miR-506 inhibits cell proliferation and invasion by targeting TET family in colorectal cancer

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ARTICLE INFO	ABSTRACT
Article type: Original article *Article history:* Received: Aug 26, 2015 Accepted: Nov 5, 2015 *Keywords:* Colorectal cancer miR-506 TET family	***Objective(s):*** Ten-eleven translocation (TET) family members have been shown to be involved in the development of many tumors. However, the biological role of the TET family and its mechanism of action in colorectal carcinogenesis and progression remain poorly understood. ***Materials and Methods:*** We measured the expression levels of TET family members in colorectal cancer (CRC) specimens, in the corresponding normal tissues and in cell lines using quantitative real-time PCR (qRT-PCR) and *in situ* hybridization (ISH). Both the protein function and the protein-independent role of TETs were investigated by cell viability assays and cell invasion assays using *in vitro* and *in vivo* models. ***Results:*** We found that all three TET genes were strongly up-regulated at the transcript level in CRC samples compared to matched normal tissues. The same results were observed in colorectal cancer cell lines. Knockdown of TETs by shTET1/2/3 showed that TET family members inhibited CRC growth and metastasis. We showed that TET family member degradation by miR-506 inhibits cell proliferation and invasion in colorectal cancer. ***Conclusion:*** Through this study, we advance our understanding of the expression levels TETs and miR-506 in CRC and further clarify the internal regulatory mechanism of miR-506 by targeting TET during CRC processes. These findings may contribute to a novel avenue for researching and developing targeted therapies for CRC.

Introduction

The Ten-eleven translocation (TET) family of proteins consists of TET1, TET2 and TET3, which are 2-oxoglutarate- and Fe (II)-dependent dioxygenases that catalyze the conversion of 5-methyl-cytosine (5-mC) to 5-hydroxymethyl-cytosine (5-hmC) and promote DNA demethylation (1, 2). In addition to their hydroxylase activity, TET proteins have also been reported to recruit O-linked N-acetylglucosamine transferase (OGT) to chromatin, which promotes gene expression and the post-transcriptional modification of histones (3, 4). The TET family plays a vital role in tumorigenesis. TET2-inactivating mutations and deletions occur in a large proportion of myeloid malignancies, such as myelodysplastic syndrome (MDS) (5, 6), myeloproliferative neoplasm (MPN) (6, 7), chronic myelomonocytic leukemia (CMML) and acute myeloid leukemia (AML) (6). TET2 frequently functions as a tumor suppressor (6-10). Decreased expression of all TET family genes or the loss of 5-hmC have been reported to be involved in large types of solid tumors. According to these reports, TET1

and TET2 act as tumor suppressors in breast, lung and liver cancers (11-14). TET1 acts as a vital oncogenic gene in MLL-rearranged leukemia by cooperating with MLL-fusion proteins (15). The mechanisms by which TET family members function and exert their action in colorectal carcinogenesis and progression have not yet been clearly described.

microRNAs (miRNAs) are small single-stranded RNAs that negatively regulate gene expression either by inducing the degradation of target mRNAs or by inhibiting the translation of mRNAs (16). miRNAs have been reported to function as oncogenes and anti-oncogenes and are usually dysregulated in tumors (17). miRNAs are involved in many cellular processes, including proliferation, differentiation, invasion, and apoptosis (18-20).

In this study, we measured the expression levels of TET family members in colorectal cancer (CRC) specimens and corresponding normal tissues by qRT-PCR and *in situ* hybridization (ISH). We induced knockdown of TETs in SW480 and SW620 cells by transfection with sh-TET1, shTET2 and shTET3 (sh-

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ctr was used as a control). Cell viability assays and cell invasion assays were used to assess cell growth and invasive ability *in vitro*. CRC cell growth and metastatic ability were evaluated in an *in vivo* xenograft tumor model in nude mice. In addition, we showed that TET is the target of miR-506 and that miR-506 inhibits proliferation and invasion by targeting TET.

Through this study, we advance our understanding of the expression levels TETs and miR-506 in CRC and further clarify the internal regulatory mechanism of miR-506 by targeting TET during CRC processes. These findings may contribute to a novel avenue for researching and developing targeted therapies for CRC.

Materials and Methods

Cell cultures and human tissues

Human CRC cell lines were obtained from the Chinese Academy of Medical Science (Beijing) and maintained at 37 °C in an atmosphere of 5% CO_2. All of the human CRC cell lines were purchased from the Beijing Institute for Cancer Research (Beijing, China). SW480, HCT15, HCT116, KM12, NCM460, DLD1 and Ls174t cells were cultured in RPMI-1640 medium (Gibco, USA) containing 10% fetal bovine serum (FBS) (PAA Laboratories, Austria), and SW620 and HT29 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum.

The clinical materials were collected for this study, and prior approval was obtained from The Third Xiangya Hospital (Changsha, Hunan, China). Written informed consent was obtained from all study participants. A total of 40 pairs of tumor tissues and their matched adjacent tissues were obtained from colorectal cancer patients between 2012 and 2013. All of the tissue biopsies used in this study were freshly frozen in liquid nitrogen and stored at –80 °C until use.

RNA isolation and quantitative real-time RT-PCR

The expression level of miR-506 was detected in both colorectal cancer tissues and cell lines. According to the manufacturer's instructions, total miRNA was extracted from cells and tissues with TRIzol reagent (Invitrogen). Reverse transcription and qRT-PCR reactions were performed with a SYBR Green-containing PCR kit (GenePharma, China). The cycling conditions were: one cycle at 95 °C for 5 minutes and 38 cycles of 95 °C for 30 se and 55 °C for 30 sec. Melting curve analysis was performed for each PCR reaction to confirm the specificity of the amplification. The expression of miR-506 was calculated based on the threshold cycle (CT), and relative expression levels were calculated as $2^{-[(CT\ of\ miR-506)-(CT\ of\ U6)]}$ after normalization with reference to the quantification of U6 small nuclear RNA (snRNA)

expression. U6 snRNA was used as an endogenous control. The 5′→3′ primer sequences used for qRT-PCR were as follows: TET1 forward, CCTAGGACA-GGCCTTTGGTG and reverse, CTGGGACAACACTCCCA-CTC; TET2 forward, AGAGAATCCACCTGCAAGCT and reverse, TGGGGTGTGGCTATCAAGTT; TET3 forward, CAACGGCTGCAAGTATGCTC and reverse, CTCGTTGGT-CACCTGGTTCT; and GAPDH forward, GACTCATGACC-ACAGTCCATGC and reverse, AGAGGCAGGGATGATGTT-CTG.

Western blot analysis

Protein was extracted from CRC cell lines with RIPA lysis buffer and a proteinase inhibitor. Protein concentrations were measured with a Protein BCA Assay Kit (Bio-Rad, USA). A total of 20 µg of protein, mixed with 2×SDS loading buffer, was loaded per lane. The proteins in the lysates were separated by 12% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore, USA). Membranes were incubated at room temperature for 1 hr with 5% skim milk powder to block nonspecific binding. Then the membranes were incubated for 12 hr at 4 °C with an antiserum containing antibodies against TET1, TET2 and TET3 (Santa Cruz, CA). A peroxidase-conjugated secondary antibody (1:1000 dilution) and ECL Western blotting detection reagents were used to visualize the target proteins (ECL New England Biolabs, USA) and the proteins were quantified with a Bio Image Intelligent Quantifier 1-D (Version 2.2.1, Nihon-BioImage Ltd., Japan). An anti-GAPDH antibody (Boster, China) was used as a control.

RNA interference

Recombinant shRNA lentiviruses containing shTET1, shTET2 and shTET3 were purchased from FulenGen (Guangzhou, China). The target sequences of TET1, TET2, and TET3 were GCAGCTAATGAAGG-TCCAGAA, TTTCACGCCAAGTCGTTATTT, and GCGA-TTGCGTCGAACAAATAG, respectively. Cells were plated in culture dishes for 24 hr and then transfected with shRNA using Lipofectamine 2000 (21). After 48 hr, the cells were harvested for use in other assays or for RNA and protein extraction.

Plasmids and transfection

To generate a miR-506 expression vector, a genomic fragment covering the region encoding pri-miR-506 and its up- and downstream region was PCR-amplified and cloned into the pLvthm vector (Addgene Inc, USA). The vectors were generated by PCR amplification and subcloned into the Bam HI/Sal I site of the pGL3–basic luciferase reporter plasmid (Promega). miR-506 mimics and scrambled oligonucleotides (GeneCopoeia, China) were also transfected into CRC cells using Lipofectamine 2000

reagent (Invitrogen). Two concentrations of miR-506 mimics (10 and 50 nM) were applied.

Luciferase reporter assay

Luc-wt, Luc-mut and Luc-ctrl plasmids were co-transfected with *in vitro*-produced miR-506 into SW480 and SW620 cells. The pMIR-REPORT β-galactosidase control vector was transfected and served as a control. At 48 hours after transfection, luciferase activity was measured in cell lysates with a dual-light luminescent reporter gene assay kit (Applied Biosystems, Germany). The results were normalized against β-galactosidase activity.

Cell viability assay

Cells transfected with shTET1, shTET2, shTET3 and sh-ctr were plated in 12-well plates at the desired cell concentrations. Cell counts were estimated following trypsinizing and analysis with a Coulter Counter (Beckman Coulter) at the indicated time points (24, 48, 72 hr and 96 hr after transfection) in triplicate. Cell number curve analyses were performed according to the recorded data at the indicated time points.

Cell invasion assays

Cells seeded onto a basement membrane matrix were cultured in the insert of a 24-well culture plate (EC matrix; Chemicon). The non-invasive cells and EC matrix were removed with a cotton swab 48 hours later, while the invasive cells, which were located on the lower side of the chamber, were stained with crystal violet and then counted and imaged.

Immunohistochemistry

Immunohistochemistry (IHC) was performed under standard procedures. For human CRC tissue microarray slides, anti-TET1 (Santa Cruz, USA), anti-TET2 (Abcam, UK), and anti-TET3 (Santa Cruz) were used. miR-506 miRCURY LNA custom detection probes (Exiqon, USA) were used for ISH. The 5'-3' sequence (enhanced with LNA) was TCCATCATTACCCGGCAGTATTA and possessed a DIG label at both the 5' and the 3'ends. Hybridization, washing and scanning were performed according to the manufacturer's instructions. The intensity of TET1/2/3 staining was scored as 0–4 according to the following standards: 0–1 (no staining), 1–2 (weak staining), 2–3 (medium staining), and 3–4 (strong staining). The percentage of TET1, TET2 and TET3 in 3 representative high-power fields of individual tumor samples and their normal adjacent samples were analyzed (As showed in Figure 1C). Those expression scores were equaled to scores of the intensities × the percentages of positive cells. The maximum was 4, and the minimum was 0. Every sample was evaluated by at least 2 pathologists who were blind to the experimental conditions. Expression scores greater than or equal to 2 were defined as high expression, while scores of less than 2 were defined as low expression.

Xenograft model in nude mice

The colorectal cancer model in nude mice was designed as previously described (22). Briefly, the transfected cells (1×10^5 respectively), including SW620/sh-TET1, SW620/sh-TET2, SW620/sh-TET3 and SW620/sh-ctr, were injected subcutaneously into the flanks of BALB/c nude mice aged 4 to 6 weeks old (5 mice per group). Tumor size was measured every 5 days. At 30 days after implantation, the mice were sacrificed, then necropsies were performed, and the tumors were weighed. Tumor volumes were determined according to the following formula: $A \times B^2/2$, where A is the largest diameter and B is the diameter perpendicular to A. To assay the effect of sh-TET1, TET2, and TET3 on tumor metastasis, 1×10^5 SW620 cells infected with sh-TET1, TET2, TET3 or sh-ctr viruses were injected into the tail veins of nude mice (five in each group). After 28 days, necropsies were performed. The numbers of micrometastases in the lung per HE-stained section in individual mice were analyzed by morphological observation. All surviving animals were handled in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The experimental protocol was approved by the medicine animal welfare committee of The Hunan Provincial People's Hospital and The Third Xiangya Hospital, Central South University (Changsha, China).

Statistical analysis

All statistical analyses were performed using SPSS19.0. A two-tailed paired Student's t-test was used for analysis. Mann–Whitney U-tests, Spearman's correlation analyses, the Kaplan–Meier method and log-rank tests were used. $P<0.05$ was considered statistically significant.

Results

TETs are significantly overexpressed in CRC

The expression levels of TET family members were examined by qRT-PCR in 40 pairs of CRC tissues and their matched adjacent normal tissues. All three TET members were significantly elevated in tumors compared to the corresponding normal tissues. Among 40 patients with CRC, approximately 90.0% ($P=0.000$, 36 of 40 patients), 87.5% ($P=0.000$, 35 of 40 patients) and 85.0% ($P=0.000$, 34 of 40 patients) of tumor tissues revealed notable increases in TET1, TET2 and TET3 levels, respectively (Figure 1A). TET mRNAs were also measured in nine CRC cell lines by qRT-PCR analysis. The results showed that TET expression levels were much higher in

SW480 and SW620 cells than in other cell lines (Figure 1B). Therefore, in the later experiments, we
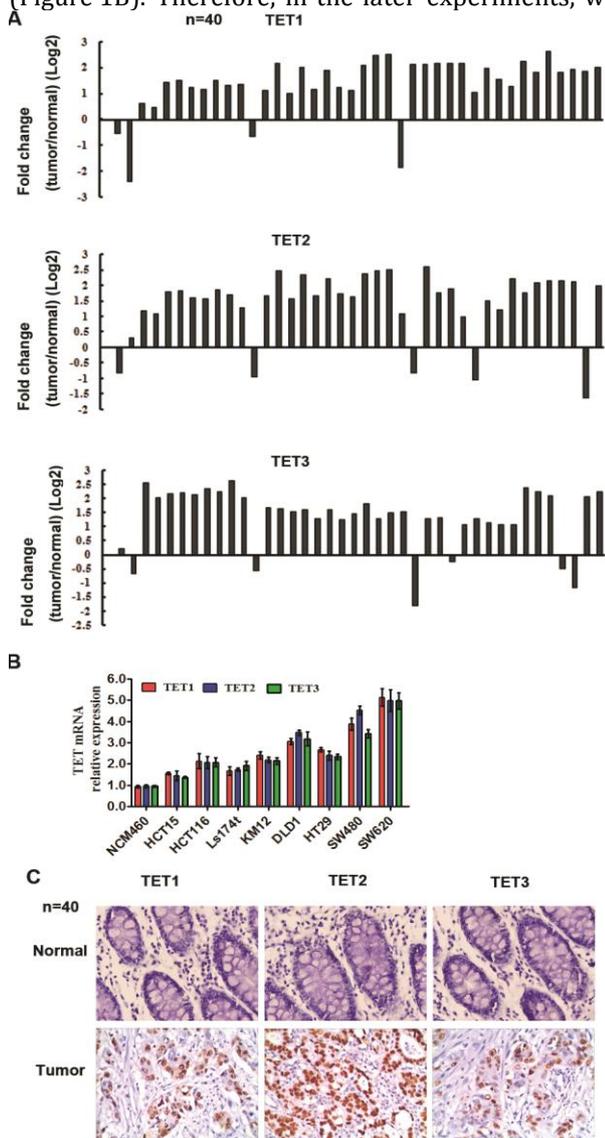
Figure 1. TETs are up-regulated in colorectal cancer. (A) qRT-PCR analysis of the expression levels of TET family members in CRC samples (n=40) and their paired adjacent normal tissues (n=40). Data are shown as log2-fold changes in CRC tissues relative to adjacent normal tissues. (B) qRT-PCR analysis of the expression levels of TET family members in 9 cell lines derived from CRC tissues. The data are presented as the mean±SD from at least three separate experiments. (C) ISH analysis of the protein expression levels of TET1, TET2 and TET3 in CRC tissues and their adjacent normal tissues. Representative images displaying TET1/2/3 expression are shown. Scale bars: 50 μm

used SW620 and SW480 cells to assess the possible role of TET members in CRC pathogenesis. Subsequently, we examined TET family proteins by immunohistochemical staining in tissue microarrays that included 40 CRC samples and 40 adjacent normal tissues. We observed that both cytoplasmic and nuclear staining of TET1/2/3 were frequently observed in tumor cells but not in normal mucosa

epithelium cells (Figure 1C).The above data indicate that there is a clear up-regulation of TETs in CRC.
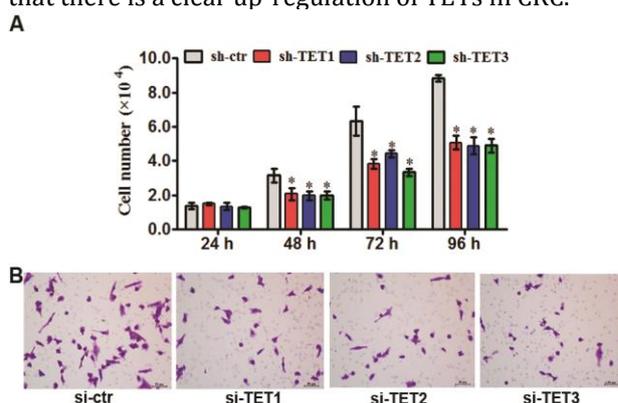
Figure 2. Knockdown of TET genes inhibits CRC cell growth and invasion *in vitro*. (A) SW620 cells were transfected with sh-ctr, sh-TET1, shTET2 or shTET3. Then they were seeded in 12-well plates at the desired cell concentration and maintained in medium containing 10% fetal bovine serum. The cells were counted at the indicated time points, and their growth rates were recorded. Experiments were performed in triplicate. (B) Invasion analyses were performed in SW620 cells that were infected with TET1/2/3 shRNA or sh-ctr RNA. Expression images displaying TET1/2/3 are shown. Scale bars: 50 μm

Knockdown of TETs reduces CRC cell growth and invasion in vitro

The biological function of TET members in CRC cells was also observed in an *in vitro* model. Sh-ctr, sh-TET1, shTET2 and shTET3 were transfected into SW620 cells. Then they were seeded in 12-well plates at the desired cell concentrations and maintained in medium containing 10% fetal bovine serum. The cells were counted at the indicated time points, in triplicate, and their growth rates were recorded. Compared with the sh-ctr treatment, cell viability assay results suggested that knockdown of any member of the TET family markedly inhibited proliferation in SW620 cells (Figure 2A). Invasion assays were used to assess cell invasive properties in a Matrigel-coated chamber. Cell migration was plotted as the average number of cells per field of view from three different experiments, as described in the materials and methods section. Cell invasion assays showed that knockdown of any member of the TET family also reduced invasion in SW620 cells (Figure 3C). Knockdown of TETs therefore reduced growth and invasion in CRC cells *in vitro*.

Knockdown of TET inhibits CRC growth and metastasis in vivo

To explore the role of TET family members in CRC tumorigenesis, SW620 cells were transfected with effective shRNAs by a lentiviral system. SW620 cells that stably expressed shTET1, shTET2 or shTET3 were subcutaneously injected into nude mice (eight in each group). At 28 days later, all mice were sacrificed and tumor weights were

measured. Compared with the control group, the

results indicated that knockdown of any member of the
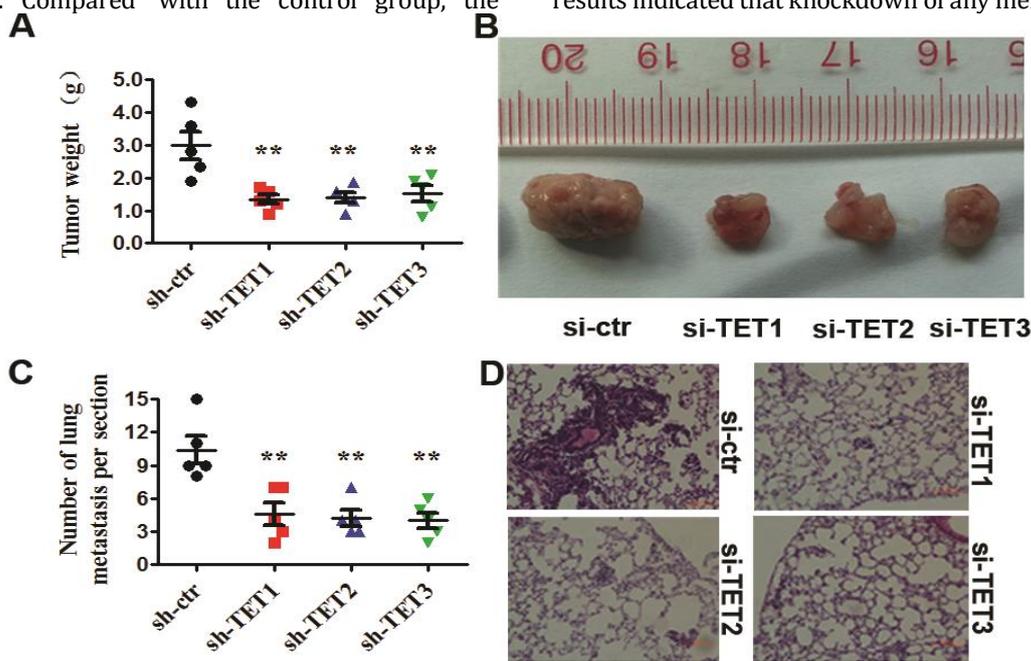
Figure 3. Knockdown of TET inhibits CRC growth and metastasis *in vivo*. (A) SW620 cells stably expressing shTET1/2/3 or sh-control were injected subcutaneously into nude mice (eight mice in each group). At 28 days after injection, all animals were sacrificed. Then necropsies were performed, and all tumors were weighed. * *P* <0.05. (B) A photograph of tumors identified in sacrificed mice. (C) The number of lung metastases in the shTET1/2/3 and sh-control groups. The number of metastatic nodules in five mice from each group were counted under a microscope. *P<0.05. (D) Representative images of lung metastases in the shTET1/2/3 and sh-control groups. Scale bars: 50 μm
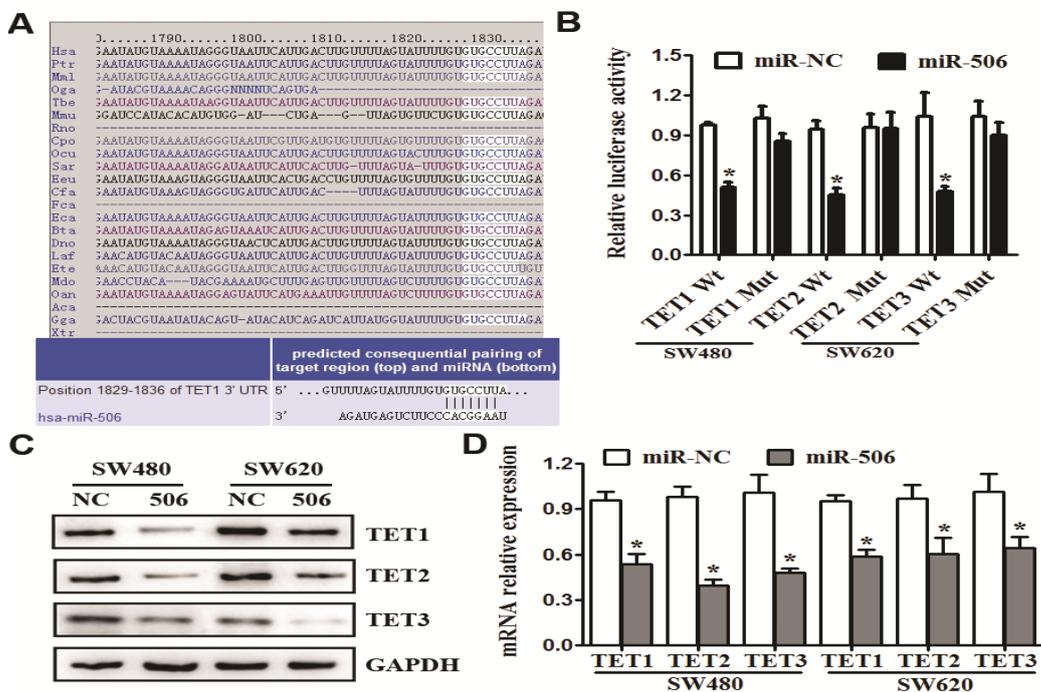
Figure 4. miR-506 targets TET family members and inhibits proliferation and invasion in CRC cells. (A) The 3' UTRs of TETs have putative binding sites for miR-506. (B) Luciferase assay of SW480 and SW620 cells, which were co-transfected with miR-506 mimics or scramble and a luciferase reporter containing the following: TET1 3'-UTR (TET1-wt), TET2 3'-UTR (TET2-wt), TET3 3'-UTR (TET3-wt) or mutant constructs in which the first four nucleotides of the miR-506 binding site were mutated: TET1-mut, TET2-mut and TET3. An empty luciferase reporter construct was used as a negative control. All data are shown as the mean ± SEM, * *P*<0.05. (C) The effect of miR-506 on

the protein expression levels of TET1, TET2 and TET3, analyzed by Western blot. GAPDH was used as a control. (D) The effect of miR-506 on mRNA expression levels of TETs, analyzed by qRT-PCR in SW480 and SW620 cells. * $P<0.05$

TET family markedly decreased tumor growth (Figure 2A). The weight of tumors was distinctly reduced in the shTET1/2/3-treated groups compared with the control group (Figure 2B). Necropsies were performed, and the number of metastatic nodules in each group of mice was counted under a microscope.

We found that ectopic expression of TET1/2/3 markedly decreased the number of lung metastases in mice (Figure 3C). Representative images of lung metastasis in the shTET1/2/3-treated and sh-ctr-treated mice are shown in Figure 3D. These results indicate that TET knockdown is associated with reduced growth and metastasis in CRC xenografts *in vivo*.

miR-506 targets TET family members, inhibits proliferation and invasion in CRC cells

To understand how TETs suppress CRC proliferation and invasion, we used algorithms (TargetScan and Miranda) to help us identify which miRNA might target TETs in CRC. miR-506 was predicted to be targeted among all candidate target genes by both algorithms (Figure 4A). Hence, miR-506 may directly target TETs. We confirmed this hypothesis in CRC cells using luciferase reporter assays. TET complementary sites were cloned downstream of the firefly luciferase gene and co-transfected with miR-506 mimics or scrambled oligonucleotides. Then luciferase activity was measured 48 hours after transfection. miR-506 significantly reduced luciferase activity in both SW480 and SW620 cells that were co-transfected with TET reporter constructs compared to cells co-transfected with scramble sequence. Mutation of the putative miR-506 sites in the 3'-UTR of TETs abrogated luciferase responsiveness to miR-506 (Figure 4B). Transfection of miR-506 mimics into SW480 and SW620 cells markedly decreased the protein levels of TETs (Figure 4C). Transfection of miR-506 mimics into SW480 and SW620 cells also decreased the mRNA levels of TETs (Figure 4D). These results may validate the hypothesis that miR-506 targets TET family members and inhibits proliferation and invasion in CRC cells.

Discussion

Several reports have shown that miR-506 plays a significant role in proliferation, growth, invasion and metastasis (23, 24). miR-506 expression is down-regulated in nasopharyngeal carcinoma (25), oral squamous cell carcinoma (26), breast cancer (27) and gastric cancer (28). The TET family plays a vital role in tumorigenesis. According to previous reports, TET1 and TET2 are tumor suppressors in breast cancer, lung and liver cancers (11-14), and TET1 also

plays a critical oncogenic role in MLL-rearranged leukemia (15). However, few studies on miR-506 and TETs in CRC have been reported. In this study, we first showed that TET levels in CRC tissues were significantly higher than levels in neighboring noncancerous tissues by using qRT-PCR and ISH. TET levels were also higher in CRC cell lines. These results suggest that abnormal expression of TETs is closely related to CRC.

Furthermore, we studied the biological effects of TET knockdown in CRC. The *in vitro* data demonstrated that TETs function as oncogenic genes in CRC and that knockdown of TETs inhibited colorectal tumor cell proliferation and invasion. Many studies have supported our findings in other type of cancers. The same inhibitory effect has also been verified in an *in vivo* xenograft model of CRC cells in nude mice. Knockdown of TET inhibited CRC tumor growth (weight) and reduced the number of lung metastases *in vivo*. Therefore, knockdown of TET in CRC inhibited tumor progression *in vitro* and *in vivo*.

Finally, the role of miR-506 in the regulation of TETs in CRC was verified. Luciferase reporter assays showed that TETs are the target genes of miR-506. Transfection of miR-506 mimics into SW480 and SW620 cells markedly decreased the protein and mRNA levels of TETs. Our research therefore demonstrates that miR-506 regulates cell proliferation and invasion in CRC by directly targeting TETs.

Conclusion

miR-506 inhibits cell proliferation and invasion in CRC by targeting members of the TET family. Our research may help to clarify the role of TETs and miR-506 in the development and progression of human CRC. Our finding advance our knowledge of the expression levels of TETs and miR-506 in colorectal cancer and further reveals an intrinsic mechanism in which TETs are regulated by miR-506. These findings may contribute to the search for novel approaches to identify CRC-targeted therapies.

Acknowledgment

Conflict interest

No potential conflicts of interest were disclosed.

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