Original Article

TET3 Protein Represses Proliferation of the MG-63 Human Osteosarcoma Cell Line by Regulating DNA Demethylation: an Epigenetic Study

(Osteosarcoma / TET3 / 5hmC)

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Abstract. Recent studies have highlighted the significant role of 5-hydroxymethylcytosine (5hmC) in carcinogenesis. However, the specific role of 5hmC in osteosarcoma (OS) remains largely unexplored. Therefore, this study aimed to investigate the function of 5hmC and TET3 in OS. In this study, we found a decreased total level of 5hmC in OS tissues. The expression of the TET3 protein was also decreased in OS. Importantly, the decreased levels of TET3 were associated with a decreased disease-free survival (DFS) rate in patients. To investigate the role of TET3 and 5hmC in OS, we manipulated the levels of TET3 in MG-63 cells. Silencing TET3 in these cells resulted in a twofold increase in proliferation. Additionally, the level of 5hmC decreased in these cells. Conversely, over-expression of TET3 in MG-63 cells led to the expected inhibition of proliferation and invasion, accompanied by an increase in 5hmC levels. In conclusion, both 5hmC and TET3 protein levels were decreased in OS. Additionally, the over-expression of TET3 inhibited the proliferation of MG-63 cells, while the suppression of TET3 had the opposite effect. These findings suggest that decreased levels of 5hmC and TET3 may serve as potential markers for OS.

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Abbreviations: 5hmC – 5-hydroxymethylcytosine, 5mC – 5-methylcytosine, CCK-8 – cell counting kit-8, DFS – disease-free survival, GFP – green fluorescent protein, OS – osteosarcoma, PBS – phosphate-buffered saline, TET – ten-eleven translocation.

Introduction

Osteosarcoma (OS) is the most commonly diagnosed primary bone tumour and primarily affects adolescents. This type of cancer is characterized by its highly malignant and invasive behaviour (Biermann et al., 2010).

The implementation of combination chemotherapy and surgical interventions has greatly enhanced the survival rates of individuals diagnosed with OS. According to findings reported by Bielack et al. (2002), the 5-year survival rate for patients without metastatic tumours has increased to 65 %. Similarly, for patients with metastatic tumours, the survival rate has improved to 20 % according to the research of Kager et al. (2003). These advancements in treatment have contributed to more favourable outcomes for individuals diagnosed with OS. Despite significant progress in improving survival rates, the underlying mechanism of OS pathogenesis remains unclear (Fang et al., 2015). This knowledge gap has hindered the development of effective therapeutic strategies, particularly in cases where chemotherapy resistance is encountered. As a result, there is a pressing need for further research aimed at elucidating the mechanisms driving OS development and identifying novel

treatment approaches to overcome chemotherapy resistance. Such advancements would greatly enhance the prospects for more efficacious management of OS.

5-Hydroxymethylcytosine (5hmC) is a stable epigenetic marker that is generated through the catalytic activity of ten-eleven translocation (TET) family proteins, including TET1, TET2 and TET3 (Guo et al., 2011b; Ito et al., 2011). This discovery of 5hmC as a distinct modification of DNA was first reported in 2009 (Tahiliani et al., 2009). The TET enzymes play a crucial role in converting 5-methylcytosine (5mC) into 5hmC, thereby contributing to dynamic changes in DNA methylation patterns and gene regulation. The identification of 5hmC and its association with TET proteins have opened up new avenues of research in the field of epigenetics.

The presence of 5mC at gene promoters is known to be associated with the repression of gene transcription. In contrast, 5hmC, as an oxidation product of 5mC, has been found to reduce the overall levels of 5mC. This reduction in 5mC levels mediated by 5hmC plays a role in the regulation of gene expression networks within cells. By modulating the balance between 5mC and 5hmC, epigenetic processes can influence the activation or suppression of specific genes, ultimately shaping the cellular function and behaviour (Guo et al., 2011a; Ren et al., 2023).

Abnormalities in 5hmC levels have been associated with a wide range of diseases, including various types of cancers, through the regulation of different oncogenic pathways (Qi et al., 2022). The loss of global 5hmC levels has been recognized as a hallmark of multiple cancer types (Hu et al., 2017). Furthermore, studies have demonstrated that the loss of TET2 protein function can contribute to the development of myeloid malignancies (Moran-Crusio et al., 2011). Although the involvement of 5hmC levels in tumorigenesis is well established, the specific roles of 5hmC levels and TET enzymes in OS remain largely unexplored. Further investigation is needed to elucidate the significance and mechanisms underlying the dysregulation of 5hmC and TETs in OS.

In our study, we observed that OS tissue obtained from our patients exhibited decreased levels of 5hmC, which coincided with a decrease in TET3 protein expression. Subsequently, we conducted additional experiments to investigate the specific role of the TET3 protein in modulating 5hmC levels and regulating the proliferation of MG-63 human OS cells. These findings shed new light on potential therapeutic strategies for OS and identify a potential epigenetic biomarker for this type of malignancy. Our research contributes to a better understanding of OS and opens avenues for further exploration in the development of targeted therapies.

Materials and Methods

Human subjects

A total of 7 patients who were diagnosed with OS and hospitalized in our medical facility over the past year were included in this study (Table 1). The study protocol was approved by the ethics committees of The Fourth Hospital of Wuhan with No. KY2023-049-02. All participants provided informed consent before their enrolment. Surgical procedures were performed to remove the tumour tissue, along with a small amount of adjacent paracancerous tissue. The tumour tissue samples were carefully preserved at −80 °C for further analysis. The study protocol adhered strictly to the guidelines outlined in the Helsinki Declaration, ensuring that ethical considerations and patient rights were respected throughout the research process.

Table 1. Patients' information

Patient ID	Age	Gender	Body weight (kg)	TNMG stage
1	47	female	59	T1N0M0G2
2	63	female	52	T2N1M0G3
3	54	female	61	T1N0M0G2
4	54	male	63	T1N1M0G2
5	44	male	76	T2N0M1G2
6	58	male	68	T1N0M0G2
7	53	male	80	T1N1M0G2

Disease-free survival calculation

To investigate the influence of the levels of TET1, TET2, and TET3 on disease-free survival, we utilized the GEPIA database (http://gepia.cancer-pku.cn/index.html)(Tang et al., 2017). The survival module within GEPIA can be used to perform this analysis. For normalization, *GAPDH* was selected as the reference gene. In terms of cutoff values, we set the cutoff-high (%) and cutoff-low (%) to 30. Additionally, we chose the SARC dataset for our analysis. By inputting these parameters into the GEPIA database, insights into the relationships between the levels of TET1, TET2, and TET3 and disease-free survival in the SARC dataset could be obtained.

Cell culture and transfection

The MG-63 human OS cell line was cultured in maintenance medium consisting of DMEM supplemented with 10 % FBS (Gibco, Life Technologies, Carlsbad, CA). The medium was refreshed every other day to ensure optimal cell growth. Prior to transfection, the cells were plated at a density of 10^4 cells/cm^2 in 60-mm dishes and allowed to adhere for 24 hours. Using mammalian expression vector pcDNA3.1, constructs were created for over-expression of genes in the cell lines. Plasmid extraction was performed using a plasmid extraction kit (Beyotime Biotechnology, cat. D0005, Nantong, China). The plasmid of interest was transfected into the cells using Lipo2000 transfection reagent (Life Technologies) following the provided protocol for transfection procedures. Transfection efficiency was assessed by trans-

fecting the pcDNA3.1 vector carrying GFP and measuring it using flow cytometry. The transfection efficiency was controlled between 10–20 %.

CCK-8 assay

After 60 hours of plasmid transfection, the medium was discarded, and the cells were washed twice with phosphate-buffered saline (PBS). The relative cell viability was assessed using a standard Cell Counting Kit-8 (CCK-8) assay for cell proliferation. For each sample, 50 µl of CCK-8 solution (C0038, Beyotime Institute of Biotechnology) was added. Following a 2-hour incubation at 37 °C, 100 µl of the solution from each sample was transferred to a 96-well plate. The absorbance at a wavelength of 450 nm was measured using a microplate reader (Spectra Therm, Mannedorf, Switzerland). This measurement enabled evaluation of cell viability based on the CCK-8 assay.

Transwell assay

The transwell inserts with 8 µm pore size were purchased from Corning Incorporated (Corning, NY, catalogue number 353090). The invasion assays were conducted using transwell membranes that were pre-coated with 24 µg/µl Matrigel (BD Biosciences, Franklin Lakes, NJ). This experiment was performed following the protocol provided by Corning (https://www.corning.com/catalog/cls/documents/protocols/protocol_DL_031_Cell_Invasion_Assay.pdf). The cells adhering to the lower surface of the membranes were stained with crystal violet, and the number of invasive cells was quantified by counting the cells in five randomly selected microscopic fields at 200× magnification and calculating the average.

Western blot

To isolate the cellular proteins, the cells were lysed using RIPA lysis buffer (Beyotime Biotechnology) and incubated on ice for 30 minutes. The lysate was then subjected to centrifugation at 12,000 × *g* for 30 minutes at 4 °C to separate the supernatant containing the total protein. For protein analysis, 50 µg of total protein was loaded onto a sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. Following electrophoresis, the proteins were transferred from the gel to a PVDF membrane. The TET3 protein was detected using an antibody (Abcam, ab139311, Cambridge, UK) at a dilution of 1 : 2000. This antibody was used to specifically detect the TET3 protein in the transferred membrane. The secondary antibody used was horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Beyotime Biotechnology, cat. A0208) at a dilution of 1 : 500. The bands were visualized using the BeyoECL Plus chemiluminescent substrate kit (Beyotime, cat. P0018S) according to the manufacturer's instructions.

Quantitative polymerase chain reaction (qPCR)

To synthesize complementary DNA (cDNA), a total of 500 ng of RNA was isolated using TRIzol reagent. Reverse transcription was carried out using HIScript II Reverse Transcriptase (Vazyme, Nanjing, China). Following the manufacturer's instructions, 1 µl of cDNA was used in a 10 µl reaction system. The reaction system consisted of 0.2 µl of forward or reverse primers, 5 µl of 2 × ChamQ SYBR qPCR Master Mix and 0.2 µl of 50 × ROX2. qPCR was performed using an ABI 7500 Fast Instrument. qPCR analysis allowed for the measurement of gene expression levels based on the amplification of cDNA using specific primers and the SYBR Green detection method. The primers used are listed in Table 2.

Dot blot

The genomic DNA used in the dot blot assay was treated with RNase for 24 hours. Subsequently, the treated DNA was spotted onto an Amersham Hybond-N+ membrane (GE Healthcare, Chicago, IL). The membrane was then fixed using UV irradiation. To prevent nonspecific binding, the membrane was blocked with 5 % BSA. The presence of 5hmC was detected using an antibody (1 : 2000 dilution, cat. 39999, Active Motif, Carlsbad, CA). Each sample was evaluated at three different concentrations: 200 ng, 400 ng, and 1000 ng. The secondary antibody used was HRP-conjugated goat anti-mouse IgG (Beyotime Biotechnology, cat. A0216) at a dilution of 1 : 500. The dots were visualized using the BeyoECL Plus chemiluminescent substrate kit (Beyotim, cat. P0018S) according to the manufacturer's instructions. To ensure equal DNA loading, the membranes were stained with 0.02 % methylene blue solution for 5–10 minutes at room temperature. The membranes were then rinsed with distilled water until the background became clear and the spots appeared blue.

Table 2. Primer information

Primer	Sequence	Length
TET1 Forward Primer	CGCTACGAAGCACCTCTCTTA	21
TET1 Reverse Primer	CTTGCATTGGAACCGAATCATTT	23
TET2 Forward Primer	ATACCCTGTATGAAGGGAAGCC	22
TET2 Reverse Primer	CTTACCCCGAAGTTACGTCTTTC	23
TET3 Forward Primer	AGTGTCCGAAAGCCCATTCAG	21
TET3 Reverse Primer	GCAAATAGCGCAAGAGAAGGTT	22

Results

5hmC and TET3 protein levels are decreased in OS tissue

To investigate the specific roles of 5hmC in the development of OS, we examined the changes in global 5hmC levels between the OS tissue and surrounding normal tissue in patients with OS. We utilized the dot-blot technique to measure the levels of 5hmC in genomic DNA samples. As depicted in Fig. 1A and B, our results revealed a significant decrease in the levels of 5hmC in the OS tissue compared to the surrounding normal tissue. To ensure that equal amounts of DNA were spotted on the membranes, we stained the membranes with 0.02 % methylene blue, as depicted in Fig. 1C. These findings suggest that there is a considerable reduction in global 5hmC levels in OS tissue, which may indicate its involvement in the pathogenesis of OS.

Given that the TET family proteins are known to catalyse the conversion of 5mC (5-methylcytosine) to 5hmC (5-hydroxymethylcytosine), we sought to investigate

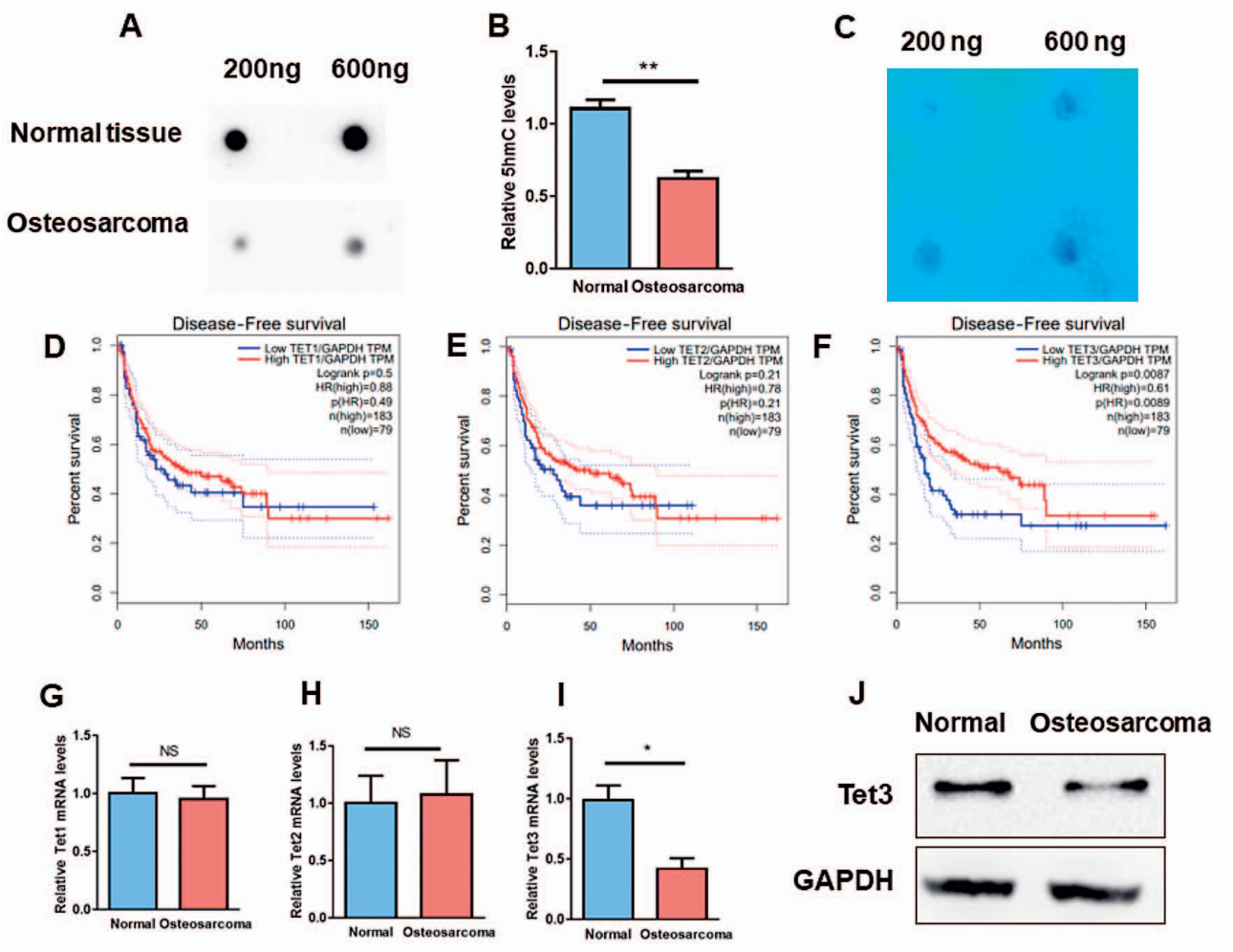

Fig. 1. 5hmC and TET3 protein levels are decreased in OS tissue.
We conducted a dot blot assay to investigate the changes in the modification level of 5hmC in OS and surrounding tissues. Subsequently, we assessed the expression levels of TET3 at the mRNA and protein levels using qPCR and Western blot analysis, respectively.
(**A**) Dot blot results showing 5hmC levels in OS tissues compared to surrounding tissues.
(**B**) Grey scanning result of picture A.
(**C**) Film with DNA spotted on the membrane, which was stained with 0.02 % methylene blue. This is to ensure equal DNA loading.
D–F shows the impact of TET3 expression on patient outcomes. We utilized the disease-free survival (DFS) rates of sarcoma patients in the high (30 %) and low (30 %) TET1 (**D**), TET2 (**E**), and TET3 (**F**) expression groups. The DFS rates were calculated using GEPIA (http://gepia.cancer-pku.cn/index.html), and a log-rank P value less than 0.05 was considered to indicate statistical significance.
(**G–H**) qPCR was used to measure the TET1 (**G**), TET2 (**H**) and TET3 (**I**) mRNA levels.
(**J**) TET3 protein levels in OS tissue. $^{*}P < 0.05$, $^{**}P < 0.01$.

the potential impact of TET levels on the disease-free survival rate of sarcoma patients. To accomplish this, we utilized data from the GEPIA database (http://gepia.cancer-pku.cn/index.html) (Fig. 1D–F). GEPIA is a valuable resource that provides gene expression data and survival data for various cancers. By analysing the expression levels of *TET* genes and correlating them with the disease-free survival rates of sarcoma patients, we aimed to elucidate any potential associations. Our analysis revealed a noteworthy finding: only high levels of TET3 significantly increased the survival rate of sarcoma patients, with a log-rank P value of 0.0089 (Fig. 1F). In contrast, TET1 and TET2 did not exhibit such a correlation.

To further investigate the expression levels of TETs (including TET1, TET2, and TET3) in OS tissue, we performed quantitative PCR. Fig. 1G and 1H show that compared with that in the surrounding tissue, the expression of TET1 and TET2 in the OS tissue exhibited minimal changes. However, the expression of TET3 dramatically decreased at both the transcriptional and translational levels (Fig. 1I–J).

Taken together, these findings indicate that the reduction in 5hmC in OS tissues is likely attributed to the decreased expression of TET3.

Over-expression of the TET3 protein inhibited proliferation of MG-63 cells

To investigate the functional role of TET3 and 5hmC in the development of OS, we conducted further research using the human OS cell line MG-63. Our initial step was to examine the mRNA levels of TETs in the MG-63 cell line, where we observed that among the three TETs, TET3 exhibited the lowest expression (Fig. 2A).

To investigate the impact of TET3 and its association with 5hmC in OS, we generated a full-length TET3 expression vector (pcDNA3.1-oeTET3) to over-express the TET3 protein in the MG-63 cell line. As expected, the protein level of TET3 in MG-63 cells transfected with pcDNA3.1-oeTET3 was significantly greater than that in the control group after 48 hours of transfection (Fig. 2B). Subsequently, we examined the overall levels of 5hmC to ascertain whether TET3 over-expression

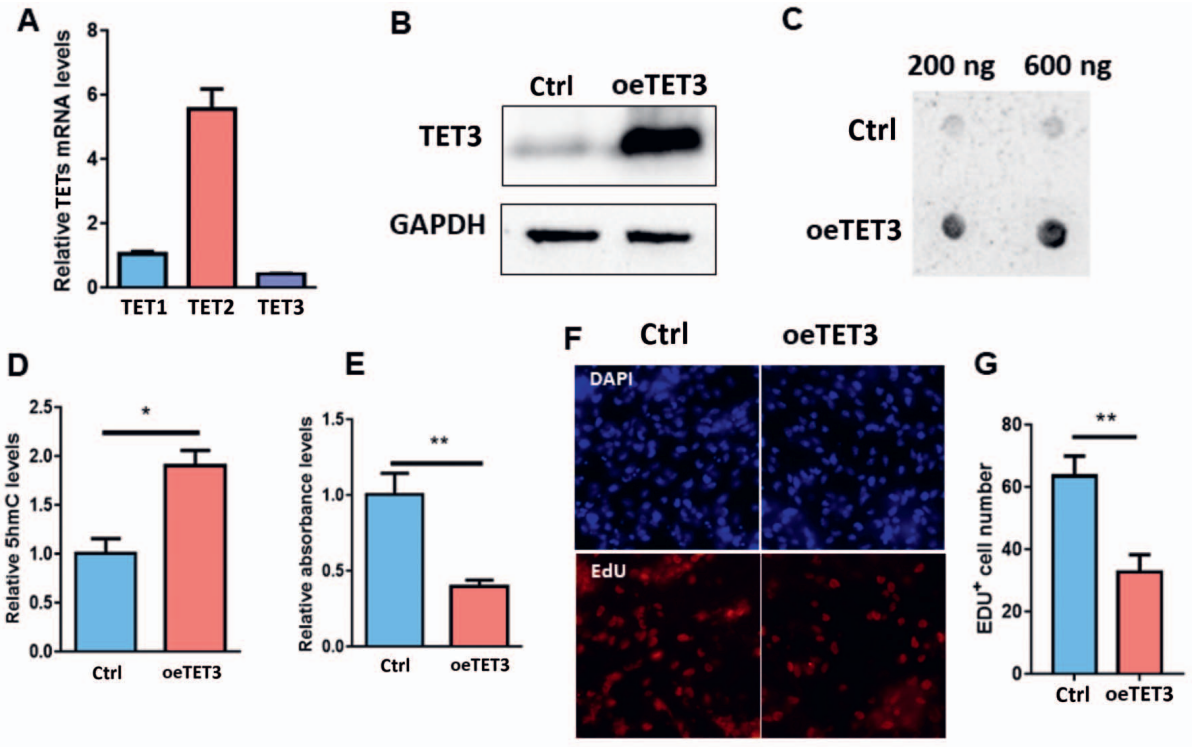

Fig. 2. Over-expression of TET3 protein inhibited proliferation of MG-63 cells.
The proliferation efficiency of the cells was verified by CCK-8 and EdU assays.
(**A**) TET mRNA levels were assayed by qPCR.
(**B**) Western blot analysis of the level of the TET3 protein after oeTET3 transfection for 48 h.
(**C**, **D**) Dot blot showing the total 5hmC level in the genomic DNA of MG-63 cells ($P < 0.05$) after oeTET3 transfection for 48 h.
(**E**) A CCK-8 assay was used to determine the proliferation rate of MG-63 cells transfected with pc-oeTET3. Ctrl represents the control group and was transfected with an empty vector.
(**F**, **G**) Proliferation of MG-63 cells assayed by EdU.
oeTET3: over-expressed TET3. $^{*}P < 0.05$, $^{**}P < 0.01$.

could positively regulate 5hmC in the MG-63 cell line. As predicted, the level of 5hmC in the genomic DNA of MG-63 cells over-expressing TET3 was greater than that in the genomic DNA of cells transfected with the control vector (Fig. 2C–D). This result strongly suggested that the over-expression of TET3 may lead to an increase in 5hmC levels in the MG-63 cell line. These findings provide valuable insights into the potential role of TET3 in modulating 5hmC levels, highlighting its significance in OS pathogenesis.

To evaluate the influence of the TET3 protein on the proliferation of MG-63 cells, we conducted a CCK-8 assay. As depicted in Fig. 2E, the proliferation of MG-63 cells was significantly inhibited upon transfection with pcDNA3.1-oeTET3, in contrast to that of the control group, which was transfected with pcDNA3.1 ($P < 0.01$). This finding clearly demonstrated that TET3 exerts a notable effect on the proliferation of MG-63 human OS cells. Furthermore, to further validate this observation, we conducted EdU incorporation experiments. The results, shown in Fig. 2F and 2G, provide additional evidence that high TET3 expression can indeed impede the proliferation of MG-63 cells.

Knockdown of TET3 promoted proliferation of MG-63 cells

Based on previous findings that the TET3 protein can modulate the level of 5hmC during various biological processes, including cellular differentiation and tumorigenesis, we aimed to investigate whether down-regulation of TET3 could lead to a reduction in 5hmC levels, potentially impacting OS pathogenesis. To achieve this goal, we constructed an shRNA expression vector (pGPU6-shTET3) designed to target TET3 mRNA and decrease the expression of the TET3 protein.

The expression level of TET3 in MG-63 cells transfected with pGPU6-shTET3 was assessed using both Western blot and quantitative RT-PCR assays. As depicted in Fig. 3A and 3B, the data clearly demonstrated an 80 % decrease in TET3 expression compared to that in control cells ($P < 0.05$), indicating successful down-regulation of TET3. As expected, when TET3 was knocked down, the level of 5hmC also decreased, as assessed by a dot blot assay (Fig. 3C–D). These results further support the notion that TET3 plays a critical role in regulating the levels of 5hmC and that its down-regulation can impact the 5hmC dynamics in OS cells.

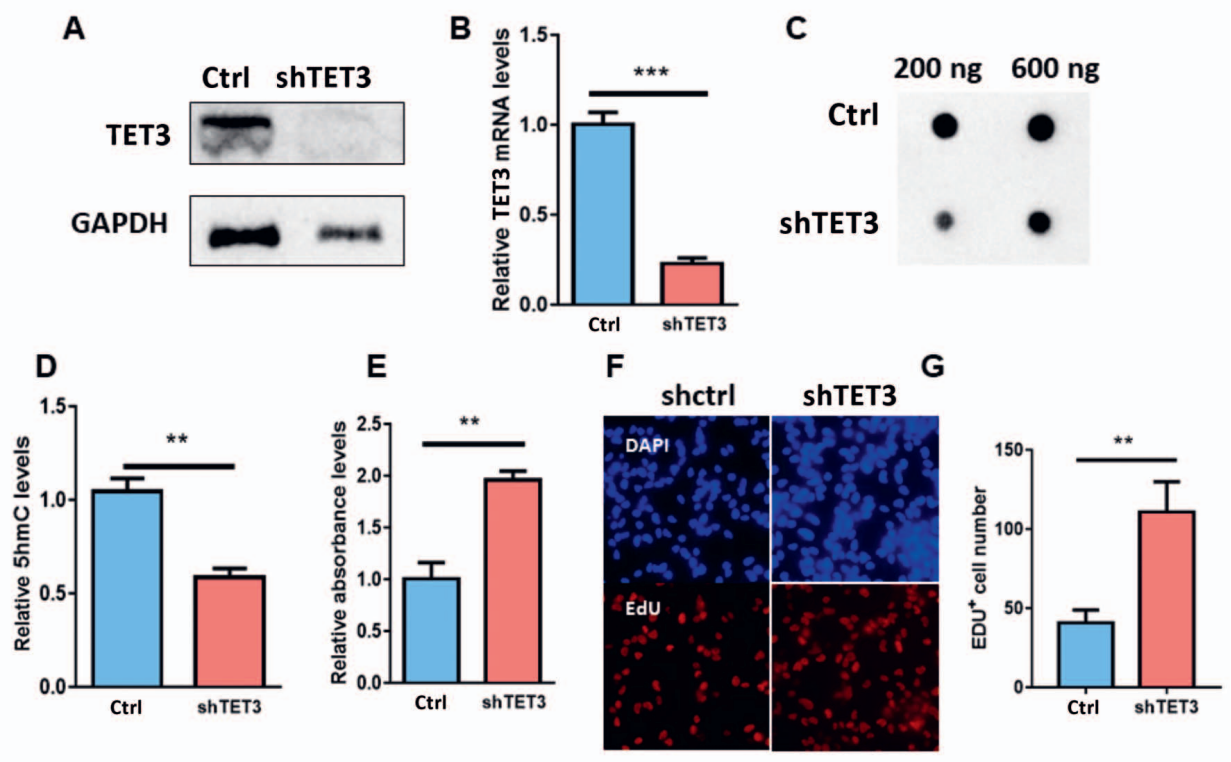

Fig. 3. Decreased TET3 promotes proliferation of MG-63 cells.
(**A**) Protein level of TET3 after transfection with shTET3 for 48 h.
(**B**) mRNA level of Tet3 after transfection with shTET3 for 48 h.
(**C**, **D**) Total 5hmC level in the genomic DNA of MG-63 cells after transfection with shTET3 for 48 h. The cells were examined by dot blot.
(**E**) A CCK-8 assay was used to examine the proliferation of MG-63 cells transfected with pc-shTET3.
(**F**, **G**) EDU assays were used to determine the proliferation of MG-63 cells. $^{**}P < 0.01$, $^{***}P < 0.001$.

To investigate the specific role of the TET3 protein in regulating 5hmC levels during OS pathogenesis, we examined whether the loss of TET3 could impact cell proliferation. To test this possibility, we conducted a CCK-8 assay to assess the effect of TET3 down-regulation on MG-63 cells. Interestingly, compared to control cells, MG-63 cells transfected with shTET3 exhibited significantly enhanced proliferation (Fig. 3E). These findings suggest that the down-regulation of the TET3 protein may not only decrease total DNA 5hmC levels but also promote the proliferation of MG-63 cells. Furthermore, the increased proliferation of MG-63 cells upon TET3 down-regulation was further confirmed by EdU incorporation experiments (Fig. 3F–G). Taken together, these results indicate that the loss of TET3 may have dual effects: decreasing the overall level of 5hmC in total DNA and promoting the proliferation of MG-63 cells. These findings provide important insights into the potential role of TET3 and its impact on both epigenetic modifications and cell proliferation in the context of OS.

TET3 influenced invasion of MG-63 cells

To further explore the functional role of the TET3 protein in MG-63 cells, we conducted a transwell assay to investigate its relationship with cell invasion. Interestingly, we observed that over-expression of TET3 via pcDNA3.1-oeTET3 transfection led to a reduction in the number of migrated cells, which indicates that TET3 potentially inhibits the invasion of MG-63 cells (Fig. 4A–B). This result aligns with our initial predictions.

To compare the effects of TET1 and TET2 on MG-63 cell invasion, we performed a similar transwell assay after suppressing the expression of TET1 or TET2 using specific shRNAs (Fig. 4C–D). Surprisingly, when TET1 or TET2 expression was down-regulated, there was little effect on cell invasion compared to that in the control group (Fig. 4E).

These findings suggest that among the three TET proteins (TET1, TET2 and TET3), TET3 appears to play a specific role in regulating the invasion of MG-63 cells,

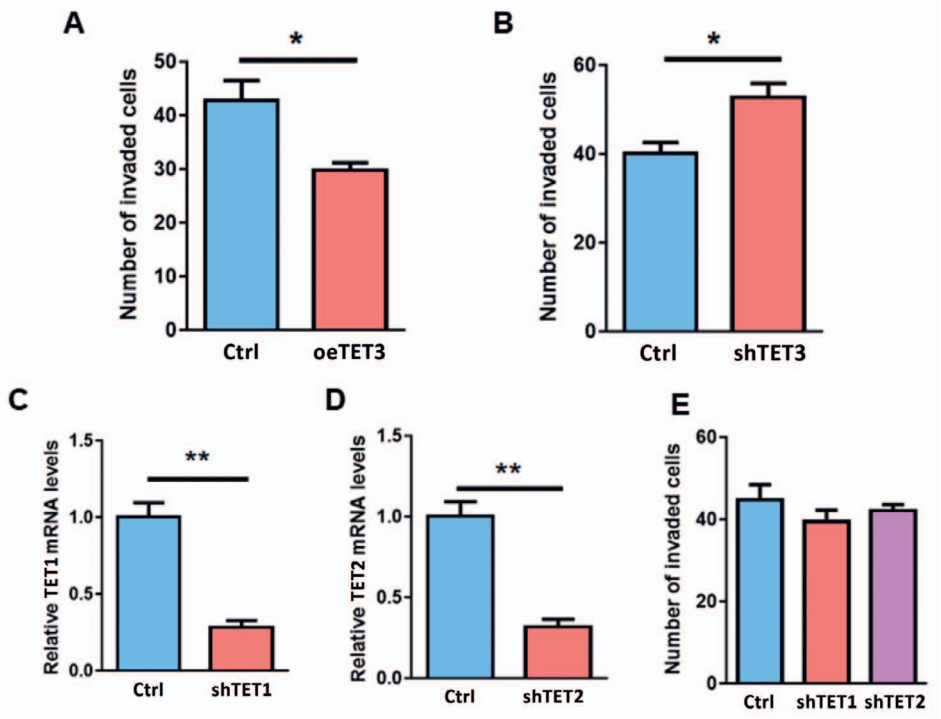

Fig. 4. TET3 influences invasion of MG-63 cells.
(**A**) Transwell assay for over-expressing TET3 (oeTET3) in MG-63 cells. The empty vector was transfected into MG-63 cells as a control group.
(**B**) Transwell assay for knockdown of TET3 (shTET3) in MG-63 cells. The empty vector was transfected into MG-63 cells as a control group.
(**C**) Relative mRNA level of TET1 in TET1-knockdown MG-63 cells (shTET1). The empty vector was transfected into MG-63 cells as a control group.
(**D**) Relative mRNA level of TET2 in TET2-knockdown MG-63 cells (shTET2). The empty vector was transfected into MG-63 cells as a control group.
(**E**) Transwell assays for knockdown of TET1 (shTET1) and Tet2 (shTET2) in MG-63 cells. The empty vector was transfected into MG-63 cells as a control group.
$^{*}P < 0.05$, $^{**}P < 0.01$.

while TET1 and TET2 may not exert a significant influence on this aspect of cell behaviour.

Discussion

Recent studies have highlighted the significant role of 5hmC in regulating gene expression, particularly during critical developmental processes and in various diseases such as embryonic development, neural stem cell differentiation, Alzheimer's disease and carcinogenesis.

Numerous lines of evidence strongly suggest that 5hmC plays a crucial role in cancer development. It is believed to be involved in the regulation of gene expression throughout the process of tumorigenesis and tumour progression (Moran-Crusio et al., 2011). The overall abundance of 5hmC undergoes noticeable changes during the development of different types of cancers. For instance, in ovarian carcinoma, 5hmC levels are significantly lower in the cancerous tissue than in the surrounding tissue (Tucker et al., 2018). This alteration in 5hmC levels can lead to changes in gene expression patterns and activation of genes associated with tumorigenesis. Consequently, 5hmC has gained recognition as a potential biomarker for certain types of cancers.

Our study provides evidence supporting the potential of 5hmC as a stable biomarker for OS. Our data consistently demonstrated that the level of 5hmC in global genomic DNA is significantly lower in OS tissue than in adjacent tissues. This trend was consistent across all samples analysed in our study, despite some individual variations in the baseline 5hmC levels. The consistent decrease in 5hmC levels during OS genesis suggests that this epigenetic modification plays a crucial role in the formation of OS. This finding underscores the potential importance of 5hmC as a key factor in OS development and supports its potential as a biomarker for this disease. The stability of 5hmC levels across samples strengthens its candidacy as a reliable biomarker. By assessing the 5hmC levels in patient samples, it may be possible to identify individuals at risk for or already affected by OS, enabling early detection and intervention.

The TET family proteins, including TET1, TET2, and TET3, play a crucial role in DNA demethylation by catalysing the conversion of 5mC to 5hmC. Although these three proteins share the same catalytic function, studies suggest that they have distinct roles in various biological processes.

For example, TET3 is particularly important for DNA demethylation in sperm and egg cells, contributing to the establishment of epigenetic patterns during early development. However, the level of TET3 decreases in the early postnatal period. In contrast, studies have shown that TET1 knockdown in mice leads to learning disorders, indicating its involvement in cognitive function. Interestingly, mice lacking the TET3 protein do not display the same cognitive impairments (Zhang et al., 2013). More recently, there has been evidence suggesting a specific functional role for TET2 in regulating the generation of adult NSCs. However, studies have not identified similar functions for TET1 or TET3 in this context.

The role of TET3 also varies in different cancer types. In some cases, it acts as an oncogene (Pulikkottil et al., 2022; Xue et al., 2023; Yang et al., 2023), while in other cases, it acts as a tumour-suppressing gene (Yang et al., 2015; Carella et al., 2020; Mo et al., 2020). However, there have been relatively few reports on the specific genes targeted by TET3 hydroxymethylation. Wang et al. (2016) reported that TET3 represses EZH2 in breast cancers. Kong et al. (2022) reported that TET3 up-regulates MUC13 via promoter hypomethylation. The details of TET3-targeted genes need to be further explored.

Mutations in TETs were found in 0.1–10 % of major types of cancer (Rasmussen and Helin, 2016). TET3 not only has frameshift mutation but also loss of expression in the tumorigenesis of GC (Mo et al., 2020). Xu et al. (2023) reported mutations of *TET* genes in 7.4 % of human lung adenocarcinomas. All these findings indicate that the TET family plays an important role in tumorigenesis.

Our study revealed a significant correlation between the TET3 level and survival rate in sarcoma patients. We observed that the expression level of the TET3 protein was substantially lower in OS tissue than in paracarcinoma tissue. In contrast, TET1 and TET2 showed minimal changes when assessed using Western blot and qPCR techniques. Considering the concurrent decrease in 5hmC levels, we concluded that the reduction in the levels of TET3 protein, but not TET1 or TET2, was responsible for the decrease in 5hmC levels in OS tissue. This finding highlights the crucial role of TET3 in modulating the global level of 5hmC during the formation of OS. These results suggest that TET3 may serve as a potential diagnostic or prognostic biomarker for OS. Patients with higher levels of TET3 may have better survival outcomes.

Our additional data revealed that the proliferation, invasion, and migration of the MG-63 human OS cell line could be influenced by controlling expression of the TET3 protein. Specifically, when TET3 expression was knocked down using the shTET3 plasmid, we observed a significant increase in the ratio of proliferation and invasion compared to that in control cells. This indicates that TET3 plays a crucial role in regulating these cellular processes in MG-63 cells. In contrast, similar effects on proliferation and invasion were not observed when TET1 or TET2 expression was controlled. This finding suggested that TET3 specifically influences the proliferation and invasion of MG-63 cells, while TET1 and TET2 may have different functions or may not be directly involved in these processes in the context of OS (Fig. 5). These findings highlight the unique and specific role of TET3 in mediating the proliferation and invasion of OS cells.

Although our study demonstrated that TET3 inhibits OS progression by increasing 5hmC levels, the specific mechanisms through which elevated 5hmC represses OS remain unclear. This is because 5hmC regulates ex-

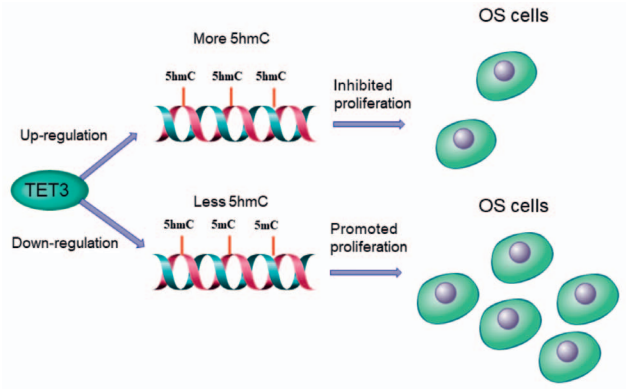

Fig. 5. Graphic summary
Up-regulated TET3 will increase the level of 5hmC on the genome by converting 5mC to 5hmC, which inhibits OS cell proliferation. Down-regulated TET3 will decrease the level of 5hmC on the genome by converting 5mC to 5hmC, which promotes OS cell proliferation.

pression of numerous genes, making it necessary to investigate this phenomenon from a systems biology perspective. Further research is required to comprehensively understand how increased 5hmC levels contribute to the repression of OS.

In conclusion, our study revealed that lower expression levels of the TET3 protein during OS pathogenesis promote the proliferation of OS cells. Moreover, we propose that 5hmC has the potential to serve as a biomarker for OS. Additionally, TET3 may represent a promising therapeutic target for the treatment of OS. Further investigations are needed to uncover the underlying molecular mechanisms involved and to develop novel therapeutic strategies based on these findings.

Ethics approval and consent to participate

The study protocol was approved by the ethics committees of The Fourth Hospital of Wuhan with No. KY2023-049-02. All participants provided informed consent before their enrolment.

Consent for publication

All the authors agreed to publish this work.

Data availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

All the authors declare that there are no competing interests.

AI declaration

This work is not based on AI tools. However, ChatGPT3.5 was used for language polishing.

Author contribution

SJH and BJC performed the experiments and drafted the manuscript. ZL edited the manuscript, performed the bioinformatic and statistical work. ACW and HTS conceived the idea and supervised the project.

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