TET3 Promotes HCC Proliferation And Metastasis Via IncRNA ARAP1-AS1

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Abstract

**Background:** Aberrations of DNA methylation and proteins involved in DNA methylation process have been demonstrated to be correlated with tumor malignancy and prognosis of patients. The present study aims to investigate the preliminary mechanism underlying the biological functions of a DNA demethylation enzyme TET3 during HCC proliferation and metastasis.

**Methods:** CCK8 assay, colony formation assay and transwell assay were performed to monitor cell proliferation, migration and invasion. RNA-sequencing (RNA-seq) was applied to screen the differentially expressed mRNA upon TET3 overexpression to investigate the downstream mediators of TET3 during HCC progression. The expression of TET3 or ARAP1-AS1 was examined by western blot or quantitative real-time PCR (qRT-PCR).

**Results:** First, TET3 expression was increased in HCC tumor tissues and positively correlated with poor prognosis of HCC patients. Next, TET3 was found to promote the proliferation and metastasis of HCC cells. RNA-seq was then performed and unveiled IncRNA ARAP1-AS1, a well-identified onco-lncRNA in several cancer types, as a candidate downstream mediator of TET3. The following results indicated that TET3 increased ARAP1-AS1 expression. And rescue experiments indicated that ARAP1-AS1 knockdown impaired the proliferation of HCC cells induced by TET3 overexpression.

**Conclusion:** TET3 promoted the proliferation and metastasis of HCC cells by regulating the expression of IncRNA ARAP1-AS1.

Introduction

Hepatocellular carcinoma (HCC) ranks the most common primary liver cancer and is one of the leading causes of cancer related death worldwide (1). Although partial hepatectomy or liver transplantation is available for patients with early-stage HCC, most of HCC patients are diagnosed at an advanced stage and miss the best treatment period owing to the lack of early symptoms and detection (2). Therefore, further understanding the molecular mechanism underneath HCC development is fundamental for the designs and inventions of more effective diagnostic and therapeutic approach in the future.

Epigenetic characteristics have been emerging as the main features of cancer (3). Epigenetic regulation, such as DNA/RNA methylation and histone modification, refers to functionally relevant changes to the genome that do not involve a change in the nucleotide sequence. As an important epigenetic modification, DNA methylation has been validated to be critical for the regulation of gene expression, histone modification and other biological processes (4, 5). Accordingly, aberrations of DNA methylation and proteins involved in DNA methylation process are significantly correlated with tumor malignancy and prognosis of patients (6).

Ten-eleven translocation enzyme (TET) family catalyzes the oxidation of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) and causes DNA demethylation (7). The TET family comprises three
members: TET1, TET2, and TET3 with different cellular locations and biological functions (8). Here we focused on TET3, which was identified to be upregulated in HCC tissues and associated with the poor prognosis of HCC patients. Increasing evidence indicates that TET3 participates in the tumorigenesis of glioblastoma, ovarian cancer, breast cancer and several other types of cancers (9, 10), but the functions of TET3 in HCC tumorigenesis remains to be elusive.

In the present study, we identified that TET3 accelerated the proliferation and metastasis of HCC cells. Mechanical assay revealed that IncRNA ARAP1-AS1 as a downstream target of TET3 played critical roles in regulating the biological function of TET3. In conclusion, our findings suggest that TET3 may exert as a potential tumor regulator to involve in HCC progression, which provided a new insight into HCC tumorigenesis.

**Materials And Methods**

**Plasmids**

FH-TET3-pEF was obtained from Addgene (#49446). pCDH-flag-TET3-puro was obtained by subcloning TET3 sequences from FH-TET3-pEF into pCDH-EF1-MCS-T2A-Puro backbone with a flag tag at the N-terminal of TET3.

**Cell culture**

The human hepatoma cell line HepG2 and human embryonic kidney cell HEK293T were purchased from the American Type Culture Collection (ATCC). The SMMC-7721 and Huh7 cell lines were obtained from the Chinese Academy of Science (Shanghai, China). All the cell lines were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (ExCell) at 37°C and 5% CO₂.

**Viral production, transduction, and siRNA transfection**

Lentivirus was produced as previous description (11) with sight modification. Briefly, lentiviral constructs were packaged into lentivirus with lipofectamine 3000 (Invitrogen) and viral supernatants were harvested at 48 h post transfection. To generate stable-expressing cells, cells were infected with the viral supernatant and selected by puromycin (1 μg / mL). siRNAs (Sangon Biotech) specifically targeting TET3 and IncRNA ARAP1-AS1 were transfected with lipofectamine 3000 (Invitrogen). Sequences of siRNAs are listed in Table 1.

| Table 1: List of siRNA sequences |
| siRNA      | Sense-strand sequence                  |
|------------|---------------------------------------|
| siTET3 #1  | GCCGAAGCUGUGUCCUCUUAAUdTdT             |
| siTET3 #2  | GAAAGAUGAAGGCUCAUUUAUdTdT             |
| siARAP1-AS1| GCCCCACAAGGACAGUGAAdTdT               |

**Quantitative RT-PCR (qRT-PCR)**

Total RNA extraction was performed with TransZol Up RNA kit (TransGen) and cDNA synthesis was performed with Transcriptor First Strand cDNA Synthesis Kit (Roche). qRT-PCR was performed with triplicate samplings on StepOne Plus real-time PCR system (AB Applied Biosystems) using the agent of Bestar SYBR Green qPCR Master Mix (DBI). 18S rRNA was conducted to standardize gene expression as reference and the relative expressions of genes were calculated with $2^{-\Delta\Delta CT}$ methods. Expression levels were calculated relative to 18S. qRT-PCR oligo sequences were listed in Table 2.

**Table 2: List of primers for qRT-PCR**

| Gene      | Primer | Sequence      |
|-----------|--------|---------------|
| TET3      | Forward| CCAGCATAACCTCTACAATGGG |
|           | Reverse| TCCTCGCTACCAAACACTCATCC |
| ARAP1-AS1 | Forward| GGGTGTCCTCCCAAGGTTTCTG |
|           | Reverse| ACTTTGCGGAGAAGGTGCTG |
| 18S       | Forward| CACCAGACTTTGCCTCCA |
|           | Reverse| AGAAACGGCTACCACATCCA |

**Western blotting**

Cell lysates were prepared in RIPA lysis buffer (Beyotime Biotechnology) containing PMSF and protease inhibitor cocktails (MedChemExpress), and the concentration of protein was measured with Easy II Protein Quantitative Kit (TransGen). Then aliquots containing 30 μg of total protein were subjected to western blotting according to the previously described protocol (11). Antibodies were as follows: DYKDDDDK-Tag (1:1000, Abmart), β-Actin (1:5000, Abcam).

**CCK8 assay**

Cells were plated in 96-well plates (2000 cells / well) and the cell viability was measured by TransDetect Cell Counting Kit (TransGen) in the following 5 days according to the manufacturer's instructions.
Colony formation assay

Cells were seeded into 6-well plates (1000 cells / well) and the medium was refreshed every three days. After 14 days cells were washed gently with PBS, fixed with 4% formaldehyde and stained with crystal violet solution (Beyotime Biotechnology).

Transwell assay

Cell strainers with 8 μm pore size were embedded in 24-well plates for transwell assay. For cell migratory assay, 2x10^5 cells suspended in serum-free medium were added into the upper chamber and 10% FBS-containing medium was added into the lower chamber. After 24 h the migratory cells were fixed with 4% formaldehyde and stained with crystal violet solution (Beyotime Biotechnology, China). Cells unpenetrated the strainer were removed by wiping with cotton stick. Images of five random fields per chamber was captured to measure the number of migratory cells. Cell invasion assay was performed with a similar way except that Matrigel (Corning-Costar, NY, USA) was coated on the upper chamber before adding the cells.

Statistical analysis

Statistical analysis of data was performed with GraphPad Prism 9.0. *p<0.05 was regarded as statistically significant. **p<0.01, ***p<0.001, ****p<0.0001. All the data were shown as means ± SD through at least three independent experiments.

Results

TET3 is upregulated in HCC tumor tissues and indicates the poor prognosis of HCC patients

According to the RNA sequencing data of an HCC sample cohort with 65 pairs of tumor and their corresponding peri-tumor tissues, which was described in our previous work (11), we identified that TET3 was significantly upregulated in HCC tumor tissues (Fig 1A). To further confirm this result, we detected TET3 expression in an expanded HCC sample cohort containing 103 pairs of HCC and the corresponding peri-tumor liver tissues by qRT-PCR. A consistent result was observed that TET3 was upregulated in HCC tumor tissues (Fig 1B). Similarly, TET3 was also identified to be upregulated in HCC according to The Cancer Genome Atlas (TCGA) RNA-Seq dataset (Fig 1C). Further Kaplan-Meier analysis of TCGA data revealed that high TET3 expression was significantly associated with poor overall survival (OS) and poor relapse-free survival (RFS) of patients with HCC (Fig 1D and E). Moreover, TET3 expression was gradually elevated from grade 1 to grade 4 in HCC according to TCGA dataset (Fig 1F). Taken together, our data indicated that TET3 is upregulated in HCC tumor tissues and its high expression is correlated with poor prognosis of HCC patients, suggesting the possible contribution of TET3 to HCC tumorigenesis.

TET3 is required for the proliferation and metastasis of HCC cells
In order to investigate the biological function of TET3 in HCC, we first knocked down TET3 in Huh7 cells with two independent siRNA sequences (siTET3 #1 and #2). qRT-PCR was performed to confirm the efficiency of these two sequences (Fig 2A). Huh7 with TET3 knockdown showed reduced proliferative capacity compared with the control cells in CCK8 assay (Fig 2B). Consistent results were observed in colony formation assay, in which TET3 knockdown significantly impaired the colony formation of Huh7 cells (Fig. 2C). To confirm the role of TET3 in HCC cell proliferation, we overexpressed TET3 in HepG2 cells and proved that TET3 was successfully overexpressed by Western blot (Fig. 3A). Overexpression of TET3 in HepG2 significantly stimulated cell proliferation (Fig. 3B) and colony formation (Fig. 3C). Similar results were obtained in SMMC-7721 cells that TET3 overexpression increased the proliferating ability and colony-formation ability (Fig. 3D-E). Taken together, the results from both our knockdown and overexpression experiments indicated that TET3 may serve as an oncoprotein to promote HCC cell proliferation.

Additionally, the effects of TET3 on cellular migration and invasion were also assessed in vitro. The transwell assays were performed and the results showed that TET3 overexpression markedly induced the migratory and invasive ability of both HepG2 and SMMC7721 cells compared with the control cells (Fig. 4A and B). Thus, our results suggested that beside proliferation, TET3 also plays an important role in promoting HCC metastasis.

**LncRNA ARAP1-AS1 is the key downstream mediator of TET3 in HCC progression**

To identify the downstream mediator(s) of TET3, we performed RNA-seq analysis in TET3 stable-expressing HepG2 and its corresponding control cells. TET3 is an epigenetic eraser which oxidizes 5mC to 5hmC. It has been reported that 5hmC is positively associated with gene expression (12). Therefore, the upregulated genes in the cells with TET3 overexpression were the reasonable downstream candidates (Fig. 5A). Accordingly, we screened the top 50 upregulated genes for their contribution to HCC proliferation and their transcriptional dependence on TET3. Our attention was captured by LncRNA ARAP1-AS1, which was upregulated to two folds in TET3-overexpressing HepG2 cells. It has been reported that ARAP1-AS1 plays important roles in promoting tumorigenesis and metastasis in several cancer types but not in HCC (13-19). qRT-PCR analysis indicated that the expression of ARAP1-AS1 was regulated by TET3, as we observed an upregulation of ARAP1-AS1 upon TET3 overexpression in both HepG2 and SMMC-7721 cells, which is ideally consistent with the RNA-seq results (Fig. 5B).

Next, we investigated whether ARAP1-AS1 was involved in TET3-dependent HCC proliferation. We transfected siRNA against ARAP1-AS1 or the control siRNA in TET3-overexpressing HepG2 and the cells were subjected to proliferation assay. The knockdown efficiency of ARAP1-AS1-targeting siRNA was verified with qRT-PCR (Fig. 5C). Rescue experiments indicated that knockdown of ARAP1-AS1 abolished the induction of proliferation by TET3 overexpression in CCK8 assay (Fig. 5D). Consistent results were observed in colony formation assay, in which ARAP1-AS knockdown reduced the colony formation capacity induced by TET3 overexpression (Fig. 5E). Taken together, these results indicated that TET3
transcriptionally regulated lncRNA ARAP1-AS1, which is required for TET3-mediated proliferation of HCC cells.

Discussion

Recently, the function of DNA methylation and enzymes involved in DNA methylation processes in HCC have attracted much attention and widen the understanding of HCC pathogenesis (20-22). Our study indicated that TET3, was upregulated in HCC tissues and was positively correlated with poor prognosis of HCC patients. Our study also verified that TET3 was required for the proliferation, migration and invasion of HCC cells. In terms of mechanism, our study identified that TET3 influenced HCC progression by regulating lncRNA ARAP1-AS1.

In human genome, 5hmC is a common epigenetic modification in promoters, gene bodies and gene regulatory elements (23). Recent studies have suggested that 5hmC modification is associated with cancer pathobiology, with the global reduction of 5hmC in both hematological and solid tumors, including colon, liver, lung, skin, prostate, and breast tumors (23-26). As one of the enzymes that catalyze 5hmC formation, the upregulation of TET3 we observed in HCC tissues is contrary to the previously described global reduction of 5hmC (22). This discrepancy might be due to the redundant function of TET family member to catalyze 5hmC formation. In two cohorts in which genomic 5hmC contents of HCC tissues were significantly decreased, the expression level of TET2 in tumor tissues was significantly decreased (22). This work indicated that among three members of TET family, downregulation of TET2 is responsible for the global loss of 5hmC in HCC. This result was consistent with the biological function of TET2 which has been previously demonstrated to repress HCC tumorigenesis (27). Meanwhile, our data indicated that TET3 was upregulated in HCC tissues and promoted the proliferation and metastasis of HCC cells.

LncRNAs have been reported to regulate tumor cell proliferation, metabolism, and metastasis, and thus have been attracted much attention as a novel and important regulator of cancer development. To address the underlying mechanism of TET3 in HCC, we performed RNA-seq in control and TET3-overexpressing HCC cells. lncRNA ARAP1-AS1 together with other upregulated genes were selected as potential downstream targets. Previous work has proved that the upregulation of lncRNA ARAP1-AS1 promotes the proliferation and metastasis in several cancer types but unknown in HCC (13-18). Our findings indicated that ARAP1-AS1 upregulated by TET3 exerts similar roles in HCC as in other cancer types. However, further studies are required to clarify the specific mechanisms underlying TET3 increase the expression of ARAP1-AS1. Moreover, increasing evidence indicates that several signaling pathways has been demonstrated to mediate biological functions of ARAP1-AS1 (14, 16, 17), but the specific effector of TET3/ARAP1-AS1 axis in regulating HCC tumorigenesis remains to be explored.

Conclusion
In this study, we demonstrated that TET3, a member of TET family which catalyzes the conversion from 5mC to 5hmC, accelerates proliferation and metastasis of HCC cells via LncRNA ARAP1-AS1. Our findings exemplified the critical role of DNA methylation proteins in HCC tumorigenesis and implied that TET3 may have attractive translational potential of HCC therapy.

**Abbreviations**

HCC: Hepatocellular carcinoma; 5mC: 5-methylcytosine; 5hmC: 5-hydroxymethylcytosine; TET: Ten-eleven translocation enzyme; LncRNA: long non-coding RNA; ARAP1-AS1: ARAP1 antisense RNA 1.

**Declarations**

**Ethics approval and consent to participate:** Written informed consents were obtained from all participants and this study was permitted by the Ethics Committee of Mengchao Hepatobiliary of Fujian Medical University

**Consent for publication:** Not applicable.

**Availability of data and materials:** The data are available from the corresponding author on reasonable request.

**Competing interests:** The authors declare that they have no competing interests.

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**Authors' contributions:** QZ, BZ and YW designed the project; QZ, XX, ZD and XZ performed the experiment; QZ, BZ, YW, WG and XL analyzed the data; QZ wrote the manuscript. All authors read and approved the final manuscript.

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**Figures**
Figure 1

<p><strong>TET3 was upregulated in HCC and correlated with poor prognosis in HCC patients.</strong> (A) TET3 was upregulated in HCC determined by RNA-seq. (B) TET3 upregulation in HCC was verified by qRT-PCR. (C) TET3 upregulation in HCC was verified according to RNA-seq data in TCGA cohort. (D and E) Kaplan-Meier analysis was used to reveal the correlation between the TET3 expression and the overall survival (D) and relapse-free survival (E) of HCC patients. (F) TET3 expression was correlated with the tumor grade of HCC patients according to TCGA data set. Abbreviations: MCHH, Mengchao Hepatobiliary Hospital.</p>
Figure 2

**TET3 knockdown inhibited proliferation of HCC cells.** (A) The knockdown effect of two siRNAs targeting TET3 (siTET3 #1 and siTET3 #2) in Huh7 was verified by qRT-PCR. (B) TET3 knockdown reduced the proliferation of Huh7 cells in CCK8 assay. (C) TET3 knockdown reduced the colony formation ability of Huh7 cells. **p < 0.01, ***p < 0.001, ****p < 0.0001. Abbreviation: siCo., nontarget control siRNA.

Figure 3

**TET3 overexpression promoted proliferation of HCC cells.** (A) TET3 overexpression in HepG2 was verified by western blot. (B) TET3 overexpression increased the proliferation of HepG2 in CCK8 assay. (C) TET3 overexpression increased the colony formation ability of HepG2 cells. (D) TET3 overexpression in SMMC-7721 was verified by western blot. (B) TET3 overexpression increased the proliferation of SMMC-7721 in CCK8 assay. (C) TET3 overexpression increased the colony formation ability of SMMC-7721 cells. **p < 0.01, ****p < 0.0001. Abbreviation: VT, the corresponding backbone vector.

Figure 4

**TET3 overexpression promoted metastasis of HCC cells.** TET3 overexpression increased the migration and invasion of HepG2 (A) and SMMC-7721 (B) cells in transwell assay. Scale bar: 100 μm. **p < 0.01, ***p < 0.001. Abbreviation: VT, the corresponding backbone vector.

Figure 5

**TET3 regulated HCC progression via lncRNA ARAP1-AS1.** (A) Top 50 upregulated genes in RNA-seq of VT/TET3-transduced HepG2 cells. (B) TET3 overexpression induced ARAP1-AS1 expression in both HepG2 and SMMC-7721 cells, which analyzed by qRT-PCR. (C) The knockdown effect of siRNA targeting ARAP1-AS1 was verified by qRT-PCR. (D) ARAP1-AS1 knockdown abolished the enhancement of proliferation induced by TET3 overexpression in CCK8 assay. (E) ARAP1-AS1 knockdown abolished the enhancement of colony formation ability induced by TET3 overexpression. **p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Abbreviations: VT, the corresponding backbone vector; siCo., nontarget control siRNA.