Quercetin mediated TET1 expression through miR-17 induced cell apoptosis in melanoma cells

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Abstract

Previous report suggested that the expression of ten-eleven translocation (TET) proteins was abnormal in different cancers. Quercetin, has been demonstrated as anti-cancer role in cancer development. To analyze the expression of TETs, quercetin treated with uveal melanoma cells in the present study. Our results suggested that the expression of TET1 was increased following treated with quercetin in OCM-1, SK-MEL-1 and B16 cells. In addition, quercetin treatment induced apoptosis and inhibited invasion. To further investigate if the expression of TET1 was associated with cell growth, apoptosis, migration and invasion, knocked down and overexpressing of TET1 was constructed. The results showed that the increased expression of TET1 induced cell apoptosis, increased 5-hydroxymethylcytosine (5hmC) and inhibited cell invasion. TET1 was a target gene of miR-17. Our results showed that inhibited expression of miR-17 increased TET1 expression in OCM-1 cells. To further confirm the effect of TET1 on melanoma tumors, nude mice was used. The results indicated that quercetin treatment increased TET1 expression and inhibited tumor growth. Taken together, these results suggested that quercetin can regulate cell proliferation and apoptosis through TET1 via miR-17 in melanoma cells.

Introduction

Uveal melanoma is a serious malignancy that spreads and grows more easily than other types of cancer [1, 2]. Previous reports have indicated that aberrant epigenetic modifications in melanoma cells, such as DNA methylation, are responsible [3]. TET proteins have ability to catalyze 5-methylcytosine (5mC) to 5hmC oxidation in global epigenetic modifications [4]. An increasing number of reports have showed that increased expression of TET1 induced cell apoptosis in triple-negative breast cancer cells [5]. Furthermore, TET2 mutations have been observed in different bone marrow malignancies [6]. A recent study indicated that TET3 mediated cell proliferation in hepatocellular carcinoma cells [7]. These reports suggested that TET proteins correlate with cancer development.

The flavonoid quercetin (3,3',4',5,7-pentahydroxyflavone) is widely distributed in drinks and foods [8, 9]. It is well documented that quercetin have role in antibacterial and anticancer through protection against mitochondrial damages [10]. Moreover, there is evidence that using high throughput screening, quercetin showed antiproliferative activity in cancer cells [11]. These reports indicated that quercetin has a role in inhibiting cancer growth.

In this study, the expression patterns of TET proteins, TET1, TET2, and TET3 were analyzed after treated with quercetin, in human uveal melanoma OCM-1 cells. Moreover, knockdown and overexpression of TET1 were used to evaluate cell apoptosis and cell growth. In addition, the expression pattern of miR-17 was used to analysis TET1 expression which was target gene of miR-17. Previous report showed miR-17 promotes cell growth and invasion in gastric cancer [12]. This indicated that miR-17 might have role in cell proliferation of cancer. In addition, little is known about how quercetin regulates TET1 expression *in vivo*. Therefore, nude mice were used to evaluate TET1 expression following quercetin treatment *in vivo*.

Materials And Methods

Animals and animal care

The animal experiments were performed by the Animal Care and Use Committee of Jilin University which in accordance with the Guidelines on Animal Care and Use of Animals in Research (Grant No. SY20191210). Ten nude mice (female, six weeks old) were purchased from the Jilin University. The mice were housed at 24-26 °C and 40% to 60% humidity. All operations were carried out under ARRIVE guidelines (<https://arriveguidelines.org>).

Cell culture and quercetin treatment

The human uveal melanoma OCM-1, human skin melanoma cells SK-MEL-1 and mouse melanoma B16 cell lines were donated by Changchun University of Chinese Medicine. The cell lines were cultured in DMEM (Gibco, USA) supplemented with 10 % FBS (Gibco) at 37°C and 5% CO₂. The cells (2×10^5 cells/mL) were treated with 50 µM quercetin (Sigma-Aldrich, USA) for 48 h.

Overexpression and knockdown of *TET1* and miR-17

Synthetic RNA oligonucleotides targeting *TET1* were purchased from GenePharma (Shanghai, China). FH-TET1-pEF was purchased from Addgene (Addgene plasmid # 49792). The mimics and inhibitor of miR-17-3p were purchased from GenePharma (Shanghai, China). OCM-1 cells were transfected with si-TET1, FH-TET1-pEF, miR-17-3p mimics, miR-17-3p inhibitor for 48 h. Control (Con) cells were transfected with nonspecific (Nc) siRNA.

Cell growth analysis

The cells were counted with the Cell Counting Kit-8 (CCK-8) assay kit (Meilunbio Dalian China) as previously described [13]. The cells (4×10^3) were cultured in 96-well plates and incubated for 48 hours. Then, CCK-8 solution (10 µL) was added to the cells for 2.5 h. The absorbance was measured at 450 nm using a microplate reader (Infinite M200, TECAN).

Cell cycle and apoptosis analysis

To analyze the cell cycle of OCM-1, PI staining was used. In brief, OCM-1 cells (1×10^6 cells/mL) were treated with quercetin for 48 h. The cells fixed with 70% ethanol for 2 h at 4 °C. The OCM-1 cells were incubated with PI and RNase A for 30 min. An AccuriTM C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) was used to analyze the cell cycles.

The procedure for cell apoptosis detection has been previously described [14]. Briefly, the OCM-1 cells were stained with Annexin V-FITC/PI after treatment for 48 h. Following incubation, the cells were collected at a concentration of 1×10^6 cells/mL. According to the manufacturer's instructions, Annexin V-

FITC and PI were added for each treated cell sample. The OCM-1 cells were incubated for 30 min. The Accuri™ C6 flow cytometer was used to analyzed.

Cell migration and invasion analysis

Wound healing assay was used to detected cell migration of OCM-1 cells. The OCM-1 (5×10^5) cells were cultured and treatment with quercetin, si-TET1 or FH-TET1-pEF. Then, the scratched line was created with no serum-containing culture medium in these cells. Cell migration was measured using the scratched area at 0 h, 24 h and 48 h.

For cell invasion assays, OCM-1 cells (3×10^4) were cultured 20 μ L Matrigel and treatment with quercetin, si-TET1 or FH-TET1-pEF. Next, OCM-1 cells were contained with 10 % FBS for 24 h. Then, 0.2 % crystal violet dye (Solarbio, China) was used to stain cells.

Melanoma models of nude mice

To build animal melanoma models, the left flank areas of nude mice were injected subcutaneously with OCM-1 cells (3×10^5 cells). The tumors were observed nude mice after seven or eight days. Then, the nude mice were divided randomly into two groups of five mice each. The treatment group was given 100mg/kg quercetin per day by intraperitoneal injection for 21 days. The Con group was given equal quantities of saline. Animals will be euthanized in advance, if they lose body weight significant significantly, become incapacitated, or have tumors larger than 1.5 cm in diameter during the period. The tumor size was measured. The length (L) and width (W) of tumors were recorded and the calculation of tumor volumes was according to $(L \times W^2 / 2)$. The allocation, the outcome assessment and the data analysis were completed with blinding.

Gene expression analysis

Total RNA was isolated from the OCM-1, B16 cells and tumor tissue using TRNzol reagent (TIANGEN, Beijing, China). The isolated RNA samples were first treated with DNase I (Invitrogen, Carlsbad, CA, USA), then reverse transcribed to cDNA using cDNA First Strand Synthesis Kit (TIANGEN, Beijing, China). Quantitative real-time PCR (qPCR) was used to measure gene expression. The sequences of primers are listed in Table S1. qPCR was performed using the BIO-RAD iQ5 Multicolor Real-Time PCR Detection System with the SYBR Green I Real-Time PCR Kit (TIANGEN, Beijing, China). The PCR conditions were 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 10 s, annealing at 60 °C for 15 s, and extension at 72 °C for 30 seconds as described by Teng et al[15]. The $2^{-\Delta\Delta CT}$ method was used to determine relative gene expression. The reference gene is *GAPDH* and U6. All experiments were repeated three times for each gene.

Western blot analysis

The proteins of cells and tumors were extracted with RIPA lysis buffer. The BCA protein assay kit (TIANGEN, Beijing, China) was used to detect protein concentrations. 10% SDS-polyacrylamide gels were used to separate proteins. Then, the proteins were transferred to PVDF membranes at 220mA. After blocking with 5% skim milk for 2 hours, the membranes were incubated with primary antibodies overnight at 4 °C. As previously reported[15], the primary antibodies were used including rabbit anti-TET1 (Abcam), anti-TET2 (Abcam, USA), anti-TET3 (Abcam, USA), and mouse anti-GAPDH (Abcam, USA). Subsequently, the membranes were incubated with HRP-conjugated secondary antibodies (anti-mouse or anti-rabbit, Invitrogen, USA) for 1 h. The proteins were detected using ECL Super Signal (Pierce, USA).

Hematoxylin and eosin (H&E) staining

The steps for HE staining are as described in our previous article[16]. Tumor tissues from the Con and quercetin groups were fixed in 4% paraformaldehyde for 48 h. Then, the samples embedded in paraffin wax and cut into 5 µm sections. The slides were stained with hematoxylin and eosin (H&E) and cancer cell infiltration was determined by observation under a light microscope.

Immunofluorescence (IF) staining

The 4% paraformaldehyde solution was used to fix OCM-1 cells for 15 min. Then, the OCM-1 cells were contained with 0.5% Triton X-100 for 30 min. The 5hmC antibody (1:400; # 51660S, Cell Signaling Technology, USA) were performed. The OCM-1 cells were incubated with anti-mouse antibody (1:400, BA1031, BOSTER, USA). The cells used 4,6-diamidino-2-phenylindole (DAPI, Meilunbio, China) with an antifade mounting medium and imaged using fluorescence microscope.

Statistical analyses

The data of qPCR and flow cytometry (FCM) were analyzed by Student's t-tests using GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA). A *p*-value of < 0.05 was considered statistically significant.

Results

TET proteins expression patterns after quercetin treatment

To investigate TET proteins expression patterns in melanoma, *TET1*, *TET2*, and *TET3* were detected in OCM-1, SK-MEL-1 and B16 cells by qPCR. The results indicated that the expression of *TET1* increased in both melanoma cells after quercetin treatment (Fig. 1A). To analyze if quercetin affected the growth of OCM-1, the CCK8 assay was performed. Our CCK8 results indicated that 100 µM of quercetin exhibited a toxic effect on OCM-1 cells. Thus, 50 µM of quercetin was used (Fig. 1B). The Western blot results of OCM-1 cells confirmed the qPCR data (Fig. 1C). To further analyze TET proteins expression by quercetin treatment, cell apoptosis and cell cycle were analyzed. Quercetin induced apoptosis compared to Con (Fig. 2A-B). Cell cycle analysis showed that quercetin treatment increased the number of G0/G1 phase cells and reduced the number of S phase cells (Fig. 2C-D). Moreover, 5hmC was increased after quercetin treatment (Fig. 3A). In addition, inhibited cell migration and invasion were observed after quercetin

treatment in OCM-1 cells (Fig. 3B-C). These results indicated that TET proteins, especially TET1, played an important role in melanoma and that quercetin regulated TET proteins expression in OCM-1 cells.

Overexpression of TET1 induced cell apoptosis

To further analyze *TET1* expression patterns in OCM-1 cells, qPCR was performed on cells in which *TET1* was knocked down or overexpressed. The results suggested that *TET1* expression was reduced after transfection with si-TET1 and was increased following transfection with FH-TET1-pEF (Fig. 4A). The CCK8 assay demonstrated that overexpression of *TET1* inhibited cell growth (Fig. 4B). The cell apoptosis result indicated that overexpression of *TET1* induced cell death (Fig. 4C-D). IF results suggested that overexpression of TET1 increased 5hmC level (Fig. 5A). Moreover, compared to Con, overexpression of TET1 inhibited cell migration and invasion (Fig. 5B-C). Thus, these results showed that the expression of *TET1* played a role in the apoptosis of OCM-1 cells.

Reduced miR-17 expression increased the expression of TET1

Bioinformatics indicated that *TET1* was target gene of miR-17 (Fig. 6A). To analyzed if the expression of *TET1* was regulated by miR-17, qPCR and western blot were performed. The results of qPCR showed reduced expression of miR-17 increased *TET1* expression in OCM-1 cells (Fig. 6B-C). Western blot confirmed qPCR data (Fig. 6D). These results suggested that miR-17 has a role to regulate *TET1* expression in melanoma cells.

Analysis of TET1 expression patterns in vivo

To analyze the TET1 expression patterns *in vivo*, OCM-1 cells were injected into nude mice. After seven days, the mice were treated with quercetin or equal quantities of saline for 21 days. Quercetin treatment inhibited the growth of tumors compared to the Con group (Fig. 7A-B). The H&E staining results showed a decreased number of melanoma cells in the quercetin-treated group compared to the Con group (Fig. 7C). In addition, TET1, TET2, and TET3 expression patterns were analyzed *in vivo*. The qPCR results showed that quercetin treatment increased the expression of *TET1*, *TET2*, and *TET3* (Fig. 8A-C). The Western blot results confirmed the qPCR tumor tissue data (Fig. 8D). These results suggested that quercetin suppressed tumor growth which might be associated with TET proteins expression, especially TET1, in melanoma cells.

Discussion

Previous studies have shown that the TET proteins family, including TET1, TET2, and TET3, have an important role in the development of cancer [17]. To determine whether the development of uveal melanoma is associated with TET proteins, we analyzed the expression of TET proteins after quercetin treatment of human OCM-1 cells. Quercetin is an anti-tumor drug, shown to have a role in many cancers, however, there is little evidence that quercetin alters the expression of TET proteins in melanoma [8]. Our results showed that increased TET1 expression after quercetin treatment in OCM-1, SK-MEL-1 and B16

cells. TET1 proteins have roles in cell apoptosis, cell cycle [5], and cell growth [18], suggesting that TET1 expression might influence the fate of melanoma cells.

To further validate the role of *TET1*, knockdown and overexpression of *TET1* were analyzed in OCM-1 cells. The data suggested that overexpression of *TET1* induced cell apoptosis, consistent with previous results [5]. A recent report suggested that *TET1* expression was associated with cell proliferation in colon cancer cells [18]. In addition, previous study reported that quercetin was able to inhibit cell proliferation in colon cancer cells [19]. Our results indicated that overexpression of *TET1* inhibited cell growth, migration and invasion in OCM-1 cells. miR-17, has been demonstrated to regulate *TET1* expression in the present study. Reduced expression of miR-17 induced cell apoptosis and inhibited cell growth in thyroid cancer [20]. Overexpression of miR-17 promotes metastasis in colorectal cancer [21]. These results suggest that miR-17 could regulate the expression of *TET1* to effect cell apoptosis and growth in these melanoma cells.

In the present study, nude mice were injected with OCM-1 cells and all mice had tumors seven days later, consistent with a previous report [22]. Our results showed quercetin increased *TET1* expression *in vivo*, which confirmed our data in OCM-1, SK-MEL-1 and B16 cells. Furthermore, quercetin treatment increased *TET1* expression and inhibited tumor growth in mice. These results demonstrated that TET1 protein played an important role in the growth of melanoma tumors in mice.

In conclusion, the results of the present study suggest that the expression of *TET1* was regulated by quercetin through miR-17 in melanoma cells. Upregulated expression of *TET1* inhibited cell growth and induced apoptosis. Moreover, increased expression of *TET1* was demonstrated *in vivo* and inhibited tumor growth after treatment with quercetin. Our results suggested an important role of TET1 expression and identified *TET1* as a novel biomarker for melanoma therapy.

Declarations

Ethics approval and consent to participate

All protocols of animal experiments in this article were approved were performed by the Animal Care and Use Committee of Jilin University which in accordance with the Guidelines on Animal Care and Use of Animals in Research (Grant No. SY20191210). The animal experiment design follows the 3R rule. Each nude mouse was anesthetized by intraperitoneal injection of 50 mg/kg sodium pentobarbital and euthanized by cervical dislocation. All operations were carried out under ARRIVE guidelines (<https://arriveguidelines.org>). All authors agreed to post this article.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article [and its supplementary information files].

Competing interests

No potential conflict of interest was reported by the authors.

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Authors' Contributions

Yongjian Gao and Dongxu Wang designed the experiments and wrote the manuscript. Yongjian Gao, Chengshun Li and Ruizhi Hou performed cell experiment and gene expression analysis. Dayong Ding and Ye Feng contributed reagents and materials. Chengshun Li carried out animal experiment. Chao Lin and Qianyun Xia analyzed the data and prepared figures. All authors reviewed the manuscript.

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Figures

Figure 1

The expression pattern of *TET1*, *TET2* and *TET3* in melanoma cells. Relative expression of *TET1*, *TET2* and *TET3* was analyzed by qPCR in OCM-1, SK-MEL-1 and B16 cells (A). The cell growth was analyzed by CCK8 assay in OCM-1 cells (B). The expression of *TET1*, *TET2* and *TET3* were analyzed by western blot in OCM-1 cells (C). The blots have been cropped. The data are represented as the mean ± SD (n = 3). * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.005$) and **** ($p < 0.001$) indicate statistically significant differences.

Figure 2

Analysis of cell apoptosis and cell cycle in OCM-1 cells. The cell apoptosis was analyzed after quercetin (A). Statistical analysis of percentage of cell apoptosis (B). The cell cycle was analyzed after quercetin (C). Statistical analysis of the percentage of cell cycle (D). The data are represented as the mean $\pm$ SD ($n = 3$). * ($p < 0.05$) and ** ($p < 0.01$) indicate statistically significant differences.

Figure 3

Analysis of 5hmC, cell migration and invasion after quercetin treatment in OCM-1 cells. The 5hmC level was analyzed after quercetin treatment at 48 h (A). The cell migration was analyzed after quercetin treatment at 0 h, 24 h and 48 h (B). The cell invasion was analyzed after quercetin treatment (C).

Figure 4

Analysis of *TET1* expression pattern in OCM-1 cells. Relative expression of *TET1* in Con, si-*TET1* and FH-*TET1*-pEF (A) using qPCR. The cell growth was analyzed by CCK8 assay (B). Statistical analysis of percentage of cell apoptosis (C). The cell apoptosis was analyzed after si-*TET1* and FH-*TET1*-pEF transfected (D). The data are represented as the mean $\pm$ SD ($n = 3$). * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.005$) indicate statistically significant differences.

Figure 5

Analysis of 5hmC, cell migration and invasion after knockdown and overexpression of *TET1*. The 5hmC was analyzed at 48 h in Con, FH-TET1-pEF and si-TET1 group (A). The cell migration was analyzed at 0 h, 24 h and 48 h after FH-TET1-pEF and si-TET1 transfected (B). The cell invasion was analyzed in Con, FH-TET1-pEF and si-TET1 group (C).

Figure 6

Analysis of TET1 expression pattern by miR-17 in OCM-1 cells. Schematic diagram of miR-17 regulate TET1 expression (A). Relative expression of miR-17 in Nc-mimics, Mimics-miR-17, Nc-inhibitor and Inhibitor-miR-17 group using qPCR (B). The expression of TET1 in Nc-mimics, Mimics-miR-17, Nc-inhibitor and Inhibitor-miR-17 group using qPCR (C) and Western blot (D). The blots have been cropped. * ($p < 0.05$) and **** ($p < 0.001$) indicate statistically significant differences.

Figure 7

Morphological and Histopathological observation of tumor tissue. Morphological observation of mouse melanoma tissue (A). Analysis of tumor volume (B). Histopathological observation of mouse tissue (C). The data are represented as the mean $\pm$ SD ($n = 3$). ** ($p < 0.01$) indicate statistically significant differences.

Figure 8

The expression pattern of *TET1*, *TET2* and *TET3* *in vivo*. Relative expression of *TET1* (A), *TET2* (B) and *TET3* (C) was analyzed by qPCR. The expression of TET1, TET2 and TET3 were analyzed by western blot *in vivo* (D). The blots have been cropped. The data are represented as the mean $\pm$ SD ($n = 3$). * ($p < 0.05$) and ** ($p < 0.01$) indicate statistically significant differences.

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