FTO-Mediated N6-Methyladenosine Modification of KLF3 Promotes Osteosarcoma Progression

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Abstract

N6-methyladenosine (m6A) methylation is the most common and abundant methylation modification of eukaryotic mRNAs, which is involved in tumor initiation and progression. This study aims to explore the functional role and regulatory mechanism of FTO in osteosarcoma progression. In this study, we detected the expressions of KLF3 in OS cells and tissues by qRT-PCR and western blot, and found that the mRNA and protein expressions of KLF3 were increased in OS cells and tissues and significantly related to tumor size, metastasis, and TNM stage and poor prognosis of OS patients. FTO promoted the proliferation and invasion and suppressed apoptosis of OS cells through CCK-8, Transwell and apoptosis assays. Further mechanism dissection revealed that FTO and YTHDF2 enforced the decay of KLF3 mRNAs and decreased its expression. Knockdown of FTO led to elevate m6A and mRNA levels. FTO-mediated m6A modification regulated KLF3 expression in the YTHDF2-dependent manner. Moreover, KLF3 overexpression abrogated FTO-induced oncogenic effects on the proliferation and invasion of OS cells. Overall, our findings showed that FTO-mediated m6A modification of KLF3 promoted OS progression, which may provide a therapeutic target for osteosarcoma.

Introduction

Osteosarcoma (OS), as the most common primary malignant bone tumor, originates from mesenchymal cells, mostly in long bone, and can also be seen in distal femur or proximal tibia. Osteosarcoma is often accompanied by micrometastasis before operation and characterized by recurrent possibility and suboptimal prognosis [1]. 15%-20% of patients with OS are diagnosed with distant metastases at the first diagnosis [2]. Even after radiotherapy and chemotherapy, the survival rate of 5-year is still less than 20% for OS complicated with lung metastasis [3]. The combination of surgery and radiotherapy and chemotherapy has made some progress in improving the prognosis of patients with OS, but the curative effect is still not ideal [4]. Therefore, we urgently need to explore the mechanism of osteosarcoma progression, find key molecular targets and improve the prognosis of osteosarcoma.

m6A methylation is a dynamic and reversible epigenetic modification of RNAs, which mainly affects the splicing, translation and stability of mRNAs and the epigenetic effects of non-coding RNAs [5,6]. The biological process of m6A is mainly regulated by three kinds of key-related kinases, m6A methyltransferase (Writers), m6A demethylase (Erasers) and methylation reading protein (Readers) [5,6]. Methyltransferase complexes composed of various writers, such as METTL3/14, WTAP and KIAA1429, catalyze the m6A modification of adenylate on mRNAs. Demethylase includes FTO and ALKBH5, and its function is to demethylate the bases modified by m6A. The main function of readers is to recognize the m6A-modified RNA, thus activating downstream regulatory pathways such as RNA processing, mRNA degradation, translation and decay [7,8]. m6A RNA methylation plays multiple roles in mammalian growth and development, including embryonic development, circadian rhythm and DNA damage response. More and more evidence shows that m6A methylation is closely related to tumorigenesis and development [6,9,10]. METTL3 promotes cell proliferation, migration and invasion of and activates Wnt/ β -catenin pathway in OS through regulating m6A methylation and LEF1 [11]. Blockade of METTL3

inversely inhibits growth and invasion of OS cells by suppressing ATAD2 [12]. ALKBH5 augments lncRNA PVT1 expression in an m6A dependent manner and facilitate OS tumorigenesis [13]. These studies show that m6A methylation plays an important role in the occurrence and development of OS, but the specific mechanism and role of m6A reader FTO has not been reported.

Krüppel-like factor 3 (KLF3), a member of the KLF family, was originally a KLF1 homolog cloned from human hematopoietic tissue. KLF3 can recruit transcriptional corepressor C-terminal binding protein and complete fusion in the target gene promoter, regulating transcription of target genes [14,15]. KLF3 plays an important role in B cell development, erythrocyte and fat production, nervous system and muscle gene regulation, cell differentiation and proliferation [14,15]. Aberrant expression of KLF3 has been detected in several cancers, such as lung cancer [16], acute leukemia [17], colorectal cancer [18], sarcoma [19]. Silencing KLF3 contributes to lung cancer metastasis and the EMT process by regulating the STAT3 signaling pathway [16]. Decreased KLF3 is associated with aggressive phenotypes and poor prognosis for colorectal cancer patients [18]. KLF3 inhibits the pro-metastatic miR-182 expression and sarcoma metastasis [19]. All these support the potential tumor-suppressor gene of KLF3. However, the underlying mechanism by which KLF3 is affected, especially m6A methylation, is virtually unknown in OS.

In the study, we initially investigated the expression of FTO and found that increased expression of FTO was related to clinicopathological characteristics and poor prognosis of OS patients. FTO elicited potent oncogenic functions in promoting the proliferation and migration of OS cells. Moreover, we determined the effect of FTO-mediated m6A modification on KLF3 expression. Overall, FTO-KLF3 may serve as a potential therapeutic target for osteosarcoma.

Methods

Detailed procedures are provided in Supplementary Materials and methods.

Results

FTO is increased expression in OS

To evaluate the expression of FTO, we investigate the expression pattern of FTO in OS cells and the normal osteoblast cells (Nhost) by qPCR. And results showed that the mRNA levels of FTO in OS cells were elevated (Fig. 1a). We also detected the mRNA levels of FTO in OS and adjacent tissues. Similarly, mRNA levels of FTO were frequently increased in cancer tissues compared with matched normal tissues (Fig. 1b). IHC staining was also used to detect the FTO expression in OS and matched normal tissues (Fig. 1c). According to the quantitative analysis of IHC staining of tissue samples, we divided the FTO expression into high expression and low expression. 64.3% of OS tissues showed high expression, whereas the low expression rate of FTO in normal tissues was only 26.2% (Fig. 1d).

We further analyzed the relationship between the FTO expression and clinicopathological parameters and postoperative survival of OS. Results from the IHC assay showed that there was a significant correlation

between the high expression of FTO and several clinicopathological features (Table 1), such as tumor size, metastasis, and TNM stage, but not with the gender and age. In order to further understand the relationship between FTO and survival of OS, according to the FTO expression, we performed Kaplan-Meier survival curve analysis. The FTO expression was negatively correlated with the 5-year overall survival of patients with OS (Fig. 1e).

FTO promotes OS cell proliferation, migration and invasion

In order to further study the effect of FTO expression on the proliferation of OS cells, FTO knockdown or overexpression was used to detect the proliferation ability of OS cells at 24h, 48h, 72h, respectively. Transfection efficiencies of FTO knockdown or overexpression were analyzed by qPCR in OS cells (Fig. 2a). Results showed that overexpression of FTO in OS cells promoted cell proliferation (Fig. 2b), while knockdown of FTO inhibited cell proliferation (Fig. 2b). As shown in (Fig. 2c), Clone formation also supported the role of FTO in contributing to proliferation of OS cells, and FTO downregulation weakened the cell proliferation. In addition, enforced expression of FTO inhibited OS cell apoptosis, however, blockade of FTO expedited apoptosis (Fig. 2d).

Scratch healing assay was used to verify the migration ability of FTO to OS cells. Amplification of FTO accelerated scratch healing of OS cells (Fig. 2e). Transwell chamber assay showed that FTO attenuates the invasion of OS cells; inversely, silencing FTO attenuated the invasive ability (Fig. 2f). These findings indicated FTO promoted proliferation, migration and invasion of OS cells.

FTO inhibits KLF3 expression in OS

As the first discovered demethylase FTO, its discovery reveals the dynamic and reversible process of m6A modification, and it can reverse the m6A modification on RNAs [22]. Previous results indicated that KLF3 is decreased expression in sarcomas [19]. It has been reported that the tumor suppressor KLF4, another member of the KLF family, could be regulated in an m6A dependent manner bladder cancer [23]. METTL3-mediated m6A methylation directly promotes the mRNA decay of KLF4 through m6A binding protein YTHDF2 [23]. Similarly, we identify whether the role of KLF3 in OS is regulated by m6A eraser FTO. Therefore, we subsequently analyzed the profile of m6A content in OS tissues. Results from m6A methylation quantification showed that m6A modification is significantly decreased in the total RNAs of OS tissues compared to adjacent normal tissues (Fig. 3a). m6A methylation quantification and dot blot assays were employed to detect the effect of FTO on m6A methylation in OS cells. As expected, m6A level was down-regulated in OS cells expressing FTO (Fig. 3b-c). Western blotting confirmed that FTO contributed to decrease KLF3 expression in OS cells, and silencing FTO conferred by siRNA inversely increased KLF3 (Fig. 3d), which were consistent with results from qRT-PCR (Fig. 3e). IHC staining also supported the decreased expression of KLF3 in OS tissues compared with normal tissues (Fig. 3f). Correlation analysis revealed that there was a significant negative correlation between FTO and KLF3 mRNA levels in OS tissues (Fig. 3g). Thus, we speculated that FTO can negatively regulate the expression of KLF3.

FTO regulates KLF3 expression in an m6A-dependent manner

The functional interaction between m6A methylase and demethylase determines the dynamic and reversible regulation of m6A modification. And m6A binding proteins can bind to mRNA containing m6A, thus affecting the fate of target mRNAs [24]. Me-RIP qPCR suggested that knockdown of FTO augmented the m6A enrichment on KLF3 mRNAs in OS cells (Fig. 3h). FTO can negatively regulate the content of KLF3 mRNAs (Fig. 3e). We carried out the stability assay of KLF3 mRNAs. The results suggested that, after treatment with Actinomycin D (Transcription inhibitor), the residual percentage of KLF3 mRNA in FTO group was significantly lower than that in control group (Fig. 3i). Previous studies have confirmed that m6A binding protein YTHDF2 affects the stability of m6A modified RNA by locating them at mRNA decay sites [24]. Transfection efficiencies of YTHDF2 knockdown or overexpression were analyzed by qPCR in OS cells (Fig. 3j). YTHDF2 attenuated the mRNA and protein levels of KLF3 (Fig. 3k-l). And YTHDF2 reduced the half-life of KLF3 mRNA (Fig. 3m). RIP-qPCR assay proved the interaction between YTHDF2 protein and KLF3 mRNA in OS cells, which was enhanced in OS cells expressing FTO (Fig. 3n). Silencing YTHDF2 abrogated FTO-mediated the decrease of KLF3 mRNA and protein levels in OS cells (Fig. 3o-p). qRT-PCR results also verified that YTHDF2 knockdown reversed FTO-induced mRNA degrading of KLF3 (Fig. 3q). Overall, Silencing FTO accelerated the YTHDF2-involved decay of KLF3 mRNA in OS cells.

KLF3 impairs the FTO-induced proliferation and invasion of OS cells

To ascertain whether KLF3 is involved in FTO function, OS cells transfected with FTO plasmids showed lower expression of KLF3, which could be reversed by overexpressing KLF3 (Fig. 4a). Moreover, FTO dramatically accelerated the proliferation of OS cells, whereas enforced expression of KLF3 reversed the FTO-induced promotion effects (Fig. 4b). Cloning formation efficiencies were significantly lower in OS cells expressing FTO and KLF3 than that of OS cells expressing FTO (Fig. 4c). FTO inhibited apoptosis, which was severely impeded in OS cells with ectopic expression of KLF3 (Fig. 4d). Analogously, KLF3 dramatically suppressed FTO-induced cell invasion (Fig. 4e). Cyclin D1 and p21 is the key protein of cell cycle regulation. FTO increased Cyclin D1 expression and decreased p21 expression in OS cells (Fig. 4f), and KLF3 could reverse the alternation. EMT also plays an important role in the process of tumor metastasis. FTO could enhance N-cadherin and Vimentin expression and suppress E-cadherin expression (Fig. 4f), which were compromised by KLF3 amplification. Thus, our findings indicated that FTO promoted cell proliferation and invasion by abating KLF3 in OS cells.

Discussion

m6A methylation, as the most important RNA internal modification in eukaryotes, was first discovered in 1974 and attracted great attention recently [6,7]. m6A methylation occurs in all stages of the RNA life cycle, from RNA synthesis to nucleation, translation regulation to RNA degradation [24]. m6A modification is closely related to the occurrence and development of cancer and participates in cell differentiation, proliferation, migration, immunity and drug resistance. Deregulation of m6A regulators in

OS is inevitable [11,13,25,26]. Miao et al. reported that METTL3 contributes to proliferation, migration and invasion of OS cells and activates Wnt/ β -catenin signaling pathway by regulating m6A modification of LEF1 mRNAs [11]. Analogously, METTL3 also promotes proliferation and invasion of OS cells by increasing ATAD2 expression via an m6A-dependent manner [12]. ALKBH5 attenuates the m6A modification of PVT1 mRNA and impedes the binding of YTHDF2 to PVT1 mRNA, which increases PVT1 expression and accelerates OS growth in vivo [13]. In addition, m6A-related enzymes are involved in regulating the pluripotency of osteosarcoma stem cells [26,27]. All these implies potential roles of m6A methylation in tumorigenesis and progression of OS. And the relationship between FTO and OS has not been reported.

In the study, our results showed that the protein and mRNA levels of FTO were increased in OS tissues and cells. Through IHC staining of OS samples, we also found that FTO expression in OS tissues was significantly higher than that in paracancerous normal tissues, and increased expression of FTO was associated with poor prognosis and aggressive phenotypes in OS. Ectopic expression of FTO promoted the proliferation, migration and invasion of OS cells and inhibited the apoptosis, and knockdown of FTO shows the opposite effects. These findings supported that FTO promotes OS progression.

KLFs are one of the key components of the mammalian Sp/KLFSp zinc finger protein family. KLF3, as a member of KLFs, can recruit transcriptional corepressor C-terminal-binding protein to promoters of downstream molecules and primarily effectuate transcriptional inhibition [15,17]. Similar to other Sp/KLFSp zinc finger protein members, KLF3 contains conserved binding sites of GC-rich motif or CACCC box, which causes the regulation diversity for target genes [28]. Therefore, KLF3 has multiple biological functions, including erythropoiesis, adipogenesis, muscle regulation, B-cell development, differentiation and apoptosis [17,29]. Aberrant expression of KLF3 is related to the occurrence and progress of various tumors, such as acute leukemia [17], sarcoma [19], lung cancer [16], colorectal cancer [18], melanoma cancer [30]. Loss of KLF3 potentiates lung cancer metastasis and EMT process through controlling STAT3 pathway [16]. DNA methylation-mediated KLF3 silencing augments the pro-metastatic miR-182 in human sarcoma cells [19]. Decreased KLF3 is associated with poor prognosis and may work as a promising predictor in colorectal cancer [18]. These suggest that KLF3 may be a potential tumor suppressor gene. It has been reported that m6A-mediated modification could regulate the methylation of other members of KLFs, such as KLF1 [31], KLF4 [32], and KLF5 [33]. RNA demethylation FTO promotes the phenotype conversion of human aortic vascular smooth muscle cells through increasing KLF5 expression in an m6A-dependent manner [33]. However, the potential mechanism of whether KLF3 is affected by m6A-mediated methylation modification have never been reported. Subsequently, we further explore whether FTO-mediated m6A modification regulates the expression of KLF3. We quantitatively detected the m6A modification of total mRNAs in OS and adjacent tissues and found that m6A content in OS samples was lower than that in adjacent samples, It is consistent with previous studies that total m6A level is decreased in OS [34]. After knocking down and overexpressing FTO, we detected the changes of KLF3 expression and found that FTO negatively regulated the mRNA and protein expressions of KLF3 in OS cells. IHC staining showed that KLF3 was down-regulated in OS tissues compared with normal tissues.

And decreasing FTO can potentiate m6A modification of KLF3 mRNAs. As m6A “reader”, the C-terminal of YTHDF2 can specifically recognize m6A-modified mRNAs to reduce the half-life of target mRNAs and decrease the stability of mRNAs [35]. We also proved the interaction between YTHDF2 protein and KLF3 mRNA in OS cells. Given that FTO positively regulates the content of KLF3 mRNA in cells, we investigated the effect of FTO-mediated m6A on the stability of KLF3 mRNAs. Our results showed that knockdown of FTO decreases the stability of KLF3 mRNA in OS cells, and deletion YTHDF2 impedes the KLF3 mRNA decay induced by silencing FTO. There was also a negative correlation between FTO and KLF3 expression in OS tissues. We concluded that FTO regulates the level of KLF3 mRNAs in an m6A-dependent manner in OS.

In vitro studies demonstrated that FTO promoted the proliferation and invasion of OS cells. However, KLF3 protected against FTO-induced promotion effects on cell proliferation and invasion. Cyclin D1 promotes cell proliferation by binding to and activating cyclin dependent kinase CDK4 unique to G1 phase. And p21 can form a complex with cyclin D/CDK and arrest the cell cycle in G1 phase. EMT concomitant with the down-regulation of epithelial markers and up-regulation of mesenchymal phenotype markers is an important process of invasion and distant metastasis of cancer. FTO up-regulated Cyclin D1 and N-cadherin level and down-regulated p21 and E-cadherin level in OS cells, and expressing KLF3 compromised the FTO-induced alternations. Collectively, FTO enhances the cell proliferation and invasion by silencing KLF3 expression in OS cells. This study focused on the effects of FTO on the proliferation and invasion of OS cells by regulating the m6A methylation of KLF3 mRNAs. Our results also supported the view that m6A modification is involved in the progression of OS. However, further researches are needed to provide direct epigenetic evidence of the FTO/KLF3 pathway in the pathogenesis of OS.

Conclusions

The expression of FTO is increased in OS and affects the prognosis survival of OS patients. FTO promotes the proliferation and invasion, and inhibits apoptosis of OS cells through regulating KLF3 expression via an m6A-dependent manner. And by revealing the previously unknown regulatory mechanisms of FTO and KLF3, it provides a new direction for the molecular mechanism of occurrence and development of OS. In addition, considering the importance of m6A modification in human cancers, it is possible to explore new strategies for the diagnosis and treatment of OS by targeting FTO with selective inhibitors.

Declarations

Ethics approval and consent to participate The study was approved by the Review Board of the Affiliated Hospital of Xuzhou Medical University. All participants provided informed consent as outlined in the manuscript.

Consent for publication All authors approved the final version of this manuscript.

Availability of data and materials All data and materials are available for verification as needed.

Competing interests The authors confirm that there are no conflicts of interest.

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Authors' contributions Conceived and designed the project: Hong-Jian Shan, Hong-Liang Chen and Zhi-Man Zhu; Performed the project: Hong-Jian Shan, Wen-Xiang Gu; Analyzed the data: Hong-Jian Shan, Gang Duan; Contributed reagents/materials/analysis tools: Hong-Jian Shan, Wen-Xiang Gu, Gang Duan, Zhi-Man Zhu, Hong-Liang Chen; Wrote the paper: Hong-Jian Shan. All authors read and approved the final manuscript.

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Compliance with Ethical Standards

Disclosure of potential conflicts of interest The authors confirm that there are no conflicts of interest.

Research involving Human Participants and/or Animals The study involving human specimens was approved by the Review Board of the Affiliated Hospital of Xuzhou Medical University. This article does not contain any studies with animals performed.

Informed consent All participants provided informed consent as outlined in the manuscript.

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Tables

Due to technical limitations, table 1 is only available as a download in the Supplemental Files section.

Figures

Figure 1

FTO is increased in OS tissues. The mRNA levels of FTO were detected in OS cells and normal osteoblast Nhost cells (a). The mRNA levels of FTO in 9 paired OS tissues and the corresponding normal tissues (b). Representative images of IHC staining were used to analyze the protein levels of FTO in OS tissues

adjacent and normal tissues (c). FTO expression was calculated in OS and normal tissues via quantitative analysis of IHC staining of FTO protein (d). Kaplan–Meier and log-rank test were used to analyze the 5-year overall survival OS patients with high or low FTO expression (e). Data represents the means $\pm$ SD. **, $P < 0.01$; ***, $P < 0.001$.

Figure 2

FTO promotes proliferation, invasion of OS cells and inhibits cell apoptosis. Transfection efficiencies of FTO knockdown or overexpression were analyzed by qPCR in OS cells (a). The cell viability of OS cells was measured by CCK8 assay after FTO knockdown or overexpression (b-c). The effects of FTO in OS cells on the clone formation (c). FTO-knockdown or -overexpression on cell apoptosis was determined by flow cytometry assays (d). OS cell motilities were analyzed by measuring the wound closure after FTO knockdown or overexpression (e). Transwell chamber assays were performed to detect the invasive ability of OS cells after FTO knockdown or overexpression (f). Data represents the means $\pm$ SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Figure 3

FTO regulates KLF3 expression in an m6A-dependent manner in OS cells. m6A methylation quantification was performed in OS and adjacent normal tissues (a). m6A quantification and dot blot assays were employed to detect the effect of FTO on m6A methylation in OS cells (b-c). The effects of FTO knockdown or overexpression on the protein and mRNA levels were determined by western blotting and qRT-PCR (d-e). Representative images of IHC staining were used to analyze the protein levels of FTO in OS tissues adjacent and normal tissues (f). Correlation analysis of FTO and KLF3 mRNA expressions in

OS tissues (Pearson correlation test (g)). Me-RIP qPCR was performed to measure m6A enrichment on KLF3 mRNAs in OS cells after knocking down FTO (h). RNA stability assay was used to detect the mRNA stability of KLF3 after FTO overexpression in cells with the treatment of actinomycin D (0.2 mM) for the indicated time (i). Transfection efficiencies of YTHDF2 knockdown or overexpression were analyzed by qPCR in OS cells (j). YTHDF2 attenuated the mRNA and protein levels of KLF3 in OS cells (k-j). The effect of YTHDF2 on the mRNA stability of KLF3 was performed (m). RIP-qPCR assay was performed to analyze the interaction between YTHDF2 protein and KLF3 mRNA in OS cells (n). The effect of silencing YTHDF2 on FTO-mediated the decrease of KLF3 mRNA and protein was performed by qRT-PCR and Western blotting (o-p). The role of FTO-induced mRNA degrading of KLF3 in OS cells was analyzed (q). Data represents the means $\pm$ SD. **, $P < 0.01$; ***, $P < 0.001$.

Figure 4

KLF3 impairs the FTO-induced proliferation and invasion of OS cells. KLF3 could reverse the FTO-induced decrease of KLF3 protein (a). KLF3 impaired the FTO-induced promotion effect on OS cell proliferation and clone formation (b-c). Flow cytometry assay was performed to investigate the role of KLF3 in FTO-involved apoptosis in OS cells (d). The effect of KLF3 on FTO-induced cell invasion (e). The effects of FTO and (or) KLF3 on the cycle regulator and EMT markers in OS cells (f). Data represents the means $\pm$ SD. **, $P < 0.01$; ***, $P < 0.001$.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Table1.xlsx](#)
- [SupplementaryMaterialsandmethods.docx](#)