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Original Article

MicroRNA-21 promotes cell proliferation by targeting tumor suppressor TET1 in colorectal cancer

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Abstract: MicroRNAs (miRNAs) regulate gene expression by binding to mRNA, and can function as oncogenes or tumor suppressors depending on the target. TET1 acts as tumor-suppressor, which is downregulated in colorectal cancers (CRC) and inhibits cell growth. However, it has not been studied as to whether miRNAs, suppressing target expression by binding to the 3'UTR, regulate TET1 expression in colorectal cancers. Here, our study found that miR-21 has matching sites on TET1. In the tumor tissue samples from 50 patients with CRC, the expression of miR-21 was upregulated compared with that in adjacent tissue samples while the expression of TET1 showed a significant decrease. In addition, miR-21 expression was negatively correlated with the expression of TET1. Moreover, low expression of miR-21 by the transfection of colorectal cancer cell lines with miR-21 inhibitors, the effect on TET1 expression was opposite to the change of miR-21 expression. Furthermore, our results indicated that miR-21 promoted proliferation of colorectal cancer cells by targeting TET1. These findings may provide a theoretical basis for clarifying the physiological and pathological role of miR-21 in colorectal cancer.

Keywords: miR-21, TET1, proliferation, colorectal cancer

Introduction

Colorectal cancer (CRC) remains an important global health problem and may lead to more than 1.2 million new cancer cases and over 600000 cancer-related deaths in 2008 worldwide [1]. So far, the prognosis of CRC patients has improved significantly due to advances in surgical techniques and the integration of radiotherapy and chemotherapy and targeted therapy [2, 3]. However, in advanced CRC patients and those with local recurrence or distant metastasis, the prognosis is still very poor [4, 5]. Therefore, it is necessary to investigate the molecular mechanisms and key regulatory factors of CRC progression and metastasis to provide biomarkers to predict the risk of local recurrence and distant metastasis [6], so that we can best choose treatment strategies and ultimately improve patient prognosis.

Ten eleven translocation enzymes (TET1, TET2 and TET3) are a family of dioxygenase, which convert 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC) and lead to CpG islands demethylation [7-9]. The deficiency or mutation of TET proteins and subsequent down-regulation of 5-hmC content is common in various kinds of human cancers. Indeed, TET1 is often absent in breast, hepatic, pancreatic and prostate cancer [10-13]. Moreover, it has been reported that TET1 was down-regulated in colon tumors from the initial stage and down-regulation of TET1 during colon cancer initiation led to repression of the promoters of WNT pathway inhibitors and resulted in a constitutive activation of the WNT pathway [14]. However, further mechanisms leading to downregulation of this important tumor suppressor in CRC need to be elucidated.

MicroRNA (miRNA), an endogenous non-coding single-stranded small RNA, exists extensively in the biological genome. It is able to pair with and bind to 3' untranslated regions (3'UTRs) of the targeted mRNA incompletely to degrade mRNA or repress the translation of mRNA, and is involved in regulating more than half of all gene

expression [15]. In addition, miRNA is an important post-transcriptional regulatory factor and plays an extensive and important role in cell proliferation, differentiation, apoptosis, tissues development, oncogenesis and other physiological processes [16]. Given the previous study that TET1 was involved in inhibiting CRC cells growth, we hypothesized that TET1 is regulated by miRNA at the post-transcriptional level. In our study, we found that the expression of miR-21 was upregulated in the CRC patient's tissue samples compared with CRC patient's adjacent-tissue samples, while the expression of TET1 showed a significant decrease. Furthermore, our findings demonstrated that miR-21 promoted proliferation of colorectal cancer cells by targeting the TET1.

Materials and methods

Patient tissues and cell culture

Tissue specimens (tumor, adjacent samples) of 50 patients with colorectal cancer were collected after informed consent and verification by a pathologist, and immediately frozen in liquid nitrogen. Fresh-frozen and/or formalin-fixed paraffin embedded samples were used for miR-21 and TET1 expression analysis. Human colorectal cancer cell lines HCT15 and HT29 were cultured in DMEM medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS (Invitrogen, Carlsbad, CA, USA) and antibiotics. All the cells were incubated in a humidified atmosphere of 5% CO_2 in air at 37°C. These cell lines were obtained from the American Type Culture Collection (Shanghai, China).

Transient transfection

The cells were cultured in a 6-well plate for 24 h and then were transfected with hsa-miR-21 mimics (miR-21), NC (miR-control), hsa-miR-21 inhibitors (anti-miR-21), inhibitor NC (anti-miR-control), which were purchased from GenePharma (Shanghai, PR China). All transfections were performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Transfection efficiency was monitored by qRT-PCR.

Plasmid construction and cell transduction

3'-UTR sequence of TET1 which was predicted to interact with miR-21 or a mutant sequence with the predicted target sites was inserted into pmirGLO vector (Promega, USA). They were named pmirGLO-TET1-wt and pmirGLO-TET1-mut. TET1 siRNA oligonucleotides and a non-specific scrambled control (si-Ctrl) were synthesized by RiboBio (Guangzhou, China). The cells were plated onto 6-well plates and were transfected with 100 ng of pmirGLO-TET1-wt or pmirGLO-TET1-mut, and miR-21 mimics (miR-21, 50 nM) by using Lipofectamine 2000 (Invitrogen Corp, CA, USA).

Luciferase reporter gene assays

HCT15 and HT29 Cells were plated into 24-well plates (3×10^4 cells/well) and co-transfected with the hsa-miR-21 mimics (miR-21), NC (miR-control) and various constructs containing the seed sequence or mutant seed sequence of TET1 3'UTR. Twenty-four hours after transfection, firefly and Renilla luciferase activities were measured using a Luc-Pair™ miR Luciferase Assay Kit (GeneCopoeia, Maryland, USA). All experiments were performed at least three times.

Isolation of total RNA and quantitative RT-PCR

Total RNA was extracted from human clinical CRC tissues (or Human colorectal cancer cell lines HCT15 and HT29) using the TRIzol method (Invitrogen, Shanghai, China) and Reverse transcription was performed using poly (A)-tailed total RNA and reverse transcription primer using reverse transcriptase kit (TAKARA, Japan) according to the manufacturers' protocol. RT-PCR analysis was performed to detect the expression of TET1 mRNA in the cells treated with miR-21 or anti-miR-21. The qRT-PCR analysis was explored to test the expression of miR-21 and TET1 in human clinical CRC tissues using Fast Start Universal SYBR Green Master (Roche Diagnostics GmbH Mannheim, Germany). The expression of specific genes or miRNAs was tested by the comparative Ct method using $2^{-\Delta\Delta Ct}$. The primers used were as follows: TET1 forward, 5'-GATGACAGAGGTTCTTGCACAT-3', reverse, 5'-AGGTTGCACGGTCTCAGTGT-3'; GAPDH forward, 5'-CATCACCATCTTCCAGGAGCG-3', reverse, 5'-TGACCTTGCCCACAGCCTTG-3'.

Western blot

Total protein was extracted cultured cells using the RIPA lysis buffer, and protein concentration was measured by the BCA assay. The proteins were separated according to size by electrophoresis, transferred to PVDF membrane, and incubated with the following antibodies: TET1

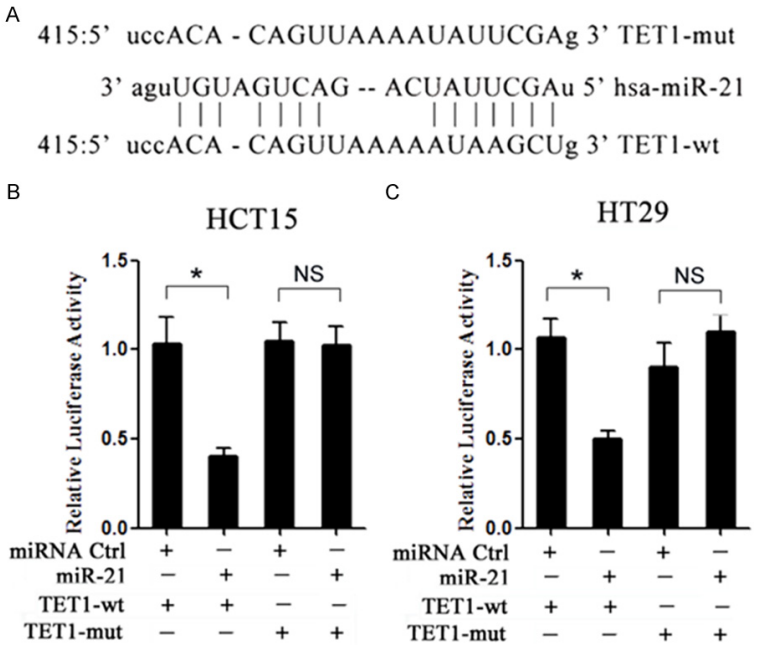

Figure 1. MiR-21 directly inhibited the expression of TET1 through targeting its 3'UTR. A. The binding site of miR-21 in 3'UTR of TET1 mRNA is shown in a model. Mutant was generated at the TET1 3'UTR as indicated. A TET1 3'UTR fragment containing wild type or mutant (wt or mut) of the miR-21-binding sequence was cloned into the downstream of the pmirGLO-control luciferase reporter gene vector. B, C. The effect of miR-21 on reporters of pmirGLO-TET1-wt and pmirGLO-TET1-mut in HCT15 and HT29 cells was measured by luciferase reporter gene assays, respectively. Statistically significant differences are indicated: $^{*}P < 0.05$; NS, no difference; Student's t test. The experiment was repeated at least three times.

antibody, rabbit polyclonal (Santa Cruz, USA); β-Actin: mouse polyclonal (CST technology, USA); All results were visualized through a chemiluminescent detection system (Pierce ECL, Thermo, USA) and then exposed in Molecular Imager ChemiDoc XRS System (Bio-Rad, USA). The integrated density of the band was quantified by Image software.

CCK-8 assay

Cell proliferation was performed with Cell Counting Kit-8 (CCK-8) (Dojindo, Tokyo, Japan). According to the instructions, Cell Counting Kit-8 reagent was added at 0, 24, 48, and 72 h respectively after seeding 4×10^3 cells per well in into 96-well plates and transfected with miR-21 inhibitor or control, and incubated at 37°C for 2 h. The OD (optical density) 450 nm value was detected by using a microplate reader (Bio-Rad, CA, USA).

EdU assay

Cells (2×10^3 cells/well) were seeded in triplicate in 96-well plates and incubated overnight. Cells were starved in DMEM without FBS for 24 hours and then incubated in DMEM containing 5% FBS. Then the EdU (5'-ethynyl-2'-de-oxyuridine) incorporation assay was performed to quantify cell proliferation using the Cell-Light™ EdU DNA Cell Proliferation Kit (Guangzhou Ribobio Co., Ltd, Guangzhou, China) according to the manufacturer's instructions. More than five random fields per well were captured, and IPP 6.0 was used to calculate the percentage of EdU-positive cells in total cells.

Statistical analyses

Data were analyzed by SPSS 11.0 and presented as mean ± SD from at least three separate experiments. The expression levels of miR-21 and TET1 in the tissues were evaluated using chi-square tests. Differences were considered statistically significant with $P < 0.05$.

Results

TET1 3'-UTR is a target for miR-21

Asangani et al. has reported that miR-21 downregulates PDCD4 protein and upregulates tumor cell invasion in cultured colon cancer cells [17], to better explore the role of miR-21 in the development of CRC, we used bioinformatics method to predict the potential targeting genes of miR-21. As the results shown in microRNA.org (http://www.targetscan.org/ and http://www.microrna.org), TET1 was the candidate gene that we picked up. We identified the binding site of miR-21 in the 3'UTR of TET1 mRNA (**Figure 1A**) and cloned the 3'UTR of TET1 mRNA and its mutants into downstream of pmirGLO-control luciferase reporter gene vector (named pmirGLO-TET1-wt or pmirGLO-TET1-mut), respectively. The luciferase reporter gene assays demonstrated that miR-21 significantly suppressed the firefly luciferase activities of pmirGLO-TET1-wt in a dose-dependent manner, whereas it failed to work when the target site was mutated in HCT15 and HT29 cells

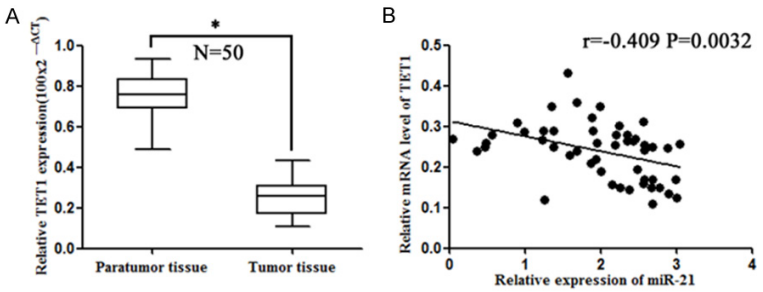

Figure 2. MiR-21 was negatively associated with TET1 in clinical CRC tissues. A. TET1 mRNA levels was examined by qRT-PCR in 50 cases of clinical CRC tissues and paired adjacent tissues. B. Correlation of MiR-21 levels with TET1 mRNA levels was examined by qRT-PCR in 50 cases of clinical CRC tissues (Pearson's correlation coefficient, r = -0.409). Statistically significant differences are indicated: $^{*}P < 0.05$; Student's t test.

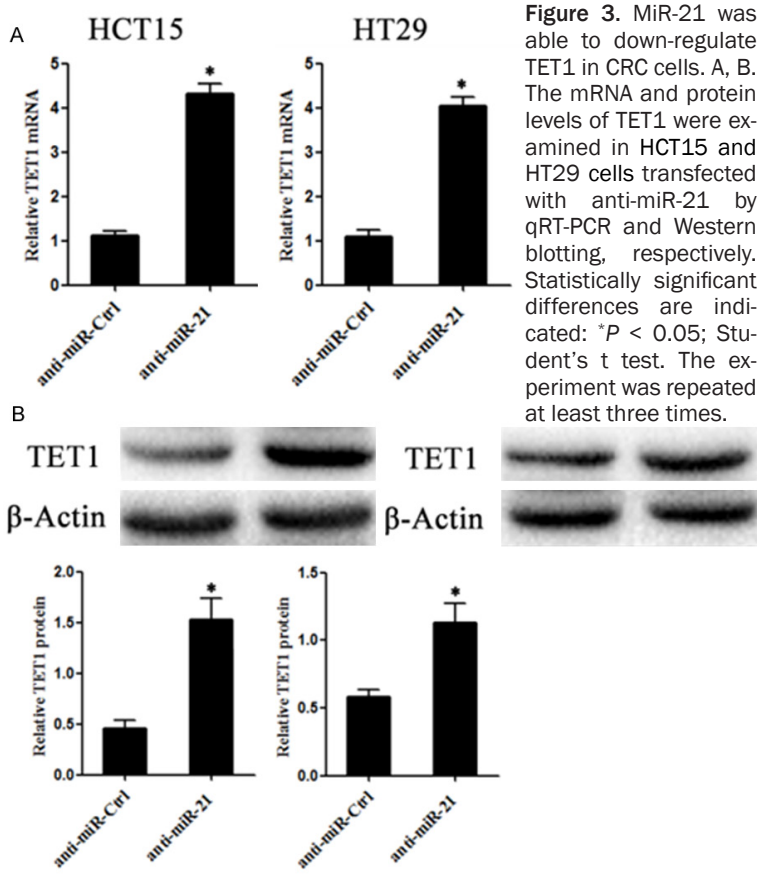

Figure 3. MiR-21 was able to down-regulate TET1 in CRC cells. A, B. The mRNA and protein levels of TET1 were examined in HCT15 and HT29 cells transfected with anti-miR-21 by qRT-PCR and Western blotting, respectively. Statistically significant differences are indicated: $^{*}P < 0.05$; Student's t test. The experiment was repeated at least three times.

(**Figure 1B** and **1C**). Thus, these data suggested that TET1 was one of the target genes of miR-21 in CRC cells.

MiR-21 and TET1 inversely expressed in clinical CRC tissues

Then, we analyzed the expression of TET1 in 50 paired clinical CRC and adjacent noncancerous tissues using qRT-PCR and normalized against an endogenous control (GAPDH). TET1 expression was highly expressed in 50 CRC patient's tissues compared with corresponding adjacent tissues (**Figure 2A**). Then we assessed the correlation between miR-21 and TET1. As expected, we found that the levels of miR-21 exhibited a significant negative correlation with the levels of TET1 mRNA (**Figure 2B**). Overall, our finding indicated that the levels of miR-21 negatively associated with those of TET1 mRNA in CRC tissues.

MiR-21 inhibited the expression of TET1 in CRC cells

To further investigate the effect of miR-21 on TET1, we performed the transient transfection of anti-miR-21 in HCT15 and HT29 cells. Our data showed that miR-21 downregulation with transfection of anti-miR-21 led to the level of TET1 mRNA obviously increasing when compared with miR-control in HCT15 and HT29 cells (**Figure 3A**). At the same time, we detected the expression of TET1 protein the transient transfection of anti-miR-21 in HCT15 and HT29 cells. Our results showed that the level of TET1 protein was substantially increased when the expression of miR-21 of was inhibited in two kinds of CRC cells (**Figure 3B**). Collectively, we concluded that miR-21 was able to down-regulate TET1 in CRC cells.

miR-21 promoted cell proliferation by targeting TET1

It has been reported that TET1 is implicated in tumor growth [14]. Therefore, we are interested in whether miR-21 inhibits proliferation of CRC cells through TET1. The EdU assay manifested that cell proliferation was decreased when the cells were treated with anti-miR-21, but TET1 downexpression could rescue inhibition of cell

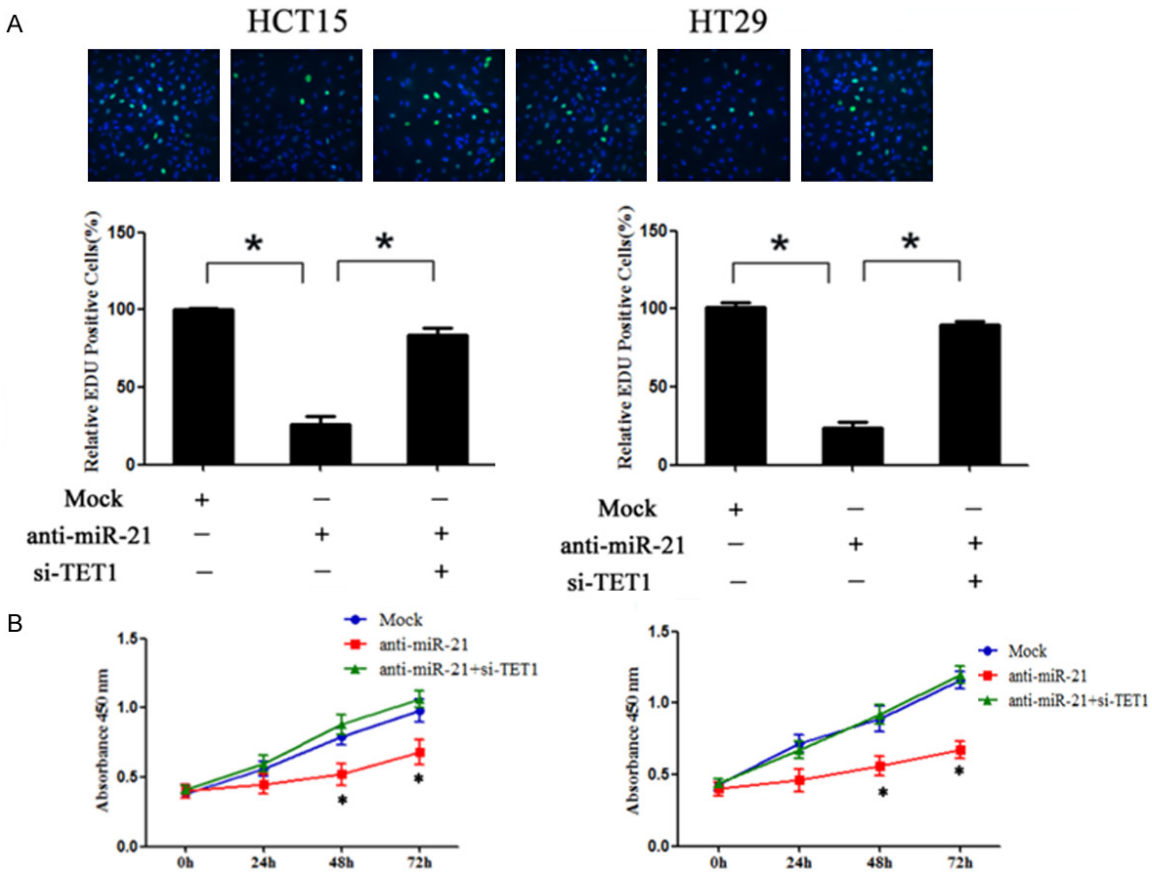

Figure 4. MiR-21 promoted the proliferation of CRC cells via targeting TET1. A. The CRC cells with bright cyan are the EdU positive cells. EdU assay showed that miR-21 downregulation decreased the EdU positive cells percentage compared to control in HCT15 and HT29 cells, and the effect of anti-miR-21 on the EdU positive cells was rescued by siTET1. Statistically significant differences are indicated: $^{*}P < 0.05$; Student's t test. The experiment was repeated at least three times. B. The CCK-8 assay showed that low-expression of miR-21 inhibited the growth of HCT15 and HT29 cells. In contrast, the decreasing HCT15 and HT29 cells growth induced by anti-miR-21 could be blocked by TET1 siRNA. Absorbance at 450 nm was measured. Statistically significant differences are indicated: $^{*}P < 0.05$; Student's t test. The experiment was repeated at least three times.

proliferation mediated by anti-miR-21 in HCT15 and HT29 cells treated with both anti-miR-21 and si-TET1 (**Figure 4A**). Results suggested that miR-21 modulated the proliferation of CRC cells relying on TET1 in part. To verify our conclusion, we also performed the CCK-8 assays. When treated with anti-miR-21 in HCT15 and HT29 cells, the proliferation capacity of CRC cells was significantly reduced other than treated with control groups or treated with both anti-miR-21 and si-TET1 (**Figure 4B**). Taken together, we concluded that miR-21 was able to promote cell proliferation through targeting TET1 in CRC cells.

Discussion

As is well known, microRNAs (miRNAs), which modulate the expression of their target genes at post-transcriptional level, have been identified to play critical regulatory roles in cancer proliferation, invasion and prognosis. They directly bind to the corresponding complementary sequences of their target genes mRNAs so that lead to target protein expression downregulation [18]. For example, miR-130a promoted gastric cancer tumorigenesis by targeting RUNX3 [19]. MicroRNA-217 functions as a prognosis predictor and inhibits colorectal cancer cell proliferation and invasion via an AEG-1 dependent mechanism [20]. In this study, we focused on exploring that the mechanism of miR-21 could promote proliferation in patients with CRC.

Methylcytosine dioxygenase TET1 as one member of TET family of enzymes (TET1/2/3)

participates in DNA demethylation by catalyzing the conversion of 5-methylcytosine to 5-hydroxymethylcytosine (5 hmC), resulting in active or passive DNA demethylation [7, 21]. Recently, TET1 is found to be over-expressed in diffuse intrinsic pontine glioma, which is a malignant pediatric brain tumor with dismal outcome [22]. It has been shown that TET1 silencing reduces 5-hmC levels and decrease cell proliferation in leiomyoma [23]. Nevertheless, another report has shown that miR-29 family decreases protein levels of TET1 through directly targeting its 3'UTR. Moreover, Neri et al. also revealed that in colorectal cancer cell, TET1 induced expression of DKK3 and DKK4, which inhibited Wnt pathway, eventually inhibited cell growth [14]. TET1 also exerts tumor suppressor function in lung, prostate, breast and gastric cancer [12, 24, 25]. The controversial function remains unclear, which may be partially explained by multifactorial etiology of cancer and the complexity of clinical tissues.

Here, we first found that TET1 is negatively regulated by miR-21 via targeting its 3'UTR. Then, we observed that high levels of miR-21 correlated with low levels of TET1 in clinical CRC samples. Additionally, we further investigated the effect of miR-21 on TET1 expression in CRC cells. RT-PCR and Western blotting analysis showed that low expression of miR-21 by the transfection of colorectal cancer cell lines with miR-21 inhibitors, respectively, the effect on TET1 expression was opposite to the change of miR-21 expression. Furthermore, our results indicated that miR-21 promoted proliferation of colorectal cancer cells by inhibiting TET1 expression. Together, the results suggested that miR-21 promoted cell proliferation of colorectal cancer cells by targeting tumor suppressor TET1.

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Disclosure of conflict of interest

None.

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