

TFAM is directly regulated by miR-23b in glioma

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Abstract. Mitochondrial transcription factor A (TFAM), a high-mobility group (HMG) protein, plays a central role in mitochondrial DNA (mtDNA) replication, transcription and inheritance. It has been shown that TFAM is associated with tumorigenesis. However, little is known regarding the post-transcriptional regulation of TFAM in glioma. In the present study, we found that the protein levels of TFAM were gradually increased, while the expression of miRNA-23b was gradually downregulated with the malignancy of glioma. Luciferase assay data demonstrated that miRNA-23b directly regulated TFAM. Furthermore, forced overexpression of miRNA-23b in U251 cells markedly inhibited the proliferation, cell cycle progression, migration and colony formation, while overexpression of TFAM significantly enhanced these biological processes. We further examined the related molecular mechanism, and found that the activity of the PI3K/Akt signaling pathway, critical for cell proliferation and migration, was suppressed in miRNA-23b-overexpressing U251 cells but was upregulated in TFAM-overexpressing cells. In addition, the expression levels of invasion-related MMP2 and MMP9 were decreased in miRNA-23b-overexpressing U251 cells but were increased in TFAM-overexpressing cells. Taken together, the present study provides a new regulatory mechanism as well as a promising therapy target for glioma.

Introduction

Glioma, the most common brain tumor, accounts for ~30% of all brain and central nervous system tumors and 80% of all malignant brain tumors (1). Over the past 10 years, the 5-year survival rate of glioblastoma patients was only 2%, and the median survival was 1 year (2,3). In fact, the prognosis for glioblastoma has not improved for an extensive period of time, due to its resistant to radiotherapy, chemotherapy and adjuvant therapies (4). As a result, development of a promising therapeutic target is urgently needed.

Mitochondria are fundamental to metabolic homeostasis (5). Mitochondrial transcription factor A (TFAM), a high-mobility group (HMG) protein, plays a central role in mitochondrial DNA (mtDNA) replication, transcription and inheritance (6). Aberrant function of mitochondria has been reported to be associated with various metabolic disorders, such as cancer (7). Thus, investigating the role of TFAM in malignant tumors may provide critical knowledge for better understanding the regulatory mechanism of cancer as well as a promising therapeutic strategy.

microRNAs (miRNAs), endogenous non-coding RNAs, regulate gene expression by binding to the 3' untranslated region (UTR) of target mRNAs and act as endogenous agents of RNA interference, resulting in either mRNA degradation or translational repression (8,9). The role of miRNAs in brain physiology and tumorigenesis has confirmed their use as promising therapy targets for glioma (10). It has been shown that miR-23b is highly expressed in tumors and is associated with tumorigenesis (11,12); however, the role of miR-23b in glioma remains unclear. The aim of the present study was to elucidate the role of TFAM and miR-23b as well as the related molecular mechanism in malignant glioma.

Materials and methods

Materials and reagents. Human glioma U251 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Fetal bovine serum (FBS) and TRIzol reagent were obtained from Invitrogen (Carlsbad, CA, USA). Dulbecco's modified Eagle's medium (DMEM) and MTT were purchased from Sigma (St. Louis, MO, USA). The cell cycle detection kit was purchased from Nanjing Jikai (Nanjing, China). All antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). SYBR-Green qPCR Mix was purchased from Toyobo (Osaka, Japan). The reverse transcription kit was obtained from Fermentas (Hanover, MD, USA). TaqMan qRT-PCR miRNA assay kit was purchased from Applied Biosystems (Foster City, CA, USA).

Cell culture. Human glioma U251 cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C with 5% CO₂.

Tissue specimen collection. All protocols were approved by the Ethics Committee of the Central South University. Informed consent was obtained from each patient. All normal brain tissues and glioma tissues were collected from patients at

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the Department of Neurosurgery, Third Xiangya Hospital of Central South University, Changsha, Hunan, China. Patients with no history of other tumors were diagnosed with gliomas and were untreated. Following surgical removal, all tissues were immediately snap-frozen in liquid nitrogen and stored until use.

RNA extraction and real-time RT-PCR analysis. Total RNA was extracted using TRIzol reagent according to the manufacturer's protocol. RNA was then reverse transcribed into a cDNA template using the reverse transcription kit. miR-23b expression was examined using the TaqMan qRT-PCR miRNA assay kit. The relative expression of miR-23b was normalized to U6. For TFAM assay, SYBR-Green qPCR Mix was then used to perform qRT-PCR. The TFAM primer is as follows: forward 5'-CCATCTACCGACCGGATGTTA-3' and reverse 5'-CAGACCTTCCCAGGGCACTCA-3'. β -actin primer is as follows: forward 5'-AGGGGCCGGAAGCTGTCATACT-3' and reverse 5'-GGCGGCACCACCATGTACCT-3'.

Western blotting. Glioma tissues or U251 cells were solubilized in cold RIPA lysis buffer. Subsequently, protein was separated with 5% SDS-PAGE. After SDS-PAGE, proteins were transferred from the gel to PVDF membranes. Membranes were blocked in 5% non-fat dried milk in PBST for 3 h and then incubated overnight with specific primary antibodies (Santa Cruz Biotechnology) with β -actin as a control. After incubation with the appropriate secondary antibody (Santa Cruz Biotechnology), immune complexes were detected using an ECL kit (Huyu Co., Shanghai, China).

Dual luciferase reporter assays. A normal and a mutated 3'-UTR of TFAM were constructed by PCR, and then inserted into the multiple cloning sites in the psiCHECK™-2 luciferase miRNA expression reporter vector.

For the luciferase assay, 20,000 cells were cultured to ~50-60% confluence in a 24-well plate. For each well, medium was replaced by 300 μ l OPTI-MEM medium (Invitrogen). Cells were cotransfected with psiCHECK™-2-TFAM-3'-UTR or psiCHECK™-2-mut TFAM-3'-UTR vector plus 50 nM miR-23b or 100 mM miR-23b inhibitor using Cellfectin II reagent (Invitrogen), according to the manufacturer's instructions. Cells were incubated with transfection reagent/DNA complex for 5 h and then refreshed with fresh complete medium. A dual-luciferase reporter assay system (Promega, Madison, WI, USA) was used to determine the luciferase activities 48 h after cotransfection. *Renilla* luciferase activity was normalized to firefly luciferase activity.

Plasmid construction and transfection. The plasmids for TFAM, miR-23b, miR-SCR as well as the retroviral supernatants were obtained from Aijia Bio (Changsha, China). U251 cells were transduced using 20 mg/ml polybrene for over 48 h.

MTT assays. For all groups, 5,000 cells/well were plated in a 96-well plate. MTT (0.5%) was added to each well and incubated for 4 h at 37°C in 5% CO₂. The reaction liquid was discarded. The formazan product was dissolved by adding 150 μ l DMSO to each well. The absorbance was detected

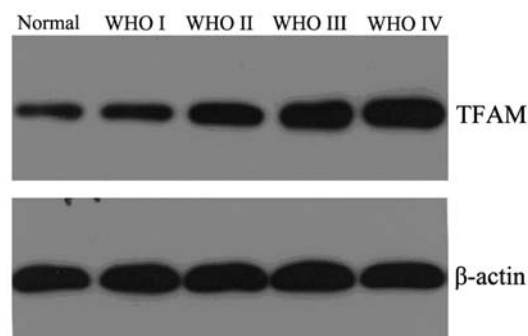


Figure 1. Protein expression of TFAM in glioma. Western blot analysis was performed to examine the TFAM protein levels in normal brain tissue and glioma tissues of different grades.

at 570 nm with a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

Cell cycle analysis. For all groups, 10⁶ cells were collected in 1X PBS and resuspended in 70% ethanol to fix overnight at -20°C. Cells were pelleted at 1,000 rpm for 5 min, washed in 1X PBS, and then pelleted at 1,000 rpm for 5 min. Cells were resuspended in 300 μ l propidium iodide (PI) staining buffer and incubated for 30 min at room temperature. DNA content analyses were performed using flow cytometry (FACSCalibur, Beckman Coulter).

Colony formation assay. For all groups, 3 ml complete medium containing 150 cells was added to each well of a 6-well plate. Plates were incubated at 37°C in 5% CO₂ for 14-21 days. After that, cells were gently washed and stained with Giemsa. Colonies containing at least 50 cells were counted.

Transwell assay. For all groups, the invasive ability of cells was determined by using 24-well Transwell chambers (Chemicon, Temecula, CA, USA). After a 24-h incubation at 37°C, the migrated cells were stained and counted.

Statistical analysis. Statistical analysis was performed using SPSS 19.0 statistical software. All data are expressed as the mean value \pm SD of triplicate experiments, and all experiments were repeated at least three times. The data were analyzed by one-way analysis of variance (ANOVA) and the Student's t-test. A P-value of <0.05 was considered to indicate a statistically significant result.

Results

TFAM protein levels are upregulated with the malignancy of glioma. The protein levels of TFAM in normal brain tissues and glioma tissues of different grades were examined by western blot analysis. Data showed that the protein expression levels of TFAM in glioma were significantly increased, when compared with those in normal brain tissues. Moreover, the protein levels of TFAM were positively correlated to the malignancy of glioma (Fig. 1).

miR-23b levels are decreased with the malignancy of glioma. The miR-23b expression levels in normal brain tissues, adjacent

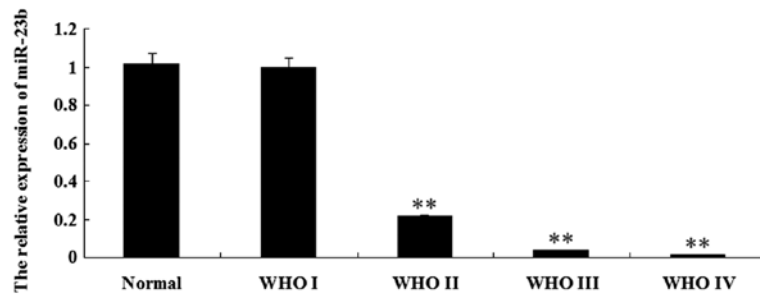


Figure 2. mRNA expression of miR-23b in glioma. Real-time PCR was used to determine the relative expression of miR-23b in normal brain tissues and glioma tissues of different grades. ** $P < 0.01$.

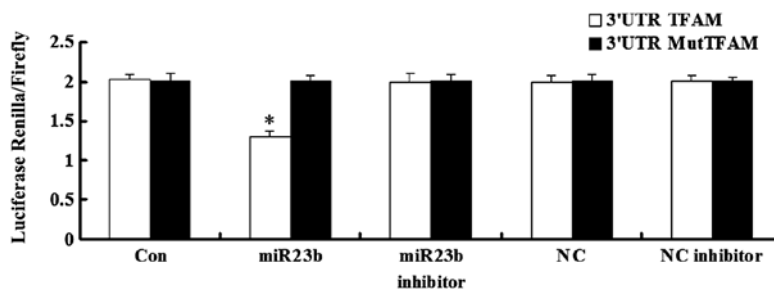


Figure 3. TFAM is the target of miR-23b. Luciferase assay was used to determine whether TFAM is the target of miR-23b. A normal and a mutated 3'-UTR of TFAM was subcloned into the psiCHECKTM-2 reporter vector. psiCHECKTM-2-TFAM 3'-UTR or psiCHECKTM-2-mut TFAM-3' UTR vector plus 50 nM miR-23b or 100 mM miR-23b inhibitor were cotransfected. Con, U251 cells; NC, U251 cells transfected with lentiviral vectors; NC inhibitor, U251 cells transfected with NC inhibitor.

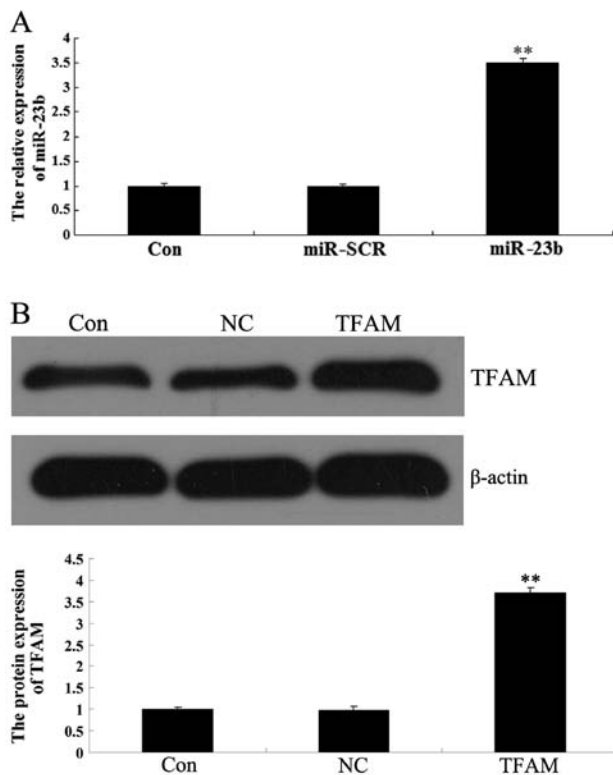


Figure 4. Expression of miR-23b and TFAM after transfection. (A) Real-time PCR was used to determine the relative expression of miR-23b in U251 cells after transfection. Con, U251 cells; miR-SCR, U251 cells transfected with miR-SCR lentiviral vectors; miR-23b, U251 cells transfected with miR-23b lentiviral vectors. (B) Western blot analysis was performed to examine the TFAM protein level after transfection. Con, U251 cells; NC, U251 cells transfected with NC virus; TFAM, U251 cells transfected with TFAM lentiviral vectors. ** $P < 0.05$.

tissues and gliomas were determined by real-time RT-PCR. As shown in Fig. 2, the expression levels of miR-23b in glioma were significantly lower than those in the normal and adjacent tissues ($P < 0.05$). Additionally, the miR-23b expression levels were negatively correlated with the malignancy of glioma.

TFAM is the direct target of miR-23b. Luciferase assay was applied to test whether TFAM is a direct target of miR-23b. As demonstrated in Fig. 3, the *Renilla*/firefly value of luciferase was significantly lower in miR-23b and 3'UTR of TFAM cotransfected cells, when compared with each control. However, the *Renilla*/firefly value of luciferase in cells cotransfected with miR-23b and mutated 3'UTR of TFAM showed no difference with that in each control. These findings demonstrated that 3'UTR of TFAM is the direct target of miR-23b.

Expression levels of miR-23b or TFAM after transfection. After transfection of miR-23b or TFAM lentiviral vectors into U251 cells, we determined the expression levels of miR-23b or TFAM, respectively. As shown in Fig. 4A, the miR-23b expression level in U251 cells was markedly increased after transfection when compared with the level in the controls ($P < 0.05$). In addition, the TFAM expression in U251 cells was significantly upregulated after transfection when compared with that in the controls ($P < 0.05$) (Fig. 4B). These data demonstrated that the transient transfection was successful.

Effects of TFAM and miR-23b overexpression on cell proliferation in U251 cells. To further study the roles of TFAM and miR-23b in glioma *in vitro*, an MTT assay was applied to determine the effects of miR-23b and TFAM overexpression

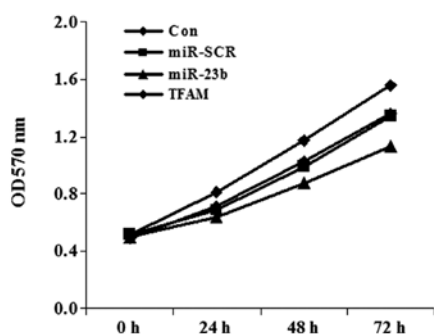


Figure 5. Effect of miR-23b and TFAM overexpression on proliferation of U251 cells. MTT was performed to determine the effect of miR-23b and TFAM overexpression on U251 cells proliferation. Con, U251 cells; miR-SCR, U251 cells transfected with miR-SCR lentiviral vectors; miR-23b, U251 cells transfected with miR-23b lentiviral vectors. TFAM, U251 cells transfected with TFAM lentiviral vectors.

on U251 cell proliferation. MTT results showed that in TFAM-overexpressing U251 cells, the cell proliferation was upregulated when compared with that in the controls. In contrast, however, in the miR-23b-overexpressing U251 cells, the cell proliferation was decreased when compared with that in the controls (Fig. 5). These findings revealed that miR-23b inhibited cell proliferation of U251 cells while TFAM promoted it.

Effects of TFAM and miR-23b overexpression on cell cycle progression in U251 cells. We further examined the cell cycle distribution in the different groups. As shown in Fig. 6, the miR-23b-overexpressing U251 cells showed the highest percentage of cells in the G2 stage when compared with other three groups, indicating that mitosis was blocked in the G2 stage. The TFAM-overexpressing U251 cells exhibited the

highest percentage of cells in the G1 and S stages, and only a few cells were in the G2 stage, suggesting that TFAM-overexpressing U251 cells were in a rapidly dividing state. These findings demonstrated that miR-23b blocked the cell cycle progression of U251 cells, entirely contrary to the effect of TFAM on human glioma U251 cells.

Effects of TFAM and miR-23b overexpression on colony-formation efficiency of U251 cells. The effects of TFAM and miR-23b overexpression on colony-formation efficiency in U251 cells were studied. As demonstrated in Fig. 7, the miR-23b-overexpressing U251 cells exhibited the lowest colony-formation efficiency, while the TFAM-overexpressing U251 cells showed the highest colony-formation efficiency, when compared with controls ($P<0.05$).

Effects of TFAM and miR-23b overexpression on the invasion of U251 cells. The changes in the cell invasive ability of U251 cells were examined after transfection with TFAM or miR-23b. As shown in Fig. 8, TFAM overexpression significantly promoted cell invasion, while miR-23b overexpression notably inhibited the invasion of U251 cells, when compared with the controls ($P<0.05$).

Effects of TFAM and miR-23b overexpression on the PI3K/Akt signaling pathway in U251 cells. Changes in the PI3K/Akt signaling pathway and invasion-related proteins in U251 cells were studied after transfection with TFAM or miR-23b. The protein levels of PI3K, p-PI3K, AKT, p-Akt, MMP2 and MMP9 were examined by western blot analysis. As shown in Fig. 9, the expression levels of PI3K, p-PI3K, AKT, p-Akt, MMP2 and MMP9 were all decreased in the miR-23b-overexpressing U251 cells, but upregulated in the TFAM-overexpressing U251 cells, when compared with the

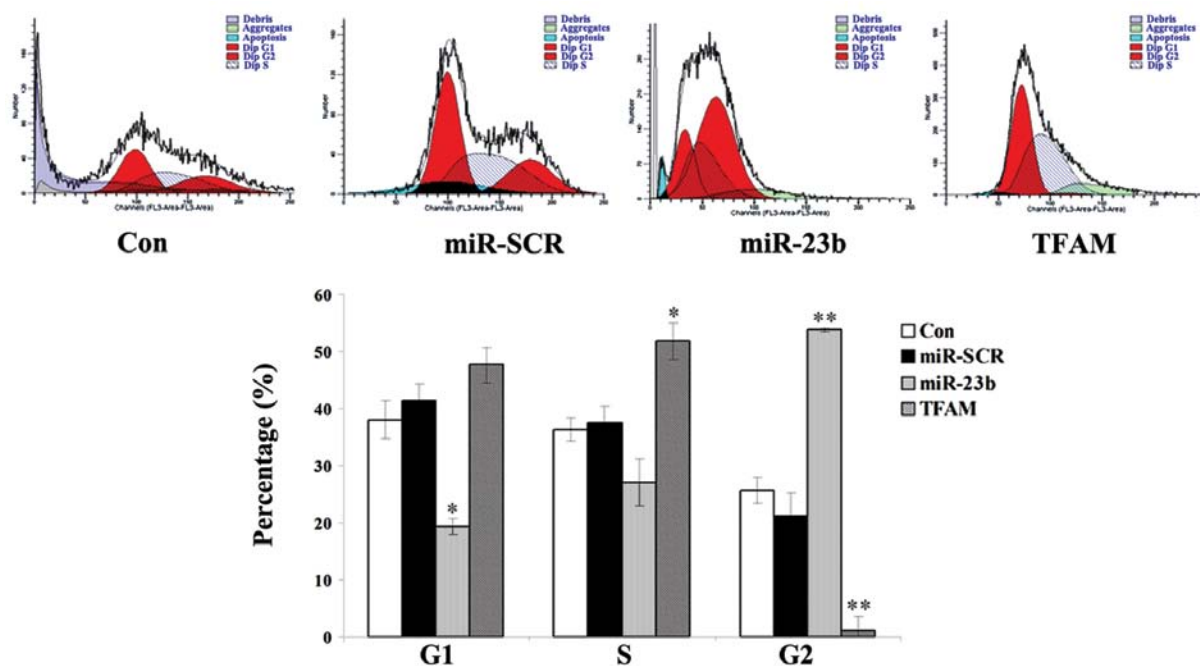


Figure 6. Effect of miR-23b and TFAM overexpression on cell cycle distribution of U251 cells. Cell cycle assay was performed to determine the effect of the overexpression of miR-23b and TFAM on cell cycle distribution of U251 cells. Con, U251 cells; miR-SCR, U251 cells transfected with miR-SCR lentiviral vectors; miR-23b, U251 cells transfected with miR-23b lentiviral vectors. TFAM, U251 cells transfected with TFAM lentiviral vectors. * $P<0.05$. ** $P<0.01$.

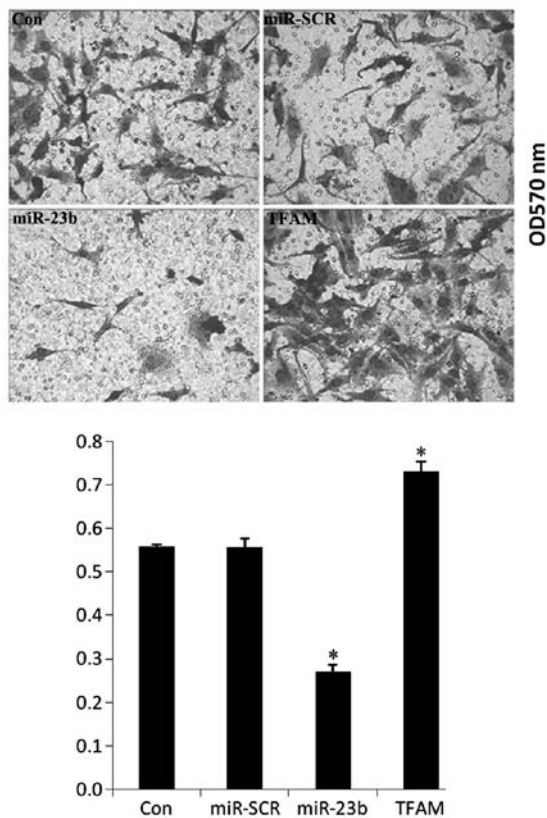


Figure 7. Effect of miR-23b and TFAM overexpression on the colony-formation efficiency of U251 cells. Clone-formation assay was performed to determine the effect of the overexpression of miR-23b and TFAM on colony-formation efficiency of U251 cells. Con, U251 cells; miR-SCR, U251 cells transfected with miR-SCR lentiviral vectors; miR-23b, U251 cells transfected with miR-23b lentiviral vectors. TFAM, U251 cells transfected with TFAM lentiviral vectors. * $P < 0.05$.

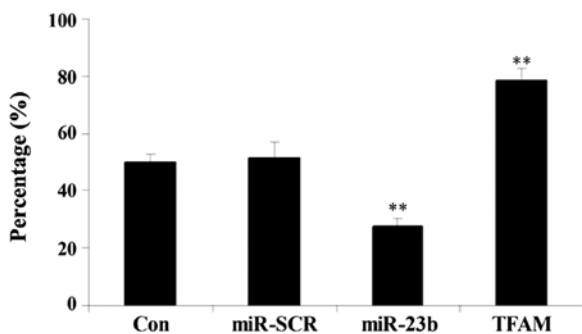


Figure 8. Effect of miR-23b and TFAM overexpression on migration of U251 cells. Transwell assay was performed to determine the effect of the overexpression of miR-23b and TFAM on migration of U251 cells. Con, U251 cells; miR-SCR, U251 cells transfected with miR-SCR lentiviral vectors; miR-23b, U251 cells transfected with miR-23b lentiviral vectors. TFAM, U251 cells transfected with TFAM lentiviral vectors. ** $P < 0.01$.

controls ($P < 0.05$), suggesting that miR-23b suppresses PI3K/Akt signaling activity while TFAM enhances it in U251 cells.

Discussion

TFAM, a transcription factor for mitochondrial DNA, is required for mtDNA replication and transcription. TFAM has

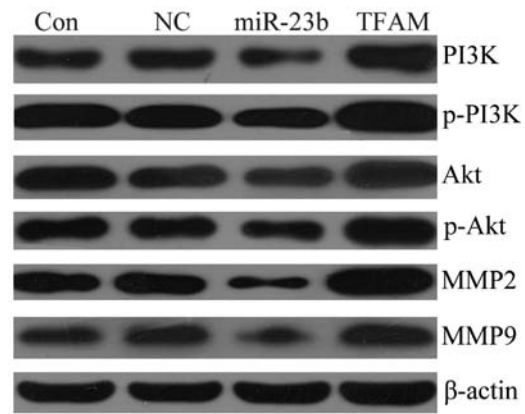


Figure 9. Effect of miR-23b and TFAM overexpression on the PI3K/Akt signaling pathway and invasion-related proteins in U251 cells. Western blot analysis was performed to examine the effect of miR-23b and TFAM overexpression on proliferative-related and migratory-related signaling pathway in U251 cells. β-actin was used as an internal reference. Con, U251 cells; miR-SCR, U251 cells transfected with miR-SCR lentiviral vectors; miR-23b, U251 cells transfected with miR-23b lentiviral vectors. TFAM, U251 cells transfected with TFAM lentiviral vectors.

been reported to participate in the regulation of cell survival, proliferation and migration. In injured rat carotid artery, vascular smooth muscle cell proliferation is dependent on the upregulation of TFAM expression (13). It has been demonstrated that TFAM regulates p21 (WAF1/CIP1), a critical regulator of cell cycle progression, as knockdown of TFAM expression was found to induce p21-dependent G1 cell cycle arrest (14,15). Moreover, TFAM is involved in the development and progression of malignant tumors. Frequent truncating mutation of TFAM has been shown to induce mtDNA depletion and apoptotic resistance in microsatellite-unstable colorectal cancer (16). However, the role of TFAM in glioma remains uncovered. In the present study, the protein levels of TFAM were significantly increased with the malignancy of glioma, suggesting that TFAM may act as an important regulator in the growth and progression of glioma.

miRNAs regulate gene expression through binding to the 3'-untranslational region (UTR) of target mRNAs and act as endogenous agents of RNA interference, and thus play a critical role in gene silencing and function (8,9). Recently, the association between miRNAs and malignant tumors has become the focus of scientific research. miR-23b has been implicated to function as a tumor suppressor in several cancer types, including breast, prostate and renal cancer (12,17,18). In fact, methylation-mediated silencing of miR-23b expression exists in glioma stem cells, and miR-23b was found to regulate cell migration and invasion via targeting of Pyk2 in migrating glioblastoma cells, indicating that miR-23b may play a regulatory role in glioma (19,20). Additionally, it has been recently demonstrated that VHL regulates the effects of miR-23b on glioma survival and invasion via suppression of HIF-1α/VEGF and β-catenin/Tcf-4 signaling (21).

In the present study, we found that the expression of miR-23b was significantly decreased in glioma tissues, when compared with normal brain and adjacent tissues. Moreover, its expression was positively correlated with the malignancy of glioma.

Furthermore, expression of TFAM was inversely correlated with miR-23b in glioma samples, suggesting that TFAM may be a direct target of miR-23b. Luciferase assay data showed that miR-23b directly deregulated the expression of TFAM. Based on these findings, we speculated that TFAM may affect the pathological process of glioma under the negative regulation of miR-23b. Further investigation was performed to test our hypothesis. Data showed that forced overexpression of TFAM enhanced cell proliferation, cell cycle progression and invasion *in vitro*, contrary to the results of forced overexpression of miR-23b in U251 cells. These findings indicate that as a direct target of miR-23b, TFAM plays a positive regulatory role in the growth and progression of malignant glioma.

The molecular regulatory mechanisms of miR-23b and TFAM in the glioma cell line U251 were further studied. The activity of the PI3K/Akt signaling pathway and the protein levels of invasion-related genes MMP2 and MMP9 were examined after upregulation of miR-23b or TFAM. Western blot data showed that the increased expression of TFAM notably promoted the protein levels of PI3K, p-PI3K, AKT, p-AKT, suggesting that PI3K/Akt signaling was activated. It has been well established that the PI3K/Akt signaling pathway plays a central role in the growth, progression and invasion of various types of cancers (22-24). Moreover, the protein expression levels of MMP2 and MMP9 were also upregulated after TFAM overexpression. MMP2 and MMP9 are two typical enzymes secreted by cancer cells and are associated with the invasion of malignant tumors, and are primarily regulated through the PI3K/Akt signaling pathway (25,26). Both the PI3K/Akt signaling pathway and MMPs were upregulated in the TFAM-overexpressing U251 cells, which contributed to the promotion of cell proliferation, cell cycle progression, clone formation and invasion of human glioma cells. In contrast, as we expected, miR-23b overexpression in glioma U251 cells significantly deregulated the protein levels of PI3K, p-PI3K, AKT, p-AKT, MMP2 and MMP9. Accordingly, we showed that the PI3K/Akt signaling pathway as well as its downstream effectors MMP2 and MMP9 participated in the regulatory network of miR-23b and TFAM in glioma U251 cells.

In conclusion, the present study elucidated the expression pattern of TFAM and miR-23b in gliomas of different WHO grades, and demonstrated for the first time that TFAM is the direct target of miR-23b. Furthermore, we revealed that the PI3K/Akt signaling pathway is involved in the TFAM regulatory network, which may contribute to the development of a promising therapeutic strategy for malignant glioma.

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