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Original Article

ART1 induces aberrant methylation of uPA promoter: a preliminary study

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Abstract: Colorectal carcinoma remains one of the most prevalent cancers with high morbidity and mortality. Arginine ADP-ribosyltransferase 1 (ART1) is one of the major mono-ADP-ribose transferases and has been shown to be involved importantly in many biological processes. DNA methylation is an important epigenetic mechanism in tumorigenesis. However, the influence of ART1 on DNA methylation contributing to this function in colorectal carcinoma cells remains unclear. The expression and activity of DNA methyltransferase 1 (DNMT1) was detected by western blotting. The binding between ART1 and DNMT1 was assessed by co-immunoprecipitation. The methylation status of uPA gene was determined by bisulfite sequencing PCR. DNMT1 expression and activity were increased as ART1 was silenced, and decreased as ART1 was over-expressed in CT26 colorectal carcinoma cells. The expression of DNMT1 decreased and uPA increased, respectively, following the treatment with 5-aza-2'-deoxycytidine in GFP-shART1 group. Invasion and metastasis were enhanced in GFP-shART1 group treated with 5-aza-dC. Similar regulation of expression of DNMT1 and uPA were confirmed in Balb/c mice. This study revealed that silencing of ART1 induced hypermethylation of uPA gene and over-expression causes hypomethylation. It probably relates to the feedback mechanism of NF- κ B to PARP1 thus mediating the expression and activity of DNMT1. The relationship between ART1 and DNA methylation might offer a new therapeutic target for the improved treatment of this major cancer.

Keywords: ART1, DNMT1, methylation, metastasis, invasion

Introduction

Colorectal carcinoma (CRC) is one of the most common tumors in a genetic view, yet it remains a major cause of cancer-caused death [1], indicating that some CRC cells are resistant to current therapies. CRC develops as a consequence of the accumulation of genetic alterations that activate proto-oncogenes. Epigenetic modifications, especially DNA methylation in certain gene promoters, are recognized as common molecular alterations in human tumors [2, 3].

DNA methylation is an important epigenetic event, in which DNA is modified by the enzymatic conversion of certain cytosine residues to 5-methylcytosine [4]. This methylation can cause changes in the structure of chromatin, and conformation and stability of DNA leading to

changes in the interactions between DNA and proteins, thereby controlling the expression of genes. Furthermore, Davis et al reported that it plays a significant role in maintaining cellular functions, and changes in the methylation status may contribute to the development of cancer [5]. The methylation reaction is catalyzed by DNA methyltransferases, helping to maintain the patterns of expressions of genes [6]. There are three types of methyltransferase enzymes, DNMT1, DNMT3a and DNMT3b, together they maintain the DNA methylation status in mammals [6]. Among them, DNMT1 regulates maintenance methylation and DNMT3 isoforms are thought to be the "de novo" methyltransferases. Recent studies suggest that DNMT1 seems to be more important than other methyltransferases. Huan X and Lan J et al reported that DNMT1 contains

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a domain showing homology to the polybromo-1 protein, which is thought to mediate protein-protein interactions [6, 7]. Many of these interactions are involved in transcriptional repression [6, 8]. It has been known that alterations in DNA methylation occur in cancer, including hypomethylation of oncogenes and hypermethylation of tumor suppressor genes [9]. Hypermethylation and hypomethylation are two contrasting types of aberrant methylation status. Previous studies have shown that hypomethylation activates certain genes that favor growth of tumors, increasing the metastatic and invasive potential of cancer cells [10, 11].

Over the past decades, research has suggested that there may be a relationship between poly (ADP-ribose) transferase-1 (PARP1) and DNMT1 [12, 13]. It has been shown that PARP-1 can bind to the promoter of the DNMT1 gene, resulting in the loss of expression of DNMT1 which leads to hypomethylation of genomic DNA [12]. Furthermore, Reale et al have reported that PARP1 can inhibit the activity of DNA methyltransferases by binding to DNMT1 to form the PARP1-DNMT1 complex [14]. PARP-1 and the DNA methylation pathway have been associated with the repression of oncogene expressions [12].

Mono ADP-ribosylation is a covalent enzymatic transfer of one ADP-ribose from NAD⁺ to specific amino-acid residues of acceptor proteins; the modification is catalyzed by cellular ADP-ribosyltransferases and certain bacterial toxins [15]. In vertebrates, what are the key enzymes are the mono-ADP-ribosyltransferases, also known as ARTs [16]. Only ART1, ART3, ART4 and ART5 have been verified in the human genome. Among the four human ARTs, ART3 and ART4 lack the signature R-S-EXE sequence, and it is still unknown whether they are catalytically active or not. Relatively, ART1 and ART5 do contain the R-S-EXE motif. ART5 has only been reported in humans but ART1 has been reported in humans and mice [15, 17]. Therefore, ART1 is the most relevant ART for study, owing to its localization and conformation.

PARP1 has been studied extensively in the context of cancer compared to ART1, and is the target of the newly approved anti-cancer drug, although they are both included in the classification of ADP-ribosylation. Our previous studies indicate that overexpression of PARP1 and

ART1 may be associated with invasion and metastasis in colon cancer [18, 19]. Our team has also demonstrated that expression of PARP1 is regulated by ART1 in colon cancer. The proposed mechanism behind this effect may involve mono-ADP-ribosylation at arginine mediated by ART1. It is reported that inhibition of ART1 decreases the expression and activity of RhoA, which regulates NF- κ B, and consequently decrease the expression of PARP-1 [20, 21]. Based on the findings that ART1 can act as one of the regulators of PARP1, we hypothesize that ART1 may be related to the expression and activity of DNMT1.

Urokinase plasminogen activator (uPA) is a member of the serine protease family that promotes conversion of inactive zymogen plasminogen to its active form, plasmin, which is regulated by growth factors, cytokines, and steroids [22]. Also, Pakneshan et al reported that uPA has some cancer-related functions and is involved in promoting invasion and metastasis of tumors [2, 23]. A previous study revealed that uPA was partially methylated in Ras-transfected cells and expression of uPA was induced upon treatment of Ras-transfectants with 5-aza-dc, an agent which diminished methylation of DNA [22]. Although it is a key mediator of the invasion and metastasis [24], it is still unclear what regulates the expression of uPA gene expression at different stages of tumor progression.

In this study, to determine the effect of ART1 on DNMT1 and on the methylation of uPA promoter regions in DNA, which may be an epigenetic mechanism that affects invasion and metastasis of colorectal carcinoma, we silenced ART1 in CT26 cells via transfection of a lentivirus-based ART1-shRNA. Further, to verify the effect of ART1 on DNMT1, we used 5-aza-2'-deoxycytidine, an epigenetic modifier, which inhibits expression and activity of DNA methyltransferases causing DNA hypomethylation and over-expression of certain genes [25].

Materials and methods

Reagents

The CT26 cell line (a murine colon adenocarcinoma cell line) was kindly provided by Professor Y-Q Wei, Sichuan University, China. CT26 cells transfected with ART1-shRNA (ART1-

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shRNA), ART1-RNA and non-target shRNA (control-shRNA) have been previously established [26]. The experimental groups either over-expressed ART1 in CT26 cells (GFP-ART1) or silenced expression of ART1 in CT26 cells (GFP-shART1). Non-transfected CT26 cells and CT26 cells transfected with an empty lentivirus (GFP-vector) were used as control groups. DNMT-1 antibody was purchased from Abcam, Ltd. (Hong Kong). Rabbit anti-mouse uPA and rabbit anti-mouse β -actin antibody were purchased from Boster Bioengineering Co. Ltd. (Wuhan, China), and polyclonal rabbit anti-mouse ART1 antibody was purchased from Santa Cruz Biotechnology, Inc. (CA, USA). DL1,000 DNA Marker was obtained from TaKaRa Biotechnology, Co. Ltd., Dakian, (China). 5-Aza-2'-deoxycytidine, from Sigma-Aldrich Co. (MO, USA), was dissolved into the concentration of 1 M in DMSO. DNeasy Blood&Tissue Kit was purchased from QIAGEN Group. EZ DNA Methylation-Gold Kit was purchased from Zymo Research Corp. BALB/c mouse were obtained from the Experimental Animal Center of the National Bio-industry Base in Chongqing, China.

Cell culture

CT26 cells (the GFP-shART1, GFP-ART1 and non-transfected cell lines) were cultured in RPMI-1640 medium (Hyclone, Logan, UT, USA) which was supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μ g/mL streptomycin (Hyclone) at 37°C in a 5% CO₂ incubator.

To confirm the effect of ART1 on DNMT1, changing the expression of uPA, CT26 cells were treated with 5Aza-dc. CT26 cells of the GFP-shART1 group were cultured to 24 h before treatment. Cells were then treated with 5Aza-dc (1 μ M; Sigma) continuously for 72 h. Meanwhile, the medium was changed and the above treatment was repeated every 24 h. The dose of 5Aza-dc (1 μ M) was chosen based on preliminary studies showing optimal reactivation of gene expression [25, 27].

Protein extract preparation

5 \times 10⁶ Cells which were washed twice with PBS were scraped to be collected into EP tubes and mouse spleen transplantation tumor tissues (100 mg) were extracted for total protein

extract. The total protein extracts were prepared with whole-cell protein lysis buffer (Beyotime, Shanghai, China). The suspensions centrifuged for 5 min at 5,000 rpm at 4°C in centrifuge tubes (Thermo Fisher Scientific, Waltham, UK). The supernatant solution was discarded and the pellets were lysed with lysis buffer for 30 min on ice. The total protein extract was obtained after centrifugation for 5 min at 12,000 rpm and 4°C. Then the protein concentration was then assessed with a BCA protein assay kit (Beyotime, Shanghai, China).

Western blot analysis

Protein and pre-stained molecular weight markers were separated in a range of appropriate voltage (80 V-100 V) by electrophoresis on 10% SDS-polyacrylamide gels and were transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). 5% Non-fat milk powder dissolved in TBST was incubated with the membranes for 2 h to be used to block heterogenous antigen. After incubation, the membranes were washed in TBST thrice and incubated respectively overnight at 4°C with primary antibodies against DNMT1 (diluted 1:300), uPA and β -actin (diluted 1:500). After the incubations with the primary antibodies, the membranes were washed three times with TBST and then secondary antibody (peroxidase-conjugated goat anti-rabbit or rabbit anti-mouse IgG) at a dilution of 1:1000 were added and the mixtures were incubated for 2 h at room temperature. The blots were visualized with BeyoECL Plus (Beyotime) for exposure after the step of being washed three times with TBST. They were exposed and imaged in a luminoscope (BioRad). The intensity of each band was analyzed with Quantity One software (BioRad).

Cell migration assay

A scratch wound assay was conducted to evaluate cell migration of cells. 5 \times 10⁵ CT26 cells (GFP-shART1) were installed in six-well plates and cultured overnight to yield a confluent monolayer. Then a channel was scratched in the middle of each plate by 10 μ L pipette tip and the displaced were washed away twice with PBS. The remaining cells were cultured with 0.5% FBS in RPMI1640 supplemented with 1 μ M 5-Aza-dC. An equal volume to 5-Aza-dC of PBS and RPMI1640 containing 0.5% FBS was used as a control medium. The cells were

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examined and images were captured at 0 h and 24 h using and NIS-Elements Image Analysis System (Nikon Corp., Tokyo, Japan) to measure the change of the width of the channel. The distances were marked at fixed intervals. Three independent measurements of the width of the wound were taken at random sites in each group and were used as statistical data. The mean values were calculated according to the following formula which determined the healing rate of the scratches. Healing rate: (initial scratch width value-corresponding points scratch width)/initial value $\times 100\%$ scratch width.

Cell invasion assay (transwell)

A Transwell Matrigel invasion assay was used to evaluate invasion by cells. The upper chamber above the polycarbonate membrane was paved with Matrigel (Becton, Dickinson and Company) and CT26 cells of GFP-shART1 (10^5 /well) were suspended in RPMI-1640 were seeded. These cells were incubated with CT26-conditioned supernatant (5-Aza-dC 1 μ M in serum-free RPMI-1640. An equal volume to 5-Aza-dC of PBS and RPMI-1640 without serum served as a control). The lower chamber was filled with 500 μ L RPMI-1640 supplemented with 10% FBS. Cells were incubated for 24 h and fixed using 4% paraformaldehyde. Then the number of cells in each well that migrated to the lower chamber was counted after staining with crystal violet. The rate of invasive inhibition was measured as follows: invasion inhibition rate = (average treated group - average untreated control group)/average value for the dosing control group $\times 100\%$.

Bisulfite sequencing PCR

Genomic DNA was obtained from CT26 cell lines using the DNeasy Blood&Tissue Kit (QIAGEN Group) in accordance with the manufacturer's instructions. Sodium bisulfite modification of genomic DNA was performed with the EZ DNA Methylation-Gold Kit (Zymo Research, Corp). In order to evaluate the methylation status of the CpG islands of the putative uPA promoter region, one set of bisulfite sequencing primers was designed. The primers B-uPA-F (5'-GGGATAGGTTGGAGAAGAATTG-3') and B-uPA-F (5'-TTCCTTTTCCTACATCCACC-3') were applied to amplify the CpG islands in the promoter region with the preparatory DNA product 254 bp in length. Following this, BSP ampli-

fications were conducted in 50 μ L reaction mixtures containing 3 μ L bisulfite-modified genomic DNA, 1 μ L dNTPs, 1 μ L primers F, 1 μ L primers R, 5 μ L Taq Buffer (100 mM Tris-HCl (pH 8.8 at 25°C), 500 mM KCl, 15 mM MgCl₂, 0.8% (v/v) Nonidet P40), 0.8 μ L Taq polymerase and 38.2 μ L H₂O, in an American ABI PCR system verity 96-well plate. The PCR cycling conditions applied to amplify the promoter region were as follows: 98°C denaturing for 4 min; 94°C for 45 sec, annealing temperature 66°C for 45 sec, 72°C for 1 min, each temperature cycle repeated twice; 94°C denaturing for 45 sec, 56°C annealing for 45 sec, 72°C for 1 min, repeated 20 times, 72°C 8 min. Then PCR products were analyzed by 3.0% electrophoresis on a 3.0% agarose gel and staining with ethidium bromide followed by visualization with ultraviolet illumination. Purified PCR products were cloned into pUC18-T (Sangon Biotech Company, Shanghai, China) as described by the manufacturer in 10 μ L reaction mixtures containing 1 mL 10 \times Ligation Buffer, 1 mL 50% PEG, 50 ng pUCm-T Vector, 0.2 pmol PCR product and 2.5 U T4 DNA Ligase. Afterwards, five individual clones were sequenced in the use of the M13 primer.

Co-Immunoprecipitation

Purchased cells were lysed on ice in RIPA buffer (Beyotime) for 15 min. After the lysates had been centrifuged at 14000 rpm at 4°C for 15 min, the supernatant solutions which contained proteins were collected and then incubated with primary antibodies of ART1 (1:200 dilution) for 2 h at room temperature. To capture the antibody-analyte complex, protein A+G agarose (Beyotime) of 100 μ L were incubated with the supernatants mentioned above overnight at 4°C. Agarose-antibody-analyte complexes were gathered by being centrifuged at the speed of 14000 rpm for 5 sec and were washed with PBS for three times and then regained with 1 \times SDS-PAGE buffer in boiling water bath for 5 min. Western blotting was used for the analysis of immunoprecipitations with the antibodies against ART1 and DNMT1. All experiments were performed in triplicate.

Model of transplantation tumor

6-to-8-week-old female BALB/c mice (18-22 g) were purchased from the Animal Center of Chongqing Medical University. All experimental procedures were conducted in conformity with institutional guidelines for the care and use of

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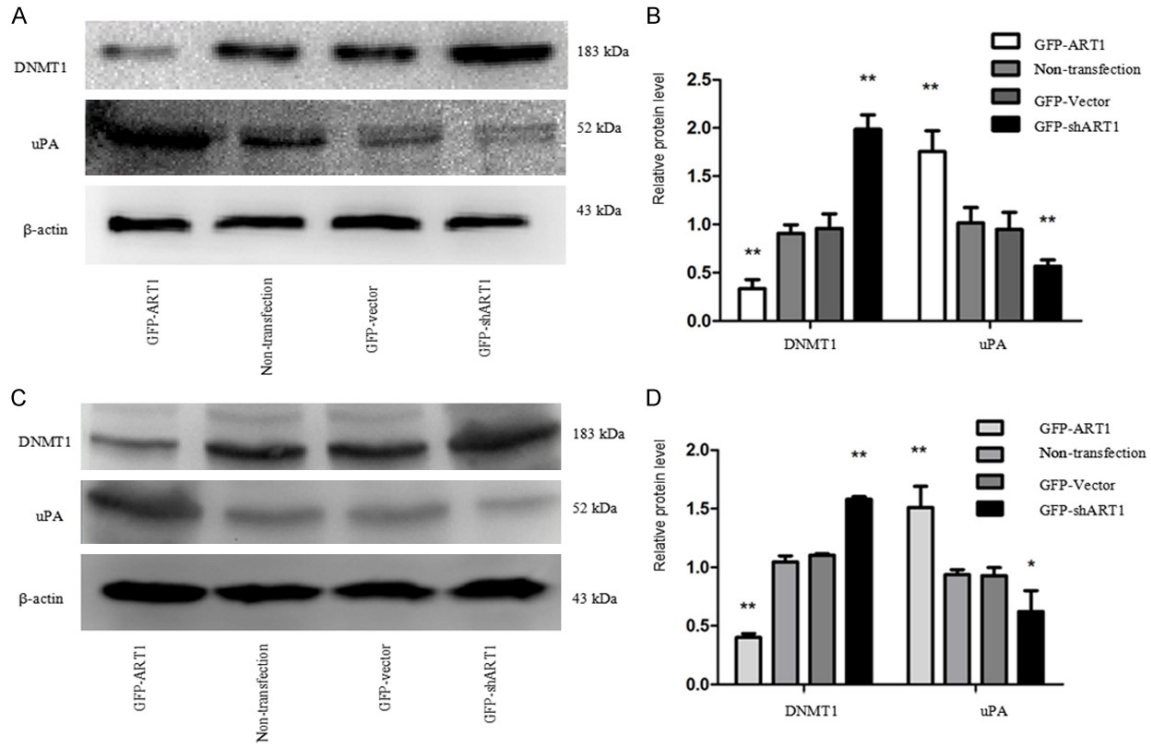


Figure 1. ART1 silencing induces the expression of DNMT1 and reduces the uPA expression in vitro and in vivo. A and C. Protein levels of non-transfection, GFP-shART1, GFP-ART1 and GFP-vector group in vitro and in vivo were determined by western blotting and images are shown representatively. B and D. Quantification of protein levels was performed by densitometry. Results represent the mean \pm SD of the expression levels from three independent experiments standardized to β -actin expression and normalized to 100% in untransfected cells. The expression of DNMT1 is statistically increased in GFP-shART1 group and decreased in GFP-ART1 group compared to other groups ($P < 0.01$), while the expression of uPA is statistically decreased in GFP-shART1 group ($P < 0.01$ in vitro and $P < 0.05$ in vivo) and increased in GFP-ART1 group ($P < 0.01$ in vitro and in vivo) relative to the expression in untransfected and GFP-vector groups.

laboratory animals in the Institutional Animal Care and Use Committee of Chongqing Medical University and conformed to the Chinese Science Institutes of Health Guide (Animal Welfare Act). To verify whether these molecular mechanisms in vivo is in accordance with those in vitro, four groups (GFP-shART1, GFP-ART1, GFP-vector and non-transfected groups) of CT26 cells (2×10^6) were administered to BALB/c mice respectively. The growth of tumors was observed daily. The mice were then executed to allow collection of the subcutaneous transplanted CT26 carcinoma at the 14th day. The isolated protein of these tumor tissues was extracted for Western blotting analysis.

To detect the effect of ART1 silencing on DNA methylation and the metastasis of the colon carcinoma in vivo, 2% chloral hydrate (0.3 g/Kg) was injected i.p. to the mice, and then GFP-shART1 CT26 cells (5×10^5) were injected into the spleen capsule of BALB/c mice. When the

size of the tumor grew to 10 d after injection, the mice of the experimental group were injected with 5-aza-dc (1.0 μ g/g dissolved in 100 μ L PBS) every week up to 3 times [28]; and the mice of the control group were injected with 100 μ L PBS. The weight of the transplanted tumor was measured and the liver metastasis nodules were calculated when mice were sacrificed at the 28th day. The sizes of the tumors were measured and calculated volumes ($V = ab^2/2$; a, the longest diameter; b, the shortest diameter). The liver metastases were assessed according to the number of metastatic nodules on the liver and the total liver weight [29]. Excised transplanted tumors (about 100 mg) of the spleen were used to extract total protein for Western blot investigations.

Statistical analysis

The results from western-blotting and RT-PCR were presented as the means \pm standard devi-

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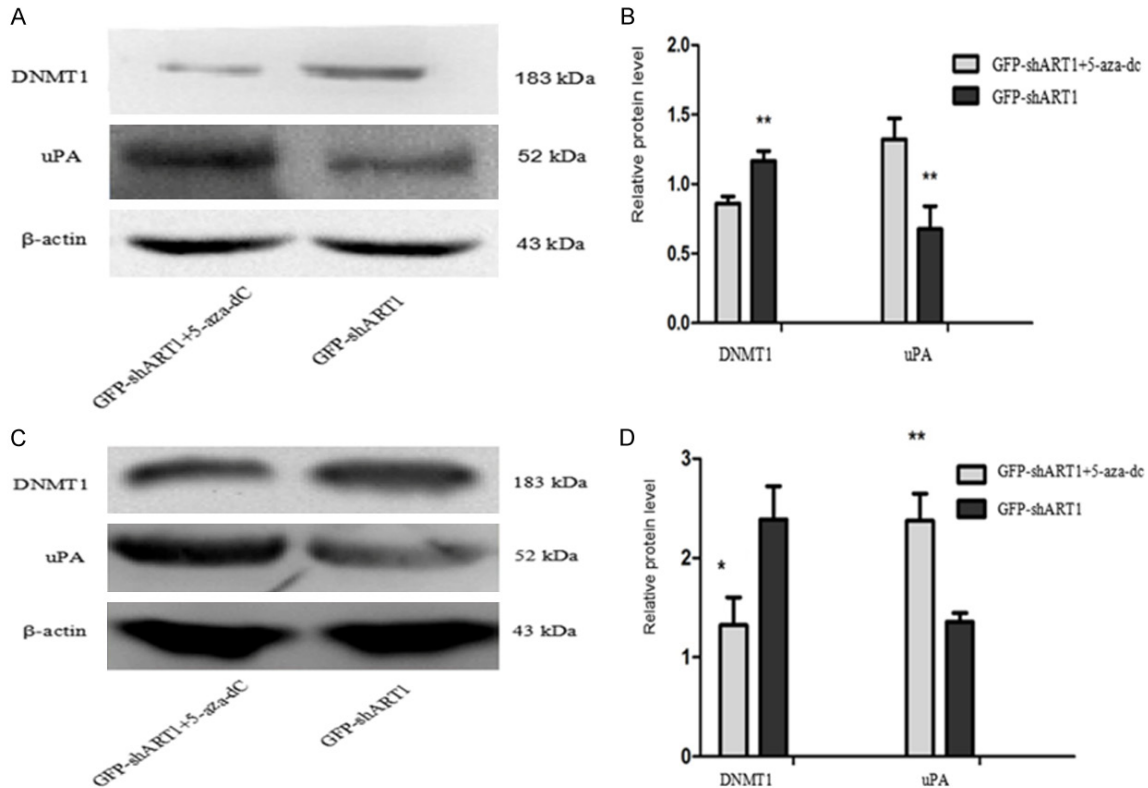


Figure 2. The effect of DNA methylation inhibition on the expression of uPA and DNMT1 in vitro and in vivo. A. CT26 cells were treated with 1 μ M of the DNA methylation inhibitor 5-aza-dC in GFP-shART1 group every 24 h for 3 days. Protein levels were detected by western blotting. Images are shown representatively. C. After GFP-shART1 CT26 cells (5×10^5) were injected into the Balb/c mice, 1 μ g/g 5-aza-dC were given to them every week up to 3 times. And the commensurable protein of subcutaneous transplanted tumor was extracted for the western blotting analysis at the 28th day. Protein levels of GFP-shART1 with 5-aza-dC and GFP-shART1 group were detected by western blotting and images are shown respectively. B and D. Quantification of protein levels was performed by densitometry. Results represent the mean \pm SD of the expression levels from three independent experiments standardized to β -actin expression. The expression of DNMT1 is statistically decreased in GFP-shART1 with 5-aza-dC group compared to the other group ($P < 0.01$ in vitro and $P < 0.05$ in vivo), while the expression of uPA is statistically increased ($P < 0.01$ relative to the expression in GFP-shART1 group).

ation (SD). Differences between different groups were analyzed with one-way ANOVA and the Wilcoxon test and correlation analysis was taken into use with Spearman's correlation analysis in SPSS 18.0 software (SPSS, Chicago, IL, USA). A probability value of $P < 0.05$ was considered to illustrate a statistically significant difference.

Results

Effects of ART1 on expression of DNMT1 and uPA in vitro and in vivo

In order to explore whether ART1 affects the expression of DNMT1 and uPA in colon carcinoma, Western blotting was used to examine

the expression of related proteins on the level of protein in vitro and in vivo. The expression of DNMT1 was increased in GFP-shART1 ($P < 0.01$) and decreased in GFP-ART1 group compared to the control and untransfected groups in vitro and in vivo ($P < 0.01$). Conversely, decreased expression of uPA in GFP-shART1 group ($P < 0.05$) and increased expression in GFP-ART1 group ($P < 0.01$) were shown compared to other groups (**Figure 1**).

Inhibition of DNA methylation regulates the expression of DNMT1 and uPA in vitro and in vivo

In order to verify the effect of ART1 on methylation of the uPA promoter and expression of uPA, the GFP-shART1 group was treated with 5-aza-

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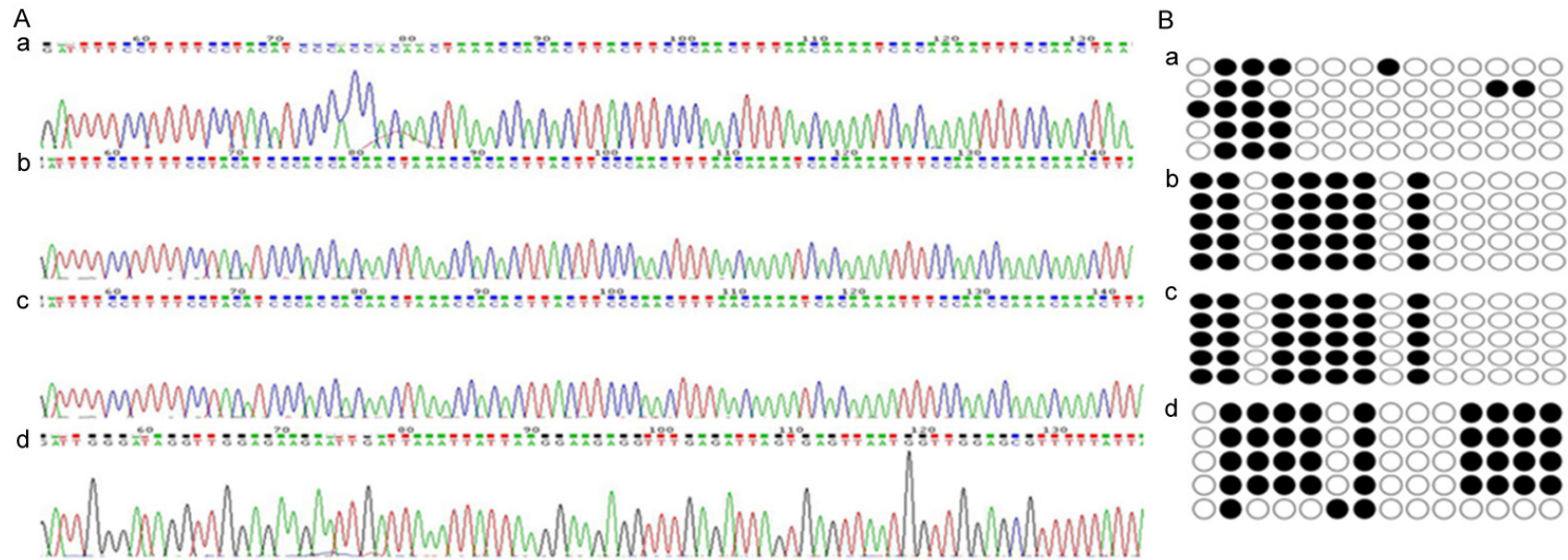


Figure 3. Effects of ART1 on the DNA methylation status of uPA gene promoter region in vitro. (A) Representative bisulfite sequencing results of uPA gene in CT26 cells were detected by BSP sequencing results that mean methylation status of GFP-ART1, GFP-vector, non-transfection and GFP-shART1 group are shown respectively as (a-d). (B) Methylation is indicated in red. Open and filled circles stand for unmethylated and methylated CpG sites, and each row stands for a single clone (a-d represent GFP-ART1, GFP-vector, non-transfection and GFP-shART1 group).

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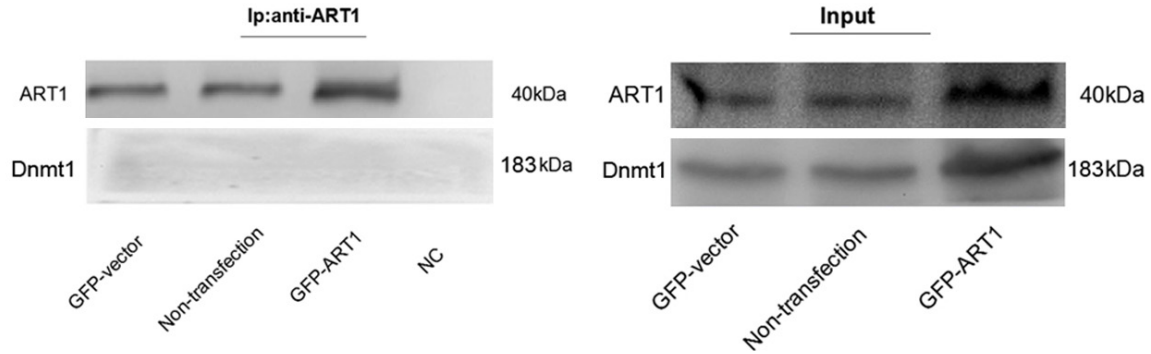


Figure 4. DNMT1 has no interact with ART1 in CT26 cells. Western blot analysis of the co-IP complex following incubation of GFP-ART1, Non-transfection and Vector CT26 cells lysates with ART1 antibody. DNMT1 was not detected in IP product of GFP-ART1 and Non-transfection group. NC; negative control group used IgG instead of ART1 antibody to incubate the lysate of GFP-ART1 CT26 cells.

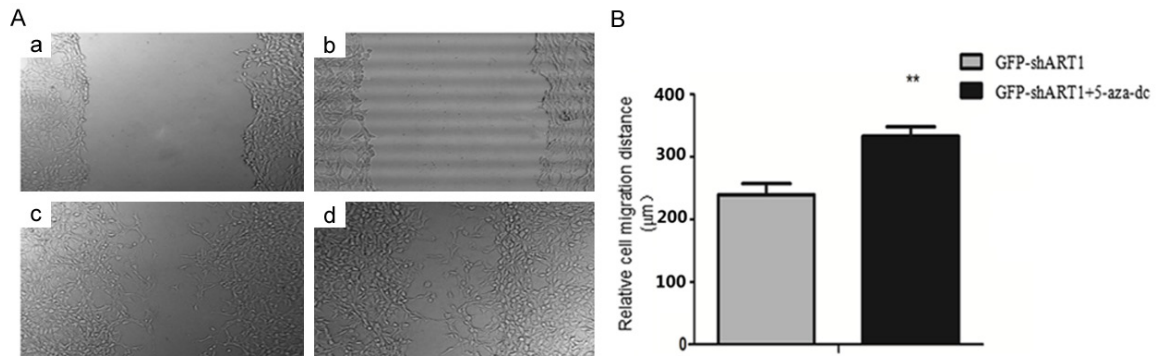


Figure 5. Effect of DNA methylation inhibitor on CT26 cells migration. A. GFP-shART1 CT26 cells were treated with an inhibitor 5-aza-dC as the experimental group. The control group was the GFP-shART1. Cell migration assay was taken to detect migration capacity. Representative images are shown above. a and c. Control group at 0 and 24 h, respectively. b and d. 5-Aza-dC treated group at 0 and 24 h, respectively. B. GFP-shART1 group treated with 5-aza-dC significantly pushed CT26 cells to heal the wound. The migration distance for 24 h in GFP-shART1 with 5-aza-dC treated group was greater than that of the GFP-shART1 group ($P < 0.01$).

dc (1.0 μM , 72 h). Western blotting was used to test the expression of DNMT1 and uPA in vitro and in vivo. It was showed that the expression of DNMT1 was reduced in the GFP-shART1 group with 5-aza-dc compared to the control GFP-shART1 group in vitro ($P < 0.01$) and in vivo ($P < 0.05$). There was an increase of the expression of uPA in the GFP-shART1 group with 5-aza-dc in contrast to control GFP-shART1 group in vitro and in vivo ($P < 0.01$) (**Figure 2**).

Effects of ART1 on the DNA methylation status of uPA gene promoter region in vitro

To investigate further the influence of ART1 on DNA methylation of the putative uPA promoter regions, Bisulfite sequencing PCR of genomic

DNA was applied. It showed that the DNA methylation status of uPA were 55.7%, 27.1%, 42.9% and 42.9% in GFP-shART1, GFP-ART1, non-transfection and GFP-vector groups respectively. Compared with non-transfection and GFP-vector group, the DNA methylation status of uPA showed an increase in the GFP-shART1 group ($P < 0.05$), while there was a clear reduction of uPA methylation status in the GFP-ART1 group ($P < 0.05$; **Figure 3**).

Relationship between ART1 and DNMT1 in vitro

Co-immunoprecipitation showed that there is no direct binding interaction between ART1 and DNMT1 (**Figure 4**).

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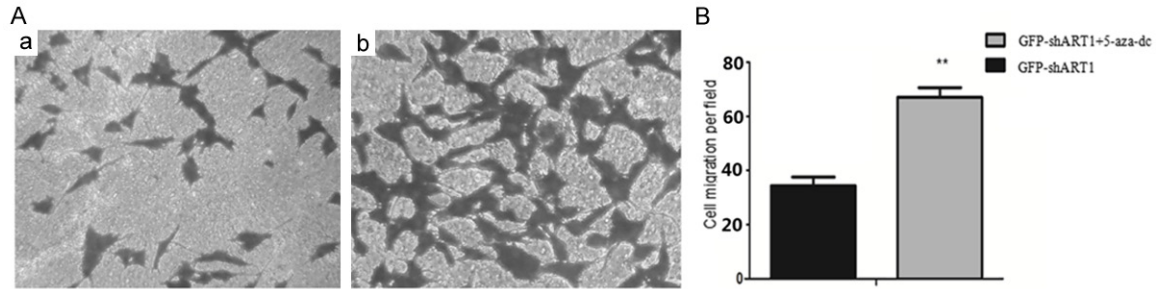


Figure 6. Effect of DNA methylation inhibition on CT26 cells invasion. A. GFP-shART1 CT26 cells were treated with an inhibitor 5-aza-dC as the experimental group. The control group was the GFP-shART1. The invasion capacity was detected by transwell assay (crystal violet staining). Representative images are shown. a. Control group; b. 5-Aza-dC treated group. B. The CT26 cells number that migrated to Chambers membrane in experimental group was greater than that of control group. ($P < 0.05$ relative to control group from three independent experiments).

Effects of DNA methylation inhibition on invasion and migration of GFP-shART1 CT26 cells in vitro

The cell migration assay was used to detect the migration capacity of CT26 cells. In the preparatory work, GFP-shART1 cells were treated with an inhibitor of DNMT1 (5-aza-dc). The results showed that the GFP-shART1 group treated with 5-aza-dc significantly stimulated CT26 cells to heal the wound and the migration distance for 24 h was increased compared to the GFP-shART1 group ($P < 0.01$; **Figure 5**). The invasion capacity of CT26 cells was detected by the Transwell assay. The number of CT26 cells that underwent invasion through Matrigel in the 5-aza-dc treated group was 67.3 ± 3.5 . The number of cells invading in the untreated control group was 34.3 ± 3.2 . The values were significantly higher in the 5-aza-dc treated group than those in the control group ($P < 0.01$; **Figure 6**).

Effects of DNA methylation inhibition on liver metastasis of GFP-shART1 CT26 cells in vivo

To verify the positive effect of DNA methylation inhibition on invasion and metastasis of CT26 cancer cells, the liver metastasis model was created in BALB/c mice by injecting CT26 cells (GFP-shART1) into the splenic capsule. The mice of the experimental group then were treated with 5-aza-dc. The neoplasm was gray and hard. Necrosis was found in some parts of the tumor whose boundaries were clear to the neighbouring tissues. Livers contained multiple, coalescing pale nodules (0.2-0.5 cm). The histological feature of different treatment

groups showed that cancer cells were arranged in sheets, round or oval. The cell nucleus was in deep colour with obvious atypia. And pathological karyokinesis could be observed. Compared to the controlled group, the weight of the liver was much higher in the experimental group ($P < 0.05$). Meanwhile the number of liver metastasis nodules showed a significant increase in experimental group ($P < 0.01$), compared with the control group. Therefore, these results strongly supported our previous theory that inhibition of DNA methylation could enhance the metastatic capacity of CT26 cells in vivo (**Figure 7**; **Table 1**).

Discussion

As Partha et al have informed, DNA methylation is a significant regulator of the transcription of genes, and its function in carcinogenesis has been a topic of debate for years [30]. In the current view, DNA methylation has a crucial role in the development of cancers. In more specific terms, hypermethylation leads to silencing of genes through repressing transcription of the promoter regions of tumor suppressor genes, while hypomethylation causes growth of the tumor through activating oncogenes [30]. Therefore, mediators of DNMT1 are of importance. It has also been reported that PARP1 could mediate the expression and activity of DNMT1 through non-covalent interactions [14].

In mammalian cells, both mono (ADP-ribosyl) ation and poly (ADP-ribosyl) ation reactions are regulated by enzymes that can reverse these posttranslational modifications [31]. In our previous study, we have demonstrated that inhibi-

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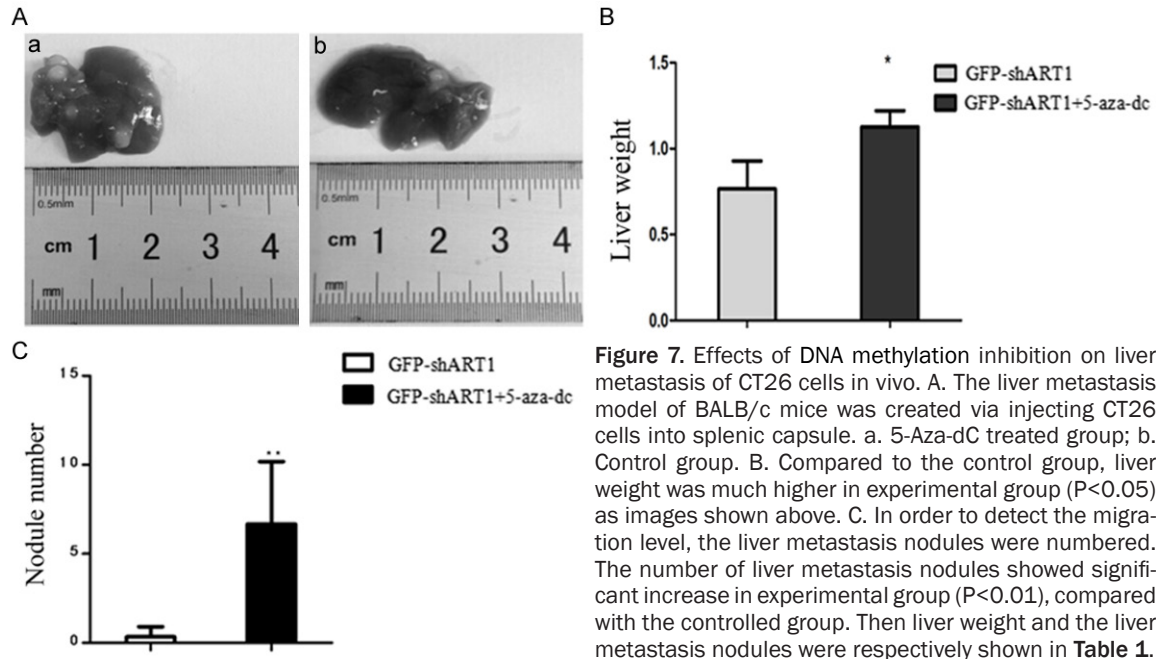


Figure 7. Effects of DNA methylation inhibition on liver metastasis of CT26 cells in vivo. A. The liver metastasis model of BALB/c mice was created via injecting CT26 cells into splenic capsule. a. 5-Aza-dC treated group; b. Control group. B. Compared to the control group, liver weight was much higher in experimental group ($P < 0.05$) as images shown above. C. In order to detect the migration level, the liver metastasis nodules were numbered. The number of liver metastasis nodules showed significant increase in experimental group ($P < 0.01$), compared with the controlled group. Then liver weight and the liver metastasis nodules were respectively shown in **Table 1**.

Table 1. Liver weight and the liver metastasis nodules were respectively shown below (SD \pm s, n=5)

Group	Liver weight (g)	Liver metastasis nodule number
5-aza-dC-treated	1.08 \pm 0.10	6.67 \pm 3.51
Untreated control	0.77 \pm 0.16	0.53 \pm 0.04

The number of liver metastasis nodules showed significant increase in experimental group compared with the controlled group ($P < 0.05$). The liver weight of 5-aza-dC treated group was higher than that of the untreated control group ($P < 0.01$).

tion of PARP1 decreases invasion and the metastatic ability of colon cancer cells [19]. Similarly, silencing of ART1 decreases invasion and the metastatic ability of colon cancer cells through down-regulating the expression of RhoA, Rock1, MMP-2 and MMP-9 [18]. In addition, it has been shown that PARP1 could be regulated by ART1 possibly via affecting the expression and activity of RhoA and NF- κ B [21]. Meanwhile, Caiafa et al demonstrated that polymers present on PARP1 bind to DNMT1 non-covalently to decrease its expression and activity [13]. As we hypothesized above, whether ART1 could regulate DNMT1 remained unknown. However, there were no data available to indicate some correlation between ART1 and DNA methylation in colon carcinoma. Thus, in this study, we examined the expression

of DNMT1 following the transfection of CT26 cells with GFP-shART1 and GFP-ART1 to elucidate the correlation between ART1 and DNMT1 in colon carcinoma. The results revealed that inhibition of ART1 could induce the expression of DNMT1 and over-expression of ART1 could decrease it in colon carcinoma. The DNA methyltransferases are available to catalyze the process of DNA methylation, and their expression and activity are closely associated with the status of DNA methylation. To confirm further the effect of ART1 on DNA methylation status, we used bisulfite sequencing PCR [32, 33] to measure the exact methylation status of certain genes. We chose the uPA gene to detect methylation status of its promoter regions. The urokinase plasminogen activator (uPA) is one of the proteases which are associated with the invasiveness of tumors and is involved in cellular migration by interacting with vitronectin and integrins [34]. Consistent with the results of expression of DNMT1, hypermethylation through silencing of ART1 and hypomethylation of uPA with ART1 over-expression were detected relative to controls. It was previously discussed that uPA expression was regulated by DNA methylation as reported by Guo et al [34, 35]. Pakneshan et al reported that up-regulation of DNMT1 caused silencing of uPA and inhibition of invasion and metastasis of breast cancer cells [34, 36, 37]. Thus we measured the

expression of the gene, and our results revealed that inhibition of DNMT1, which may cause hypomethylation of uPA promoters, brought about up-regulation of uPA gene expression in mouse CT26 cells, and increasing of DNMT1 might cause the opposite effect. Therefore, we could initially conclude that ART1 might induce aberrant methylation status of the uPA promoter so as to regulate the expression of uPA which could participate in the progresses of tumor invasion and metastasis.

From the current findings, it may be reasonable to hypothesize that there might be two explanations for the effect of ART1 on DNMT1. On one hand, just as there is a relationship between PARP1 and DNMT1, ART1 might have interaction with DNMT1 thus causing its expression and regulating its activity. On the other hand, their interaction might be not direct but through some regulatory pathway. We confirmed the former situation and demonstrated that ART1 and DNMT1 have no direct interaction between them. In a previous study, we demonstrated that the expression of PARP1 could be mediated by ART1 in mouse colorectal carcinoma CT26 cells [21]. We also reported that inhibition of ART1 could decrease the expression and activity of RhoA, which stands downstream of the mono (ADP-ribosyl) ation regulated by ART1 [20]. Yau et al pointed out that MIBG (metaiodobenzylguanidine, an inhibitor of ART1) can inhibit the Rho effector, implying the participation of mono (ADP-ribosyl) ation in a Rho-dependent signaling pathway [38]. Among the Rho proteins, RhoA can regulate NF- κ B-dependent transcription [39]. It is also revealed by Huang et al that inhibition of RhoA could downregulate the NF- κ B through the decrease of phosphor-I κ B α in prostate carcinoma cells [40]. We have previously demonstrated that inhibition of NF- κ B could downregulate the expression of PARP1 through a feedback mechanism [41]. MIBG which contemporaneously downregulates the expression of PARP1 and ART1 to clarify the effect between ART1 and PARP1. Therefore, we conclude that ART1 may mediate expression of PARP1 by mediating the expression and activity of RhoA, thus regulating NF- κ B-dependent transcription. Meanwhile, some reports suggested a link between PARP-1 and DNMT1. In their model, PARP1 could prevent the enzymatic activity of DNMT1 through non-covalent protein-protein interactions [13].

Similarly, it has been demonstrated that PARP-1 binds to the promoter of the DNMT1 gene and affects DNA methylation status by regulating the expression and activity of the DNMT1 [12]. Hence it could be concluded that the expression of PARP1 was probably regulated by ART1 through the feedback mechanism of NF- κ B to PARP1 thus mediating the expression and activity of DNMT1 through non-covalent interactions. And in our current study, we used an inhibitor of DNA methyltransferase (5-aza-dc) in the GFP-shART1 group and observed how the expression of uPA changed. Expression of uPA increased upon treatment with the demethylating agent 5-aza-dc in the GFP-shART1 group. Then we tested the capacity for invasion and metastasis, a significant increase was shown. And according to some studies, DNMTs could be inhibited by 5-aza-dc in cancer cells. In detail, DNMT1 was nearly all inhibited while DNMT3a and DNMT3b was part of losing the activity [42]. It meant that 5-aza-dc had the most effective inhibition on DNMT1 among DNMTs. Some reports also suggested that the decrease of DNMT1 could cause DNA hypomethylation of certain cancer-related genes [13]. Therefore, it could be concluded that inhibition of DNMT1 might be related to CT26 cells migration motility. In our previous study, we have demonstrated that inhibition of ART1 could decrease invasion and metastatic ability in mouse CT26 cells [18]. Therefore, as there is a relationship between ART1 and DNMT1, the effect of DNMT1 on cancer invasion and metastasis could be mediated by ART1. Altogether, ART1 might participate in invasion and metastasis in mouse CT26 cells through the ART1-DNMT1 pathway.

The above conclusions have been translated effectively in vivo in our BALB/c mice models. 5-aza-dc was applied to the experimental group, causing hypomethylation of uPA gene promoters and then over-expression of uPA [43]. Opposing effect of 5-aza-dc on growth and invasion of tumors has been reported [25], an issue which remains to be resolved.

In summary, the novelty of the present work is that it not only elaborates how PARP1 is mediated by ART1 but also reveals ART1 causes aberrant methylation of the uPA promoter. It probably related to PARP1-DNMT1 complex, thus participating in tumorigenesis. Although further

studies may be required, the data presented here offered a dependable mechanism for ART1 mediating DNMT1 in colon cancer cells. We expect that our study may be a new approach to clarifying ART1 in the ART1-DNMT1 pathways as a promising therapeutic strategy for colorectal carcinoma.

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Disclosure of conflict of interest

None.

Authors' contribution

Xi Yang and Prof. Yalan Wang designed the experiments; Xi Yang, Ming Li, Jia-Yue Han, Xu Han, Liang Chen, Huan Chen, Wen-Ping Hu and Xiao Lin performed the experiments; Xi Yang and Yi Tang analyzed the data; Prof. Yalan Wang provided reagents/materials; Xi Yang wrote the paper, Prof. Michael D. Threadgill edited the text and contributed to the discussion.

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