Original contribution

Methylation status of T-lymphoma invasion and metastasis 1 promoter and its overexpression in colorectal cancer☆,☆☆

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Summary
T-lymphoma invasion and metastasis 1 has been implicated in tumor invasion and metastasis. However, the regulatory mechanisms underlying aberrant T-lymphoma invasion and metastasis 1 expression in human colorectal cancer have not been well defined. To investigate the relationship between methylation status and expression levels of T-lymphoma invasion and metastasis 1 gene, methylation-specific polymerase chain reaction, and immunohistochemistry staining were performed in 232 matched samples of human colorectal cancer tissue and normal colorectal mucosa. Results showed that T-lymphoma invasion and metastasis 1 protein was overexpressed in colorectal cancer, especially in metastatic cases (\(P < .001\)). The degree of T-lymphoma invasion and metastasis 1 promoter methylation was a little lower in cancer tissues than in matched normal mucosa (\(P < .05\)), and the expression level of T-lymphoma invasion and metastasis 1 was inversely related to the methylation status in cancer tissues (\(P < .001\)). Colon cancer cell lines HT29 and LS174T were treated with demethylating agent 5-aza-2′-deoxycytidine, resulting in promoter hypomethylation accompanied by reexpression of T-lymphoma invasion and metastasis 1 mRNA and protein. In contrast, colon cancer cell lines SW620 and LoVo were treated with hypermethylation agent S-adenosylmethionine, resulting in T-lymphoma invasion and metastasis 1 promoter hypermethylation, accompanied by suppression of T-lymphoma invasion and metastasis 1 expression and inhibition of cell growth, plate colony formation, and migration. The present study demonstrates that overexpression of T-lymphoma invasion and metastasis 1 is associated with hypomethylation status of T-lymphoma invasion and metastasis 1 promoter region in colorectal cancer tissues. It suggests that promoter hypomethylation of T-lymphoma invasion and metastasis 1 may play a role in colorectal cancer development.

We disclose that there are no financial and personal relationships with other people or organizations that could inappropriately influence (bias) our work.

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1. Introduction

T-lymphoma invasion and metastasis 1 (TIAM1) is one of the guanine nucleotide exchange factors for Rac, a member of Rho GTPase family. TIAM1 activates Rac1 by catalyzing the exchange of inactive guanosine 5′-diphosphate–bound Rac for active GTP-bound Rac, which in turn activates downstream pathways that can lead to increased migration and invasion, critical properties in the development of the metastatic phenotype [1-4]. Recent studies have demonstrated that overexpression of TIAM1 is associated with a more malignant potential in human breast carcinoma, colon carcinoma, prostate carcinoma, and retinoblastoma [5-8]. Furthermore, increased expression of TIAM1 in prostate carcinomas and hepatocellular carcinomas predicts poor prognosis of patients [7,9]. In agreement with these studies, we have found that increased TIAM1 expression was highly related to the metastatic potential of human colorectal cancer in vitro and in mouse model in our previous studies [10-12].

Because aberrant up-regulation of TIAM1 is associated with cancer invasion and metastasis, it is therefore important to elucidate the mechanisms responsible for the increased TIAM1 expression during tumor progression. However, the molecular mechanisms underlying overexpression of TIAM1 in human cancers remain largely unknown. Up to now, only Engers et al [13] studied the mutation status of TIAM1 in renal cell carcinoma. In their study, a point mutation A441G was found in 2 of the 30 primary renal cell carcinoma tissues and 2 of the 5 renal cell carcinoma cell lines. Unfortunately, the expression level of TIAM1 was not detected in the primary renal cell carcinoma tissues, and thus, no relationship between TIAM1 mutation and expression could be found in their study. Because the low frequency of point mutation in their finding cannot fully explain abnormal TIAM1 expression in cancer, we believe that genetics is not the exclusive regulatory mechanism contributing to aberrant TIAM1 expression in human cancers.

Research indicates that aberrant gene expression is regulated by 2 main mechanisms, genetics and epigenetics. Genetic alterations, as the main regulatory mechanism in vivo, have been extensively explored, but epigenetic modifications have not attracted much attention until recently. Epigenetic alterations are believed to modulate gene expression without changes in the primary DNA sequence. Aberrant DNA methylation is a common epigenetic mechanism in gene regulation and frequently occurs in initiation and development of human neoplasia [14]. Inactivation of tumor suppressor genes by hypermethylation and activation of tumor progression factors by hypomethylation or demethylation are both frequent epigenetic events in the progression of human cancers.

Because TIAM1 has a CpG island at its promoter, we sought to identify an epigenetic mechanism that may regulate expression of this gene. In our preexperiment, we treated TIAM1-negative cell line HT29 with the demethylation agent 5-aza-2′-deoxycytidine (5-aza-dC) and found that HT29 cells obtained the ability to reexpress TIAM1 mRNA and protein. Thus, we hypothesized that CpG hypomethylation of TIAM1 promoter might be related to increased TIAM1 expression in colorectal cancer. To test this hypothesis, in the present study, the expression level of TIAM1 and the methylation status of TIAM1 promoter were analyzed in human colorectal cancer samples and matched normal colorectal tissues. In highly invasive human colon cancer cell lines LoVo and SW620, the methyl donor S-adenosylmethionine (SAM) was used to reverse the hypomethylation status of TIAM1 promoter, followed by detecting its effects on cell proliferation and migration.

2. Materials and methods

2.1. Cell line preparation

The human colon cancer cell lines HT29, LS174T, LoVo, and SW620 were obtained from America Type Culture Collection (Manassas, VA, USA). They were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 100 U/mL of penicillin/streptomycin. All the cultured cells were grown in a 5% CO₂-humidified atmosphere at 37°C.

2.2. Patients and tissue samples

A total of 116 patients with colorectal cancers who had undergone routine surgery at Nanfang Hospital (Guangzhou City, Guangdong Province, China) between 2006 and 2008 were enrolled in this study. They were 73 men and 43 women, with a mean age of 49 years (range, 20-92 years). They were not pretreated with radiotherapy or chemotherapy before surgery. A total of 232 samples of colorectal cancer tissue and paired distant normal colorectal mucosa were collected, with each pair taken from the same patient. All the samples were fixed in 10% formalin, embedded in paraffin, sectioned consecutively at 4 μm, and stained by hematoxylin and eosin. The histologic types were assigned according to the criteria of the World Health Organization classification system by 3 pathologists independently in a double-blinded manner. The clinicopathologic parameters of the patients in this study were obtained by a medical history review. Written consent forms were signed by all the well-informed patients according to the ethical protocols of Nanfang Hospital, Southern Medical University.
2.3. Immunohistochemical detection

Immunohistochemical (IHC) was performed with the ultrasensitive streptavidin-peroxidase technique according to the manufacturer’s protocol. Briefly, representative 4-μm sections of the paraffin-embedded tissue specimens were incubated in 3% hydrogen peroxide for 10 minutes at room temperature, then in a 1:10 dilution of fetal bovine serum (Genetime, Guangzhou, China) for 30 minutes at room temperature. After incubation at 4°C overnight with a 1:400 dilution of rabbit antihuman TIAM1 polyclonal antibody (C16, Santa Cruz Biotechnology, Santa Cruz, CA), the sections were treated with horseradish peroxidase (HRP)-labeled antirabbit IgG (1:200 dilution; DAKO, Glostrup, Denmark) at room temperature for 30 minutes and developed with 3-′-diaminobenzidine–hydrogen peroxide (Maixin, Fuzhou, China) and counterstained with Mayer hematoxylin. As a negative control, representative sections were subjected to the same immunostaining procedure, except that the primary antibody was replaced by phosphate-buffered saline (PBS). When IHC signals were present, the cytoplasm was stained brown. Immunohistochemically stained tissue sections were scored separately by 2 pathologists blinded to the clinical parameters. The scoring approach to assessing TIAM1 immunostaining accorded with the protocol of Masunaga et al [15] and Soumaoro et al [16]. Tumors with a final staining score of 3 or higher were considered as positive.

2.4. Detection of methylation status in TIAM1 CpG island

2.4.1. Prediction of CpG islands

CpG islands in TIAM1 gene sequence were predicted using software on the following 2 Web sites: http://www.ebi.ac.uk/emboss/cpgplot/#andNewCpGSeek and http://www.urogene.org/methprimer/index1.html. A CpG island was defined as a DNA fragment with a length of at least 200 base pairs (bp), a GC content of more than 50 %, and a ratio of more than 0.6 between the observed and expected CpGs.

2.4.2. Nucleic acids extraction and sodium bisulfite modification of genomic DNA

DNA was extracted by a standard phenol-chloroform method. DNA from all tissue samples was also digested by protease K and extracted by phenol-chloroform. Sodium bisulfite modification was carried out by CpGenome DNA Modification Kit (Chemicon, Temecula, CA) to convert the unmethylated cytosines to uridines and leave the methylated cytosines unchanged. The modified DNA samples were finally stored at −20°C for further use.

2.4.3. Methylation-specific polymerase chain reaction

The methylation status of the 5′ CpG island of TIAM1 in tissue samples was determined by methylation-specific polymerase chain reaction (MSP) as described previously [17]. The sequences of all primers were as follows: MF: 5′-GCGAGATGATAGGTAGTTTCGC-3′, MR: 5′-ACGCACGCAGAAATATCCCGG-3′, with the expected product size 246 bp long; UF: 5′-TATTGTGAGTAGTAGG TAGTTTTGT-3′, UR: 5′-TATAAACACACACACAAATA TATCCACA-3′, with the expected product size 254 bp long. The annealing temperature was 60°C for methylation-specific PCR and 56°C for unmethylation-specific PCR. Methylated control DNA (CpGenome DNA Modification Kit; Chemicon) was used as a positive control, whereas DNA isolated from human placenta served as a nonmethylated control. Negative control samples without DNA template were incubated for cross-contamination assessment for each set of PCR. All MSP assays were repeated at least twice to validate the results. The PCR products were electrophoresed on 2% agarose gel with ethidium bromide, visualized, and purified under ultraviolet light. When a sample was amplified only by M-primers or U-primers, it was regarded as full methylation or full unmethylated. When a sample can be amplified by both M-primers and U-primers, it was regarded as partial methylation [18].

2.5. Demethylation with 5-aza-dC and hypermethylation with SAM

2.5.1. Cell lines and cell culturing

HT29 and LS174T were used in 5-aza-dC treatment assay. LoVo and SW620 were used in SAM treatment assay. S-adenosylhomocysteine (SAH), as unmethylated analogue of SAM, was also used. Exponentially growing cells were cultured in the RPMI-1640 medium containing 2 μmol/L 5-aza-dC, or 250 μmol/L SAM, or 250 μmol/L SAH for 6 days. The medium was changed the other day with the same concentration of 5-aza-dC. Cells were harvested and washed with PBS before nucleic acid extraction or fixation in 4% paraformaldehyde for immunocytochemistry analysis. PBS was used in the control group.

2.5.2. Nucleic acids extraction

Cells were harvested and washed with PBS before adding lysis buffer. After addition of protease K (25 mg/mL), the lysed cells were incubated at 37°C overnight. DNA was extracted by a standard phenol-chloroform method. DNA from all tissue samples was also extracted by phenol-chloroform after digestion by protease K. Total RNA was prepared using the Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions.

2.5.3. Semiquantitative reverse transcription PCR

Three micrograms of total RNA was reverse transcribed by M-MuLV reverse transcriptase (MBI, Vilnius, Lithuania) with Oligo(dT)16 as a primer. Semiquantitative reverse transcription PCR (RT-PCR) was performed using a sense primer (5′-AAGACGTACTCGGGCATGTTCC-3′) and an antisense primer (5′-GACCCAAATGTCGAGTCGAGTC-3′) specific to TIAM1, with its annealing temperature at 58°C. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was
amplified as an internal control using sense primer (5′-AATCCCATCACCATTTCCA-3′) and antisense primer (5′-CCTGCTTACACCACCTTCTTTG-3′). The expected product was 252 bp for TIA1 and 380 bp for GAPDH. PCR products were analyzed by 2% agarose gel electrophoresis. Signal ratio was assessed by Kodak Digital Science 1D Image Analysis Software (Eastman Kodak, Rochester, NY, USA).

2.5.4. Immunocytochemistry

Immunocytochemistry staining was performed on cellular smears. Before adding primary antibody, cellular smears were fixed by 4% paraformaldehyde/PBS for 10 minutes and pretreated with 0.5% Triton X-100 for 15 minutes. The following steps were the same as described in the above IHC detection.

2.5.5. Western blotting analysis

Cells were washed twice with cold PBS and lysed on ice in Radio immunoprecipitation assay (RIPA) lysis buffer (1× PBS, 1% BP40, 0.1% sodium dodecyl sulfate, 5 mmol/L EDTA, 0.5% sodium deoxycholate, and 1 mmol/L sodium orthovanadate) with protease inhibitors. The protein lysates were resolved on 6% sodium dodecyl sulfate polyacrylamide gel, electrotransferred to polyvinylidene fluoride membranes (Immobilon P; Millipore, Bedford, MA), and blocked in 5% nonfat dry milk in Tris-buffered saline, pH 7.5 (100 mmol/L NaCl, 50 mmol/L Tris, and 0.1% Tween-20). Membranes were immunoblotted with anti-TIAM1 polyclonal antibody (Santa Cruz Biotechnology) and anti-GAPDH antibody (Santa Cruz Biotechnology) at 4°C overnight. Then they were added respectively with the horseradish peroxidase–conjugated secondary antibodies. Signals were detected by enhanced chemiluminescence (Pierce, Rockford, IL).

2.5.6. Sodium bisulfite modification of genomic DNA and methylation detection

Genomic DNA was modified by sodium bisulfite, and methylation status of TIA1 gene in the 5-aza-dC–treated cell lines was detected by MSP as described above.

2.6. Effects of SAM on cell growth and migration

2.6.1. In vitro cell growth assay

LoVo and SW620 cells (1 × 10^3) were grown in 96-well plates and treated with 250 μmol/L SAM and SAH for 0 to 6 days. On each day, 20 μL of 5 g/L 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, St Louis, MO, USA) was added to each well and incubated at 37°C for 4 hours in a humidified incubator with 5% CO2. Then MTT was removed, and 150 μL dimethyl sulfoxide was added to each well. The plate was shaken on the microplate shaker, and the absorbance of the wells was measured at 570 nm with a Microplate Autoreader (Bio-Rad, Hercules, CA, USA). The experiment was repeated thrice.

2.6.2. Plate colony formation assay

About 1 × 10^2 cells were added to each well (3 cm in diameter) of a 6-well culture plate, and each cell group contained 3 wells. After incubation at 37°C for 2 weeks, the cells were washed twice with PBS and stained with Giemsa solution. The number of colonies containing 50 cells or more was counted under a microscope (plate colony formation efficiency = [number of colonies/number of cells inoculated] × 100).

2.6.3. Boyden chamber migration assay

This assay was performed using the method described previously [19]. SAM- and SAH-treated LoVo and SW620 were included. For quantification, the cells were counted under a microscope in 5 randomly selected fields (original magnification, × 200).

2.7. Statistical analyses

All statistical analyses were performed by SPSS13.0 for Windows (SPSS Inc, Chicago, IL). A Wilcoxon test was used to determine the differences between colorectal cancer and matched normal tissue in expression and methylation status of TIA1, respectively. A Spearman correlation coefficient (r) was calculated to assess the significance of association between methylation status and expression level of TIA1. A Mann–Whitney test was used to analyze the correlation between TIA1 expression level and clinicopathologic parameters, and a χ^2 test was used to determine the correlation between methylation status and clinicopathologic parameters. One-way analysis of variance was applied for analyzing cell proliferation, plate colony formation, and migration between different cell groups. The threshold value was established at .05 (2-tailed).

3. Results

3.1. Overexpression of TIA1 in colorectal cancers and metastasis

Expression levels of TIA1 protein were significantly higher in colorectal cancers than in matched normal mucosa as assessed by IHC (Fig. 1A and B). Of the 116 samples of colorectal cancers, 22 (19.0%) were TIA1 negative; 35 (30.1%), faintly stained; and 59 (50.9%), intermediately or strongly stained. By contrast, of the 116 samples of normal mucosa, TIA1 was undetectable in 47 (40.5%), faintly expressed in 45 (38.8%), and intermediately or strongly in only 24 (20.7%). The expression differences between colorectal cancer tissue and normal mucosa were significant (Wilcoxon test: Z = −4.608, P < .001). The expression of TIA1 protein was stronger in cancer tissues with metastasis than without metastasis (Mann–Whitney test: Z = −2.584, P = .01). Similarly, expression of TIA1 protein was higher in the lymph node metastasis than in the matched colorectal
cancer mucosa (Wilcoxon test: $Z = -3.115$, $P = .002$; Table 1). Thus, TIAM1 expression was correlated to tumor metastasis. Furthermore, we also found that TIAM1 expression was positively related to the differentiation status of colorectal cancer (Mann-Whitney test: $Z = -2.673$, $P = .008$). However, no statistical relationships were found between TIAM1 expression and other clinicopathologic parameters (Mann-Whitney test: $P > .05$; Table 2).

### 3.2. Promoter hypomethylation associated with overexpression of TIAM1 in colorectal cancers

One CpG island, containing 256 CpG sites, was identified in the 5′-flanking of TIAM1 gene, covering the region −781 to +1401, relative to the transcription start site. The methylation status of the CpG sites was examined by the MSP method in all the 232 samples and confirmed by DNA

<table>
<thead>
<tr>
<th>Cases</th>
<th>TIAM1 expression intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Colorectal normal mucosa</td>
<td>116†</td>
</tr>
<tr>
<td>Colorectal cancer</td>
<td>116†</td>
</tr>
<tr>
<td>Colorectal cancer with metastasis</td>
<td>49†</td>
</tr>
<tr>
<td>Lymphatic metastasis</td>
<td>49†</td>
</tr>
</tbody>
</table>

NOTE. Wilcoxon test: †$Z = -4.608$, $P < .001$; †$Z = -3.115$, $P = .002$. 

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**Table 1**  TIAM1 expression in colorectal normal mucosa, colorectal cancer, and lymphatic metastasis

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**Fig. 1**  Representational protein expression and methylation status of TIAM1 in colorectal cancers. A, Representational protein expression of TIAM1 in colorectal cancers as detected by IHC. TIAM1 was highly expressed in colorectal cancer, except in signet-ring cell carcinoma. B, The expression of TIAM1 protein is higher in colorectal cancer samples than in matched normal mucosa. Wilcoxon test, $P < .001$. C, Representational methylation status of TIAM1 promoter in the same colorectal cancers as shown in panel A. The methylation status was inversely related with TIAM1 expression. D, Representational DNA sequencing of methylation-specific PCR results. Abbreviations: N, normal colorectal mucosa; T, colorectal cancer tissue; Ctl, blank control of PCR system; M, methylated products; U, unmethylated products; MP, positive control of methylation; UP, positive control of unmethylation; O, the original sequence. The numbers represent different cases.
sequencing analysis (Fig. 1C and D). Of the 116 samples of cancer tissue, none was fully methylated, 44 (37.9%) were partially methylated, and the other 72 (62.1%) were unmethylated. Of the 116 matched samples of normal tissue, 7 (6%) were fully methylated, 43 (37.1%) were partially methylated, and 66 (56.9%) were unmethylated. A lower incidence of TIAM1 promoter methylation was revealed in colorectal cancer tissues than in matched normal tissues (Wilcoxon test: Z = −2.244, P = .025).

The expression level was negatively correlated to the methylation status in the 116 samples of colorectal cancer (r = −0.377, P < .001) just as in the total samples (r = −0.494, P < .001; Table 3). Interestingly, of the 49 cases of colorectal cancer with lymph node metastasis, we found that TIAM1 was strongly expressed but unmethylated in 25, whereas it was faintly expressed but hypermethylated in 15 cases. The expression of TIAM1 was significantly correlated to the methylation status of TIAM1 promoter in the colorectal cancer with metastasis (r = −0.653, P < .001; Table 4).

In addition, the methylation level of TIAM1 was negatively associated with the differentiation status of colorectal cancer (χ² test: P < .001) and with patients’ age (χ² test: P = .028). Unexpectedly, the relationship between methylation status of TIAM1 and tumor metastasis was not statistically significant, although the methylation status seemed to have a tendency of association with cancer metastasis (χ² test: P = .087). No correlations were found between the methylation status of TIAM1 and other clinicopathologic parameters in the 116 colorectal cancers (χ² test: P > .05; Table 5).

### 3.3. Reexpression and promoter demethylation of TIAM1 induced by 5-aza-dC treatment in colon cancer cell lines

TIAM1 was reexpressed in colon cancer cell lines HT29 and LS174T after treatment with 5-aza-dC as detected by RT-PCR, immunostaining, and Western blot (Fig. 2B-D). As expected, the methylation status of TIAM1 promoter was altered from full methylation to partial methylation in cell line HT29, whereas it altered from partial methylation to unmethylation in cell line LS174T (Fig. 2A).

### 3.4. Effects of SAM on TIAM1 promoter methylation, expression, and migration capacity of colon cancer cell lines

TIAM1 was overexpressed in metastatic colon cancer cell lines LoVo and SW620. These 2 cell lines were then used in SAM and its unmethylated analogue SAH treatment assay. Both the mRNA and protein expression of TIAM1 were markedly suppressed in cell lines after treatment with SAM as detected by RT-PCR, immunostaining, and Western blot (Fig. 3B-D). Furthermore, SAM induced hypermethylation of TIAM1 promoter in both cell lines as detected by MSP. TIAM1 promoter was changed from unmethylation to partial methylation in both cell lines (Fig. 3A). However, the similar effects were not observed with SAH (Fig. 3A-D).

### Table 2 Relationship between TIAM1 expression and the clinical pathologic features

<table>
<thead>
<tr>
<th>Cases</th>
<th>Expression intensity</th>
<th>Z</th>
<th>P</th>
</tr>
</thead>
<tbody>
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<td>Age y</td>
<td>&lt;60 y</td>
<td>18</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>≥60 y</td>
<td>4</td>
<td>12</td>
</tr>
<tr>
<td>Sex</td>
<td>Male</td>
<td>14</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>8</td>
<td>18</td>
</tr>
<tr>
<td>Location</td>
<td>Colon</td>
<td>10</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>Rectum</td>
<td>12</td>
<td>29</td>
</tr>
<tr>
<td>Differentiation</td>
<td>High-middle age</td>
<td>12</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>Metastasis</td>
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<td>22</td>
</tr>
<tr>
<td></td>
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<td>9</td>
<td>24</td>
</tr>
</tbody>
</table>

NOTE: Mann-Whitney test.

### Table 3 Relationship between TIAM1 promoter methylation and its expression in total 232 samples

<table>
<thead>
<tr>
<th>Cases</th>
<th>Expression intensity</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unmethylation</td>
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<td>55</td>
</tr>
<tr>
<td>Partial methylation</td>
<td>87</td>
<td>53</td>
</tr>
<tr>
<td>Full methylation</td>
<td>7</td>
<td>0</td>
</tr>
</tbody>
</table>

NOTE: r = −0.494.

### Table 4 Relationship between TIAM1 promoter methylation and its expression in 49 colorectal cancer samples with metastasis

<table>
<thead>
<tr>
<th>Cases</th>
<th>Expression intensity</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Low (+/-)</td>
<td>26</td>
<td>15</td>
</tr>
<tr>
<td>High (++/+++)</td>
<td>23</td>
<td>8</td>
</tr>
</tbody>
</table>

NOTE: r = −0.625.
To determine whether SAM and SAH treatments have any effects on cell growth and invasive, MTT assay, plate colony formation assay, and Boyden chamber migration assay were performed. The results showed that SAM treatment inhibited the proliferation capacity, plate colony formation efficiency, and migration capacity of metastatic cases. The data are summarized in Table 5.

### Table 5: Relationship between TIAM1 methylation status and the clinical pathologic features

<table>
<thead>
<tr>
<th>Cases</th>
<th>Methylation status</th>
<th>( \chi^2 )</th>
<th>( P )</th>
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<tbody>
<tr>
<td>Age</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>&lt;60 y</td>
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<td>45</td>
<td>36</td>
</tr>
<tr>
<td>≥60 y</td>
<td>35</td>
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<td>8</td>
</tr>
<tr>
<td>Sex</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>73</td>
<td>44</td>
<td>29</td>
</tr>
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<td>Female</td>
<td>43</td>
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<td>15</td>
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<tr>
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<td>Colon</td>
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<td>Differentiation</td>
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<tr>
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<td>49</td>
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</table>

**NOTE.** \( \chi^2 \) test.

Fig. 2  Reexpression and demethylation of TIAM1 in 5-aza-dC–treated colon cancer cell lines HT29 and LS174T. A, Results of MSP in colon cancer cell lines after treatment with 5-aza-dC. TIAM1 promoter was changed from full methylation to partial methylation in HT29, it was changed from partial methylation to unmethylation in LS174T after 5-aza-dC treatment. B, Results of RT-PCR of TIAM1 and GAPDH in colon cancer cell line after treatment with 5-aza-dC. The expression of TIAM1 mRNA was restored in HT29 and LS174T after 5-aza-dC treatment. C and D, Results of Western blot and immunostaining of TIAM1 in colon cancer cell line after treatment with 5-aza-dC. Expression of TIAM1 protein was restored in HT29 and LS174T after 5-aza-dC treatment. Abbreviations: Ctl, blank control of PCR system; MP, positive control of methylation; UP, positive control of unmethylation; M, methylated products; U, unmethylated products; -, without 5-aza-dC treatment; +, with 5-aza-dC treatment.
LoVo and SW620 cells in parallel to the silencing of TIAM1 (Fig. 4A-D). However, SAH treatment has no such effects (Fig. 4A-D).

4. Discussion

In this study, we found increased expression of TIAM1 in colorectal cancer, consistent with our previous studies [10-12]. It is more important that such overexpression was found to be associated with hypomethylation of TIAM1 promoter. Besides, reexpression and hypomethylation of TIAM1 were induced by demethylation agent 5-aza-dC in TIAM1-negative colon cancer cell lines. The findings suggest that hypomethylation of TIAM1 promoter may be one of the regulatory mechanisms underlying overexpression of TIAM1 in colorectal cancer.

The expression level of TIAM1 has been widely studied in human cancers. Most previous reports indicated that up-regulation of TIAM1 was associated with malignant behavior in a variety of tumor types [2-12], suggesting that TIAM1 was identified as an invasion-promoting gene with oncogenic effect. In the current study, our similar finding of overexpression of TIAM1 in a large number of paired colorectal cancer samples confirms an oncogenic or metastasis-promoting function of TIAM1 in colorectal cancer. Contrary to the above finding, loss or decrease of TIAM1 expression was observed in renal cell cancers and breast cancers to be associated with poor prognosis and tumor progression [13,20]. Overexpression of TIAM1 was shown in metastatic melanoma to promote tumor cell proliferation but inhibit cell motility and invasion, although why TIAM1 affected the metastatic but not the nonmetastatic cells was unclear [21]. It is hard for us to elucidate this contradictory and complex behavior of TIAM1 in human cancers. It is possible that the oncogenic function of TIAM1 could be specific to different tissues or human individuals, or this might be because of a cause or causes totally beyond current knowledge. Anyhow, at present, further research should be carried on in much more human tissues, in all types of cancer, and in a larger number of tissue samples.

Despite the comprehensive studies of TIAM1 expression, the regulatory mechanisms underlying aberrant TIAM1 expression in cancers remain largely unclear. It is widely accepted that aberrant methylation of promoter CpG islands is a kind of regulatory mechanism of silencing tumor suppressors or activating oncogenes. Little attention, however, has been paid to the effect of aberrant methylation on TIAM1 expression levels. In investigating whether
methylation of \textit{TIAM1} promoter might participate in modulating \textit{TIAM1} expression in colorectal cancer, the present study observed a lower level of methylation status of \textit{TIAM1} promoter in colorectal cancer tissues when compared with the matched normal mucosa. It is noticeable that there was a correlation between promoter hypomethylation and \textit{TIAM1} overexpression, implying that promoter demethylation up-regulated \textit{TIAM1} expression in colorectal cancer. This finding was further validated by our subsequent in vitro test in cell lines HT29 and LS174T. All these confirm our initial speculation that methylation may play a role in modulating \textit{TIAM1} transcription in colorectal cancer. To our knowledge, this is the first attempt to explore a possible mechanism leading to the abnormal \textit{TIAM1} expression levels in colorectal cancer, though still leaving much to investigate.

CpG islands hypermethylation, a cause of transcriptional silencing, has been considered as the predominant mechanism leading to inactivation of genes in sporadic colorectal cancer in the past decades. A great number of groups have been engaged in the research of the association between hypermethylation of promoter CpG islands and the expressions of tumor suppressor genes, cell cycle regulatory genes, and DNA mismatch repair genes in carcinogenesis [22]. On the contrary, much fewer studies have addressed the role of abnormal demethylation or hypomethylation of promoter and exon 1 CpG islands in cancers, though it was indicated that demethylation of CpG islands might restore or activate gene expression, oncogene expression in particular [23]. Recent research confirmed that overexpression of many genes was induced by hypermethylation of CpG islands in a variety of cancers, for instances, \textit{Bcl-2} gene exon 1 in B-cell chronic lymphocytic leukemia, \textit{MAGE-1} gene 5′-region in melanomas, \textit{S100A4} gene intron 1 in pancreatic carcinoma, \textit{SNCG/BCSG1} gene exon 1 in breast cancer and ovarian cancer, \textit{Maspin} gene promoter in gastric cancer, and \textit{uPA} gene promoter in breast cancer [24-29]. All these genes were reactivated by hypomethylation in cancers, and most of them were related to cancer metastasis. In agreement with these studies, our present data verify the important role of DNA hypomethylation in progression and aggression of colorectal cancer. We support the notion that aberrant hypomethylation or demethylation, accompanied by increased expression of affected genes, is also a frequent epigenetic event in human cancers.

In the present study, SAM treatment assay reversed the hypomethylation status of \textit{TIAM1} promoter in highly invasive LoVo and SW620 cells, resulting in the reduction of \textit{TIAM1}
expression both at mRNA and protein levels. The reversal of hypomethylation is associated with suppression of the proliferation and migration capacity of these cells. In contrast, SAH, the inactive analogue of SAM, showed no similar effects on TIAM1 expression and cell migration. These data suggested that the hypomethylation of TIAM1 in metastatic LoVo and SW620 cells is responsible for its activation. Further experiments are required to fully elucidate the mechanisms how SAM affects the state of TIAM1 methylation, its expression, and tumor cell migration. Because SAM is a nonspecific hypermethylation agent, further studies are required to seek for other genes that silenced by SAM but therefore responsible for inhibition of metastasis. Our study implied that inhibition of DNA hypomethylation at late stage of carcinoma might provide a therapeutic way for suppressing cancer migration and metastasis.

As observed by other researchers and us, DNA hypomethylation or demethylation activates tumor-promoting genes and metastasis-promoting genes. This can be of value to clinicians. So far, hypermethylation has been an anticancer target in clinical therapy, because it is believed to be closely related to carcinogenesis. 5-aza-dC, one of DNA methylation inhibitors, has been used in several clinical trials, especially for myelodysplastic syndrome and acute myeloid leukemia [30]. However, because 5-aza-dC is a nonspecific demethylation agent, it can lead to hypomethylation of genes uniformly, involving tumor suppressor genes and oncogenes. Consequently, tumor-promoting genes and metastasis-promoting genes can be activated, most likely resulting in cancer progression and metastasis [31-33]. Therefore, we think that it should be prudent and cautious when and how to apply demethylation agents in clinical therapy. Possible adverse effects of undue demethylation in clinic therapy should be well aware of. It is worthy using demethylation agents at the early stage of carcinogenesis when they mainly activate tumor suppressor genes, whereas at advanced stages of cancer, inappropriate administration of demethylation agents might lead to activation of metastasis-promoting genes. In short, it is a challenging task how to balance the advantages and disadvantages of demethylation agents.

However, we also observed several exceptional cases regarding the association between TIAM1 expressions with methylation status. Overexpression of TIAM1 was found to be accompanied by hypermethylation, but little or undetectable expression by hypomethylation or unmethylation. This might be attributed to other factors regulating TIAM1 expression, such as mutation, absence of necessary transcription factors, and others. The inevitable contamination of interstitial cells might be a likely cause. It is puzzling that we failed to find a statistical relationship between TIAM1 methylation and cancer metastasis, although the methylation status of TIAM1 was significantly related to its expression in colorectal cancer accompanied by metastasis. We speculate that this may contribute to the limited amount of metastasis tissues or to the fact that we did not detect the methylation status in samples of lymph node metastasis. In addition, this may be related to the general MSP method, which, as a qualitative but not a quantitative technique, cannot distinguish between various percentages of methylation.

In conclusion, our study clarifies one of the mechanisms involved in overexpression of TIAM1 in colorectal cancer. Increased TIAM1 expression is associated with hypomethylation of TIAM1 promoter in colorectal cancer. It can be inferred that TIAM1 hypomethylation may have an effect on the development and metastasis of colorectal cancer. We hope that this first study on increased TIAM1 expression and hypomethylation of TIAM1 promoter in colorectal cancer will attract more attention addressing the association between hypomethylation and cancer progression and metastasis, as well as its potential significance for clinic therapy.

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References