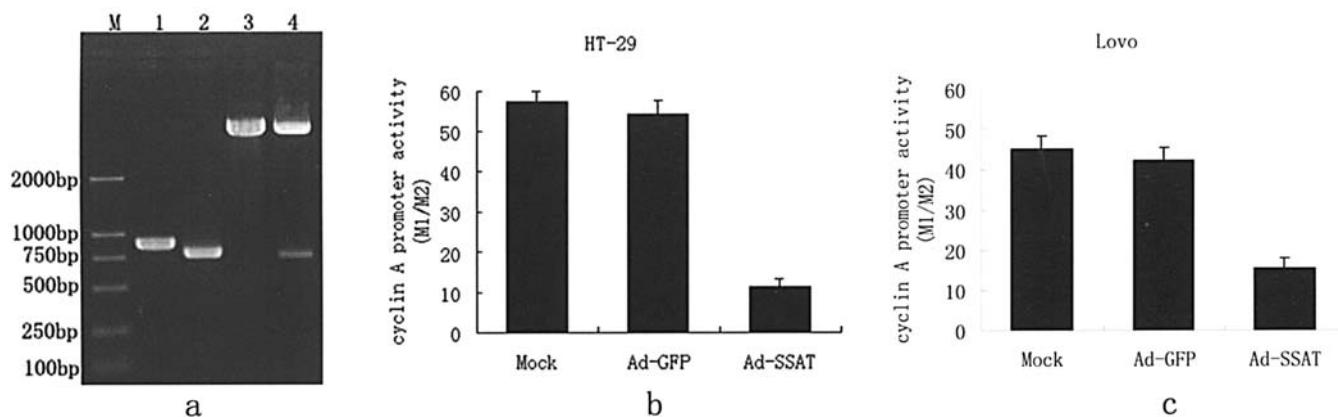


Figure 5. Construction and promoter activity of a cyclin A reporter plasmid. (a) Restriction digestion of pGL3-cyclin A-luc reporter plasmid. Lane M, marker; lane 1, cyclin A PCR product; lane 2, cyclin A PCR product digested by *SacI* and *SmaI*; lane 3, pGL3-basic digested by *SacI* and *SmaI* and lane 4, pGL3-cyclin A digested by *SacI* and *SmaI*. (b) Cyclin A promoter activity in HT-29 cells and (c) cyclin A promoter activity in LoVo cells. The HT-29 and LoVo cells were transfected with pGL3-cyclin A-luc reporter gene constructs and the pRL-TK control vector. At 24 h after transfection, cells were infected with Ad-GFP and Ad-SSAT for 72 h. Cyclin A promoter activity was measured as described in Materials and methods. Each bar represents the mean \pm SD of three experiments.

protein level by Western blot analysis. The level of cyclin A protein decreased in the Ad-SSAT-infected cells when compared with cells infected with Ad-GFP or treated with PBS (Fig. 4d and e). There were no obvious changes in the Cdk2 protein levels in the three groups.

After demonstrating that Ad-SSAT suppressed the levels of cyclin A mRNA and protein in HT-29 and LoVo cells, we evaluated the effect of Ad-SSAT on the cyclin A promoter. The transcription of the cyclin A gene is regulated by the E2F-1 pathway, which is active in colorectal cancer cells. Therefore we designed a luciferase reporter plasmid containing enough of the cyclin A promoter to include the E2F response elements but not the full-length promoter region. The cyclin A promoter region was amplified by PCR, and the PCR product was visualized by agarose gel electrophoresis. A band of ~867 bp was obtained (Fig. 5a, lane 1). The purified PCR product was digested with *SacI* and *SmaI*, which produced a 762 bp band containing the promoter region from -516 to +245 (Fig. 5a, lane 2). Digestion of the resulting pGL3-cyclin A plasmid by *SacI* and *HindIII* produced a fragment equal in size to the promoter region (Fig. 5a, lane 4), which suggests the PCR product was successfully inserted into the pGL3-basic null vector. The sequencing results further confirmed the exactness of the fragment's sequence and orientation (data not shown).

The cyclin A promoter luciferase reporter plasmid was transfected into the HT-29 cells and a luciferase activity assay was carried out. The results are shown in Fig. 5, where M1/M2 represents the relative luciferase activity. The results showed that the 762-bp fragment possessed a promoter activity (57.5 ± 2.58) which was ~800-fold stronger than that of the pGL3-basic (0.07 ± 0.001).

We examined whether pGL3-cyclin A could be regulated by Ad-SSAT. Luciferase activity assay results showed that cyclin A promoter activity decreased by ~80% in Ad-SSAT-infected HT-29 cells and ~65% in Ad-SSAT-infected LoVo cells, compared to cells infected with Ad-GFP or treated with PBS (Fig. 5b and c). These data indicate that Ad-SSAT

inhibited cyclin A expression by suppressing the promoter activity.

Ad-SSAT inhibited the expression of nuclear factor E2F-1. After confirming that Ad-SSAT inhibited the activity of a minimal cyclin A promoter containing E2F response elements, we analyzed the effect of Ad-SSAT on the expression of E2F-1, which is the important upstream regulator of cyclin A. The mRNA and protein levels of E2F-1 in Ad-SSAT-infected cells decreased relative to those in cells infected with Ad-GFP or treated with PBS. These data indicate that Ad-SSAT inhibited E2F-1 expression.

Discussion

Polyamines are essential for normal cell growth, and aberrant polyamine metabolism is known to play an important role in the development of tumors (15). Colorectal cancer cells show many biochemical alterations compared to normal colorectal cells, but one of the most consistent changes is the increase in intracellular polyamine content (16). Depletion of the intracellular polyamine pools invariably inhibits cell growth. Although this is usually accomplished by inhibiting polyamine biosynthesis, this may be more effectively achieved by activating polyamine catabolism at the level of SSAT, a strategy that has been validated in MCF-7 breast carcinoma cells (12).

SSAT is the principal catabolic enzyme responsible for the regulation of intracellular polyamine contents in mammalian cells. SSAT transfers an acetyl group from acetyl-CoA to the N¹ positions of spermidine and spermine. The corresponding N¹-acetyl derivatives are either excreted outwards, or undergo further metabolism by polyamine oxidase, ultimately yielding putrescine, which is the opposite pathway to polyamine synthesis. SSAT is thought to play a key role in maintaining a properly balanced ratio of polyamine biosynthesis and breakdown in cells and in preventing the overaccumulation of polyamines that may become cytotoxic (16-18). Whereas

the SSAT activity is normally very low in cells and tissues, the enzyme is strongly induced by increases in intracellular polyamine concentration and in response to terminally alkylated polyamine analogues that are currently undergoing clinical trials as cancer chemotherapeutic agents (6,19).

In this study, Western blot analysis demonstrated that Ad-SSAT significantly increased SSAT protein levels in HT-29 and LoVo cells. Furthermore, in parallel with the substantial increase in SSAT expression, Ad-SSAT significantly inhibited colorectal cancer cell growth. Polyamines are known to play a key role in maintaining a high-cell proliferation rate. An increased SSAT expression accelerated the polyamine catabolism, and polyamine levels decreased accordingly. A reduction in polyamines may therefore contribute to the suppression of cancer growth.

To further understand the underlying mechanism of growth inhibition of the tumor cells, the expression levels of cell cycle and cell cycle-related proteins were examined. Flow cytometry showed that Ad-SSAT affected the S-phase progression of colorectal cancer and induced S-phase arrest. We measured the levels of cyclin A and Cdk2 in HT-29 and LoVo cells to study the mechanism whereby Ad-SSAT arrested the cells in S-phase. Cyclin A is required for cell cycle S-phase entry. Cyclin A starts to accumulate during S-phase and is abruptly destroyed before the metaphase. Cyclin A-associated CDKs play a critical role in initiating DNA replication and the subsequent S-phase entry, which they trigger by phosphorylating components of the DNA replication machinery (20). The synthesis of cyclin A is controlled mainly at the transcriptional level by E2F and other transcriptional factors. The prevailing dogma for how E2F transcriptionally activates target genes such as cyclin A is that pre-existing E2Fs are released from inactive complexes of E2F and hypophosphorylated retinoblastoma (RB). Activated CDKs can also hyperphosphorylate and inactivate the RB tumor suppressor protein. It is believed that RB hyperphosphorylation causes the release of E2F transcriptional factors from pre-existing RB-E2F complexes (21). These functional E2F transcriptional factors then bind to specific promoter regions to induce target genes whose products are necessary for S-phase entry. Among the six members of the E2F family of transcriptional factors, E2F-1 possesses oncogenic properties *in vitro* (22) and *in vivo* (23). E2F-1 is an important upstream regulator of cyclin A, and the E2F-1 pathway is active in colorectal cancer cells. The investigation of Yue *et al* has shown that E2F-1 plays an important role in colorectal carcinogenesis and in the up-regulation of E2F-1 expression. They have also shown that this up-regulation correlates with progression through Dukes' stages of colorectal cancer, indicating that an elevated E2F-1 expression is associated with the invasive and aggressive behavior of colorectal cancer (24). Using a luciferase reporter plasmid containing a minimal cyclin A promoter with E2F response elements, we showed that Ad-SSAT reduced the cyclin A promoter activity. Therefore, we presume that Ad-SSAT down-regulates cyclin A transcription by inhibition which prevents the E2F-1 activation of the cyclin A promoter. Consistent with this hypothesis, we found that the Ad-SSAT treatment reduced the levels of E2F-1 mRNA and protein in HT-29 and LoVo cells. The inhibition of E2F-1 may explain the transcriptional inhibition of cyclin A.

In summary, our data provide evidence that the expression of SSAT mediated by Ad-SSAT leads to a significant growth suppression of colorectal cancer cells. The inhibitory effects of Ad-SSAT on HT-29 and Lovo cells are mediated through the down-regulation of the signaling pathway involving E2F and cyclin A. The inhibition of this pathway causes the cell cycle to arrest in S phase, which finally results in the suppression of proliferation of colorectal cancer cells. These results suggest that the expression of SSAT mediated by Ad-SSAT may be a potential therapeutic approach for colorectal cancer types. However, further studies are needed to examine this potential therapy *in vivo*.

Acknowledgements

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