Original Article Reversing hypomethylation of TIAM1 promoter inhibits TIAM1 gene expression and cell proliferation and migration in colorectal cancer

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Abstract: Objective: To investigate the reversing effect of hypomethylation status of T lymphoma invasion and metastasis 1 (TIAM1) gene promoter on TIAM1 expression and evaluate the biological behavior of colorectal cancer cells in vivo and in vitro. Methods: DNA methyltransferase was used to hypermethylate the predicted TIAM1 promotor. Then the luciferase activity of the unmethylated TIAM1 promoter (UP-T) and hypermethylated TIAM1 promoter (MP-T) was examined by Dual Luciferase Reporter assay. We constructed plasmids with the PGL3.0-Basic-hypermethylated-promoter-TIAM1-cDNA vector and PGL3.0-Basic-unmethylated-promoter-TIAM1-cDNA vector and then transfected them into cell lines HT29, LS174T and SW480. Expression of TIAM1 protein were examined by Western blot. Subsequently, the biological behavior of the transfected cells were assessed by MTT, plate colony formation and in-vitro invasion analysis. SW480 cells labeled with green fluorescent protein (SW480/GFP) were treated with S-adenosylmethionine (SAM, methyl donor), S-adenosylhomocysteine (SAH, non-methyl donor), respectively. A visualized orthotopic animal model was built by injecting the above three group cells into nude mice subcutaneously and intravenously. Then the effects of aberrant DNA methylation on the biological behavior of colorectal cancer cells were detected in vivo. Results: The hypermethylated TIAM1 promoter showed lower luciferase activity than that of the unmethylated promoter. Expression of TIAM1 protein was statistically decreased in MP-T transfectant. Furthermore, a significant suppressed proliferation, migration and invasion ability were observed in MP-T transfectant cells as compared with those in the UP-T transfectant cells (P<0.05). Hypermethylation of SW480/GFP cells resulted in decreased proliferation, migration and invasion abilities in vivo. Conclusion: Aberrant methylation of TIAM1 promoter was closely related to aberrant TIAM1 expression in colorectal cancer. Reversing hypomethylation status of TIAM1 promoter can lead to inhibition of cell growth, migration and decreased invasion capacity of colorectal cancer in vivo and in vitro, which implied a potential therapy pathway for colorectal cancer clinically.

Keywords: TIAM1, colorectal cancer, DNA methylation, cell proliferation

Introduction

Colorectal cancer (CRC) is one of the most common cancers and the most prevalent cancerrelated causes of death all over the world. Due to the continuance of the current trends, it is of great important to elucidate the molecular mechanisms underlying the initiation and development of CRC. Up to now, many oncogenes and tumor suppressor genes are known to be the key factors in controlling cell fate. A balance between these key factors is essential for cell growth, differentiation and death. The relationships between CRC/Wnt signaling pathway and CRC/Rac signaling pathway have already been validated [1-3].

T lymphoma invasion and metastasis 1 (TIAM1) was firstly isolated and cloned from high-invasive mutant T lymphoma cells of mice and the name was given according to its characteristics in T lymphoma cells by Habets in 1994 [4]. It is not only one of the known guanine nucleotide (GDP/GTP) exchange factors (GEFs) for Rho GTPases (e.g., Rac1), but also a Wnt-responsive gene that affects tumor development [5]. The expression and function of TIAM1 have already been studied in breast cancer, renal carcinoma, chondrosarcoma, T lymphoma, and colorectal cancer [6-12].

Our previous studies have demonstrated that the expression of TIAM1 protein was significantly higher in colorectal cancer than in matched normal mucosa, and the expression level of TIAM1 protein is closely associated with lymph node metastasis [11-14]. We further explored the mechanism underlying overexpression of TIAM1 in colorectal cancer. Consequently, hypomethylation of TIAM1 promoter was detected in colorectal cancer tissues, indicating that abnormal methylation of TIAM1 promoter is account for aberrant TIAM1 expression [14]. Since DNA methylation is a reverse process, in this study, we further hypothesized that hypermethylation of TIAM1 promoter might greatly affect cell growth and migration in colorectal cancer in vivo and in vitro. Thus, we constructed a new plasmid with hypermethylated TIAM1 promoter to explore its effects on TIAM1 expression and cellular biological behavior in colorectal cancer cells. We also build a visual mice model to explore DNA hypermethylation on tumor cell growth and migration in vivo.

Materials and methods

Ethics statement

The study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of Southern Medical University (Permit Number 2006-0015). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Cell culture

Three human colorectal cancer cell lines (HT29, LS174T and SW480) were procured from American type culture collection (Manassas, VA, USA) and cultured in RPMI-1640 medium containing 10% heat-inactivated FBS (fetal bovine serum). All cells were cultured at 37° C in a humidified atmosphere of 5% CO₂.

Amplification of TIAM1 promoter

TIAM1 promoter sequence was predicted online (http://www-bimas.cit.nih.gov/molbio/prosc-

an/analysis.html) and all the CpG sites were analyzed by the website (http://www.urogene. org/methprimer/index1.html). DNA was extracted from normal colorectal tissue using a DNA Extraction Kit (Takara, Kyoto, Japan). DNA concentration and quality was evaluated by agarose gel electrophoresis. PCR amplification was performed using the forward primer (5'-GGTACCGTACTCAC CCGCGACGGT-3') and reverse primer (5'-AAGCTTCTGCTCAGGGTCTAA CATCC-3') with an initial denaturation step at 95°C, followed by 35 cycles of denaturation at 95°C, annealing at 52°C and extension at 72°C. PCR products were purified from 1.0% agarose gel.

Hypermethylation of TIAM1 promoter sequence

TIAM1 promoter region was treated with M.SssI methyltransferase enzyme (NEB, Ipswich, MA, USA) at 37°C overnight. The product was purified using a Fragment Purification Kit (Takara, Kyoto, Japan) according to the manufacturer's protocol. Then the DNA sequences of hypermethylated-TIAM1-promoter and unmethylated-TIAM1-promoter were modified by the Methyl Detector Bisulfite (Active Motif, Carlsbad, CA, USA). Bisulfite sequencing PCR was performed using the forward primer (5'-AGGGAGTTTTT-AATAAAGTAGTTTTGG-3') and the reverse primer (5'-ACTCAAAATCTAACATCCTCTAACA-3'). The PCR products were sequenced by an ABI-377 instrument to verify the sequence was methylated or unmethylated.

Construction of promoter plasmid

Both the hypermethylated promoter and unmethylated promoter were digested with HindIII and Kpn I enzymes, and then inserted into pGL3.0-Basic vector (Promega, Madison, WI, USA), respectively. Subsequently, the unmethylation-promoter-pGL3.0-Basic (UP-B) plasmid and hypermethylation-promoter-pGL3.0-Basic (MP-B) plasmid were sequenced by an ABI-377 instrument.

Dual-luciferase reporter assay

Transfection was performed with Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA). 1×10^5 cells were seeded in 24-well plates. When cells reached 75% confluence, PGL3.0-SV40 Renilla luciferase reference control plas-

mid (Promega, Madison, WI, USA) were transfected with UP-B or MP-B plasmid according to the manufacturer's instructions. Luciferase activity was measured in cell lysates 24 hours after transfection using the Luciferase Assay System (Promega, Madison, WI, USA). All experiments were performed at least 3 times.

Construction of TIAM1 plasmid

The cDNA of TIAM1/C1199HA expression plasimd was digested by EcoRI restricted enzyme and subcloned into the *EcoR*I site of UP-B vector and MP-B vector. The vectors were then sequenced by an ABI-377 instrument to confirm whether the direction of the inserted TIAM1 fragment was correct.

Plasmid transfection

The MP-B and UP-B plasmids were respectively transfected into colon cancer cells HT29, LS174T and SW480 by lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the reagent manufacturer's protocol. The cells were used in the following assays 48 hours after transfection.

Proliferation assays

1×10³ cells were cultured in 96-well plates shaken on a mocroplate shaker for 1 to 7 days. 20 μ l of 5 g/L MTT [3-(4,5-dimethylthiazol-z-yl)-2,5-diphenyltetrazolium bromide] was added to each well on each day, and the plate was incubated at 37°C for 4 h in a humidified incubator with 5% CO₂. Afterwards, MTT was removed from each well and replaced with 150 μ l DMSO (Sigma, St Louis, MO, USA). Measuremet of the absorbance was conducted at 570 nm by using a Microplate Autoreader (Bio-Rad, Hercules, CA, USA). The experiment was replicated three times.

Plate colony formation assay

The plate colony formation assay was performed in a 6-well culture plate. About 1×10^2 cells were added to each well and each cell group took up three wells. After two weeks' incubation at 37°C, the number of colonies containing \geq 50 cells was counted after two washes with PBS and Giemsa staining under a microscope [plate colony formation efficiency = (number of colonies/number of cells inoculated) $\times 100\%$]. The experiment was replicated three times.

Migration assay

About 2×10^4 cells were added to the upper compartment of the transwell plate and 200 µl 10% FBS in free medium (Gibco, Invitrogen Carlsbad, CA, USA) was added to the lower compartment. The transwell plate was fixed with methanol after 18 hours. The cells at the upper side were then cleaned carefully with a cotton swab. For quantification, the cells on the lower side were stained with Giemsa liquor and counted in five randomly chosen fields (×200) under a microscope. The experiment was replicated three times.

Western blot analysis

Cells washed twice with cold PBS were lysed on ice in RIPA buffer (1×PBS, 1% BP40, 0.1% SDS, 5 mM EDTA, 0.5% sodium deoxycholate and 1 mM sodium orthovanadate) using protease inhibitors. The protein lysates were resolved on 8% SDS polyacrylamide gel followed by electrotransfer onto polyvinylidene fluoride membranes (Millipore, Billerica, USA). After blocking in 5% nonfat dry milk/Tris-buffered saline (100 mM NaCl, 50 mM Tris and 0.1% Tween-20, PH7.5), the membranes were immunoblotted using anti-TIAM1 polyclonal antibody (Santa Cruz Biotech, CA, USA) and anti-β-actin antibody (Santa Cruz Biotech, CA, USA) at 4°C overnight. Then they were reacted with horseradish peroxidase-conjugated secondary antibodies and finally detected using the ECL (enhanced chemiluminescence) kit (Thermo Scientific, DE, USA).

In vivo function assay

To assess in vivo tumor growth, Colon cancer cell line SW480 labeled with green fluorescent protein (SW480/GFP) was treated with S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH) and phosphate-buffered saline (PBS), respectively. Then 1×107 cells were iniected subcutaneously into nude mice through the left or right flank (n=4 per group). The condition of the mice and growth of the resultant primary tumors were monitored every day afterwards. Calipers were used to estimate the volume of tumors from day 5 to day 28 after the injection. To assess in vivo metastasis, 5×105 cells were injected into 6- to 9-week-old Balb/C mice through tail veins (n=4 per group). All mice were euthanized using deep inhalation of isoflurane according to animal care guidelines after



Figure 1. Analysis of TIAM1 promoter sequence and its activity. A: CpG Island analysis in TIAM1 promoter. CpG island was predicted by methprimer software and labeled in blue color. B: Verification of the methylation status of TIAM1 promoter with and without Sss.I treatment by bisulfite sequencing. The unmethylated TIAM1 promoter was shown above while the hypermethylated TIAM1 promoter shown below. C: The luciferase activity of hypermethylated TIAM1 promoter in CRC cell lines. U, unmethylated TIAM1 promoter, M, hypermethylated TIAM1 promoter. Each bar represents the mean \pm S.E. of three replicates. The asterisk denotes statistically significant differences between the indicated samples (*P<0.05).

6 weeks, and various organs were removed from the thoracic, peritoneal and retroperitoneal cavities. All removed organs were rinsed, fixed and subjected to pathological examination. The number of tumor colonies was counted under a dissecting microscope.

Statistical analysis

Statistical analyses were performed using SPSS13.0 (SPSS Inc., Chicago). One way ANOVA

and χ^2 test were used to compare the statistical difference of cell proliferation, plate colony formation ability, and migration rate between different cell groups. A *P* value of 0.05 or less was considered significant (two tails).

Results

Promoter CpG analysis

The TIAM1 gene promoter region was predicted and analyzed by Bioinformatic software. There



Figure 2. Effects of TIAM1 promoter with different methylation status on the biological behavior of CRC cells and TIAM1 expression in vitro. A: Proliferation assay; B: Plate colony formation assay; C: Migration assay; D: TIAM1 protein expression by Western blot. Each bar represents the mean \pm S.E. of three replicates. The asterisk denotes statistically significant differences between the indicated samples (**P*<0.05).

was one CpG island within TIAM1 promoter region, containing 105 CpG sites (**Figure 1A**). After Sssl Methyltransferase enzyme treat-

ment, the methylation status of CpG sites at the promoter region was examined by BSP. The results showed that 16 CpG sites were hyper-



Figure 3. Effects of TIAM1 promoter with different methylation status on the biological behavior of CRC cells in nude mice model. A: Gross observation of tumor size. B: Statistical chart of tumor size. Each bar represents the mean ± S.E. of three replicate samples. C: Microscopic observation of tumor. D: Metastasize in lung. E: HE of metastasize in lung.

methylated after Sssl Methyltransferase enzyme treatment (**Figure 1B**).

Luciferase activity of unmethylated TIAM1 promoter and hypermethylated TIAM1 promoter

The luciferase reporter assay of cultured cells was performed to measure the activity of hypermethylated TIAM1 promoter and unmethylated TIAM1 promoter. The results indicated that the activity of hypermethylated TIAM1 promoter was 2.5 folds lower than that of unmethylated TIAM1 promoter (P<0.05; Figure 1C). The positive control containing SV40 promoter and enhancer sequences resulted in strong expression of luciferase activity in many types of mammalian cells. The activity of unmethylated TIAM1 promoter was as strong as that of positive control (P>0.05). Additionally, our results demonstrate that the predicted TIAM1 promoter fragment is sufficient to trigger maximum activity of the TIAM1 gene.

Effects of hypermethylated TIAM1 promoter and unmethylated TIAM1 promoter on the biological behaviors of CRC cell lines in vitro

As compared with HT29, LS174T and SW480 cells that transfected with MP-T plasmid, the cells transfected with UP-T plasmid showed a significantly enhanced cell proliferation as determined by MTT assay (P<0.05) (Figure 2A). No significant difference was observed between MP-T group and mock group (P>0.05) (Figure **2A**). Furthermore, the cell growth ability was assessed by colony formation assay and the cell growth ability was significantly higher in UP-T group than that in MP-T group (P<0.05) (Figure 2B). No significant difference was found between MP-T group and mock group (P>0.05) (Figure 2B). The results indicated that hypermethylated TIAM1 promoter inhibited the proliferation ability of colorectal cancer cells when compared with unmethylated TIAM1 promoter group. Invasion through the extracellular matrix (ECM) is an important step in the progress of tumor metastasis. The ECMatrix serves as a reconstituted basement membrane matrix of proteins. The number of cells that migrated through the ECMatrix was counted and the results showed that the MP-T group possesses a remarkable decrease in cell invasion as compared with UP-T group (P<0.05) (**Figure 2C**). No significant difference was observed between MP-T group and mock group (P>0.05) (**Figure 2C**). The results indicated that hypermethylated TIAM1 promoter inhibited the migratory and invasive ability of colorectal cancer cells when compared with unmethylated TIAM1 promoter group.

Effect of hypermethylated TIAM1 promoter and unmethylated TIAM1 promoter on TIAM1 expression

The result of western blotting analysis showed that TIAM1 protein expression was significantly higher in UP-T group than in MP-T group (**Figure 2D**). The MP-T group exhibited no significant difference with the mock group. These data indicated that hypermethylation of TIAM1 promoter decreased TIAM1 protein expression, furthermore low expression of TIAM1 protein was associated with decreased proliferative, migratory and invasive ability of colorectal cancer cells in vitro.

Effects of hypermethylated TIAM1 promoter and unmethylated TIAM1 promoter on tumor growth and metastasis in vivo

To evaluate the effect of different methylation status of TIAM1 promoter on tumor growth in vivo, hypermethylated TIAM1 group (SAM-treated group) cells, unmethylated TIAM1 group (SAH-treated group) cells and mock cells (PBStreated group) were implanted subcutaneously into nude mice. The growth of the resultant primary tumors were monitored soon afterwards. Palpable tumors were detected on day 7 after injection in mice of all the three groups, but were smaller in mice injected with cells from hypermethylated TIAM1 group than from mock group at day 28 (**Figure 3A-C**).

To assess the effect of different methylation status of TIAM1 promoter on cell metastasis in vivo, the above cells from the three groups were injected in the experimental mice model through tail veins. As we observed, 25% of mice injected with cells from SAH-treatment group and mock group (n=1 of 4 cases) developed lung metastasis. However, no metastasis was observed in mice from the SAM-treated group. No other tumor metastasis was detected in all the three groups. A significantly higher number of metastatic lesions were found in SAH-treated and mock group than SAM-treatment group (**Figure 3D** and **3E**). All these results corroborate with the inference that hypermethylation of TIAM1 promoter inhibited TIAM1 expression and also profoundly decreases tumor growth and metastasis in vivo.

Discussion

The alternation of methylation patterns in the promoter region of genes is one of the most important pathways that regulating gene expression at transcription level [15]. There are two distinct aberrant methylation forms: hypomethylation and hypermethylation. Hypomethylation of gene promoter is transcriptionally active while hypermethylation of gene promoter is transcriptionally silent.

The role of DNA hypermethylation in carcinogenesis has been widely studied [16-18]. Hypermethylation of tumor suppressor genes usually results in the down-regulation of gene expression and decrease of cell proliferation and migration capability [16-18]. On the contrary, hypomethylation of CpG islands located in the promoter or exon 1 region restores or activates oncogene expression, such as Bcl-2 gene in B-cell chronic lymphocytic leukemia, MAGE-1 gene in melanomas, S100A4 gene in pancreatic carcinoma, SNCG/BCSG1 gene in breast cancer and ovarian cancer, Maspin gene in gastric cancer, and uPA gene in breast cancer [19-24].

The process of aberrant DNA methylation is reversible, which is of great value to cancer therapy clinically. Up to now, studies have demonstrated that reversing hypomethylation status of oncogenes by methylation drugs, such as folic acid, leads to the silence of oncogene expression, and then inhibits tumor cell growth [25, 26].

TIAM1 was one of the most important colorectal cancer-related genes. It was upregulated in the intestines of conditional adenomatous polyposis coli (APC) mutant mice and increased TIAM1 expression is correlated with the metastatic potential of human colorectal cancer [5]. In our previous study, we have observed hypomethylation of TIAM1 promoter and overexpression of TIAM1 protein in colorectal cancer tissues [14], which may account for invasive and metastasis potential of colorectal cancer. Since DNA methylation is a reversible process, in this study, we hypermethylated TIAM1 promoter to suppress TIAM1 expression and subsequently explore its function in colorectal cancer in vivo and in vitro. We firstly detected the activity of the hypermethylated and unmethylated TIAM1 promoter by dual luciferase reporter assay. The luciferase activity was strongly suppressed in hypermethylated TIAM1 promoter group, suggesting hypermethylation of TIAM1 promoter decreased gene expression. We then constructed a new plasmid by connecting PGL3.0-Baisc vector, unmethylated TIAM1 promoter or hypermethylated TIAM1 promoter, and TIAM1 cDNA together. The PGL3.0-Baisc vector contains no promoter, which is very suitable for assessing the activity of ectogenic promoter. We transfected the above plasmids into different colorectal cancer cell lines. TIAM1 protein was lowly expressed in LS174T, SW480 and HT29 cells when transfected with the hypermethylated promoter plasmid, while highly expressed in these cells when transfected with the unmethylated promoter plasmid. Significant difference of TIAM1 expression was found between hypermethylated promoter group and unmethylated promoter group, indicating that hypermethylation of TIAM1 promoter do inhibit TIAM1 expression. Subsequently, the biological capabilities of the transfected cells were further investigated in vitro. Both the proliferation and migration ability of MP-T transfectant cells were significantly decreased as compared with UP-T transfectant cells. In UP-T group, hypomethylation of TIAM1 promoter increased TIAM1 expression, which leads to a high capability of growth and migration in colorectal cancer in vitro. On the contrary, hypermethylation of TIAM1 promoter suppressed TIAM1 expression and consequently inhibited cell growth and migration in colorectal cancer in vitro.

In this study, we also treated SW480 cell with SAM, a methyl group donor, to reversed hypomethylation status of TIAM1 promoter in colorectal cancer cell, and further explored the effects on cell biological behavior in vivo. SAH, a metabolite or isoform of SAM, harboring no methyl group, was used as an unmethylation control. In our previous work, SAM treatment reduced TIAM1 expression and further suppressed cell growth and migration in vitro [14]. It was no surprise, SAM treatment decreased cell growth, migration and invasion in vivo in our present study, suggesting that SAM reversed hypomethylation status of TIAM1 promoter and subsequently inhibit the malignant behavior of colorectal cancer cell in vivo and in vitro.

As we all know, SAM treatment increases DNA methylation level and then alters genes expression. As a common co-substrate involved in methyl group transfers, SAM is involved in more than 40 metabolic reactions. Transmethylation, transsulfuration, and aminopropylation are the metabolic pathways that recruit SAM. These processes are complex and complicated. Since SAM is a nonspecific hypermethylation agent, many genes would be hypermethylated after SAM treatment. To avoid the interference of other genes, we constructed a new plasmid by connecting a basic vector without promoter, with a predicted unmethylated TIAM1 promoter or hypermethylated promoter and TIAM1 cDNA together. The new plasmid can be used to investigate the effects of aberrant methylation of only TIAM1 promoter on TIAM1 gene expression and colorectal cancer behavior. Furthermore, this method can be applied to study abnormal DNA methylation of other genes in addition to TIAM1.

Aberrant DNA methylation is now recognized to be a common hallmark of human cancers. The pattern of DNA methylation plays an important role in regulating various genes expression and function. One of the features is the inactivation of tumor related genes by hypermethylation of the CpG islands located in their promoter regions. The promising chemotherapeutic drugs can be applied to these promoter sites. Current research has shown that epigenetic drugs could be applied in adjuvant therapy for currently accepted treatment methods such as radiation and chemotherapy, or could enhance the effects of the current treatments. Epigenetic drugs will be an important weapon in the war against cancer. Therefore, we need to continue our research to find more specific DNA-hypermethylating agents to determine whether they will be successfully used in cancer therapy combing with other epigenetic drugs.

In conclusion, our study further verifies that aberrant DNA methylation is one of the mechanisms involved in aberrant TIAM1 expression in colorectal cancer. We reverse hypomethylation status of TIAM1 promoter and consequently inhibit cell growth and migration in vivo and in vitro. It can be inferred that reverse of DNA hypomethylaiton status of tumor related genes may supply a potential alternative for clinical therapy.

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Dislosure of conflict of interest

None.

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